The Role of the Transforming Growth Factor Beta (TGF-β) Family in Diabetic Foot Ulcer (DFU) Recovery

Thesis submitted by

Samuel David HUGHES

B.Sc. (*Hons*)

For the degree of Masters of Philosophy (Medical and Molecular Sciences).

In the College of Medicine and Dentistry

July 2023

Acknowledgements

I would like to thank many people for their contributions to the completion of this thesis.

To my supervisors: Associate Professor Haleagrahara Nagaraja, Dr. Venkat Vangaveti, and Professor Usman Malabu, thank you for your time, patience, expertise, and having my back when others did not.

To my Mum, thank you for listening to me rant about my issues. Your patience and understanding were invaluable.

To my best friend Lisa, you provided me with advice, laughs, and someone to talk to when I needed to. You were there in some of my darkest hours, and words can not describe how appreciative I am for our friendship.

To Dani, we may not have spoken as often, but your wisdom, patience, and good humour were greatly appreciated. Thank you for giving me a sense of comradery when I was not able to find it elsewhere.

To Michael, thank you for giving me a place to stay and work in relative peace. The laughs and advice were just the cherry on top.

To Ben, thanks for the chats over Mario Kart. They were welcome distractions for a tired brain.

COPYRIGHT STATEMENT

STATEMENT OF CONTRIBUTION OF OTHERS

Nature of Assistance	Contribution	Names
Intellectual Support	Proposal Writing	Associate Professor Haleagrahara Nagaraja, Dr. Venkat Vangaventi, Professor Usman Malabu
	Data Analysis	Dr. Venkat Vangaventi
	Statistical Support	Dr. Venkat Vangaventi, Emeritus Professor Rhondda Jones
	Editorial Assistance	Associate Professor Haleagrahara Nagaraja, Dr. Venkat Vangaventi, Professor Usman Malabu
Financial Support	Fee Waiver Stipends	Research Training Program Fee Offset (2021), Australian Government Research Training Program Stipend (2021), College of Medicine and Dentistry Top-up Scholarship

ABSTRACT

Diabetic foot ulcer (DFU) is a problematic, costly, long-term complication associated with Diabetes mellitus (DM), with an annual cost of treatment to the Australian public of $1.6 billion. Current treatment for these chronic wounds involves surgical debridement, wound offloading, moist dressing application, disease control, vascular assessment, and infection control. A third of DM patients are at risk of developing DFU and, even when treated, 40% of DFU cases can reoccur within a year. Growth factor signalling, a component of the wound healing response, is one of the many biological processes that are altered by hyperglycaemia. The transforming growth factor-β (TGF-β) family of growth factors participate in normal pancreatic function and diabetic pathology. Both DFU and the causal pathology show changes in TGF-β signalling, indicating a strong role for the growth factor in DFU pathology. This study aimed to determine the levels of TGF-β1 and bone morphogenetic protein-7 (BMP-7), a member of the TGF-β family, in DFU patients, and then determine if these levels were related to ulcer healing, diabetes improvement, or other laboratory parameters involved with DM and DFU. To investigate this, serum from 10 patients that participated in the Galvus Anti-Inflammatory Effects in Diabetic Foot Ulcer (GIED) trial were analysed for TGF-β1 and BMP-7 using enzyme-linked immunosorbent assays (ELISA). Serum TGF-β1 increased from 7686 pg/mL to 11226 pg/mL and levels of BMP-7 improved from 42.37 pg/mL to 49.23 pg/mL. This improvement in growth factors levels was independent of Galvus treatment. However, no relationship was observed between surrogate markers of ulcer healing, diabetes improvement, and normalised values for either growth factor. Analysis of covariance found a relationship between DFU patients without diabetic neuropathy, microvascular complications, dyslipidaemia, or hypertension and TGF-β1 values at the end of the trial period. Additionally, 12 week BMP-7 levels were higher in patients receiving insulin than those who were not, an effect that was not seen with oral anti-hyperglycaemics or co-treatment with insulin and oral anti-hyperglycaemics. Correlation analysis showed a potential relationship between intake values for both proteins and inflammation, both pro- and anti-inflammatory actions. Furthermore, correlations were observed between 12 week values and laboratory values that suggest wound normalisation and anti-inflammatory effects. This work is the first study to look at BMP-7 in DFU. Despite

the small size of the study, there appears to be a role for the TGF-β family in DFU recovery, particularly around the regulation of inflammation and possibly treatment response.

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANCOVA: Analysis of Covariance

BMP-7: Bone Morphogenetic Protein-7

DFU: Diabetic Foot Ulcer

DM: Diabetes Mellitus

DN: Diabetic Neuropathy

DPP4: Dipeptidyl Peptidase-4

eGFR: Estimated Glomerular Filtration Rate

ELISA: Enzyme Linked Immunosorbent Assay

GIED: Galvus Anti-Inflammatory Effects in Diabetic Foot Ulcer

GLP-1: Glucagon-Like Peptide-1

Hb: Haemoglobin

HbA1c: Glycated Haemoglobin

HCl: Hydrochloric Acid

HDL: High Density Lipoprotein

LDL: Low Density Lipoprotein

mm: millimetres

mRNA: Messenger Ribonucleic Acid

NaOH: Sodium Hydroxide

NPWT: Negative Pressure Wound Therapy

PAD: Peripheral Artery Disease

ROS: Reactive Oxygen Species

SA: Surface Area

T1DM: Type 1 Diabetes Mellitus

LIST OF ABBREVIATIONS

T2DM: Type 2 Diabetes Mellitus

TGF-β: Transforming Growth Factor-β

VLDL: Very Low Density Lipoprotein

WBC: White Blood Cells

CHAPTER 1: INTRODUCTION

1.1 Diabetes: Epidemiology, Pathology and Complications

Diabetes mellitus (DM) is a medical condition that has become a health and financial burden of global proportions (Zimmet et al., 2016, Tan et al., 2019a). The global population of DM patients was 451-476 million in 2017, doubling from 211.2 million in 1990 (Cho et al., 2018, Lin et al., 2020). This number is set to increase with 570.9 million patients anticipated to suffer from DM by 2025 and a predicted 693 million DM patients by 2045 (Cho et al., 2018, Lin et al., 2020). Furthermore the global incidence of DM was 22.9 million in 2017, a rate which is set to increase with an estimated 26.6 million people to develop DM by 2025 (Lin et al., 2020). Approximately 4.9% (1.2 million) of Australians self-reported suffering from diabetes in 2017-2018, with DM contributing 10.5% of total deaths for Australia in 2018 (AIHW, 2020). In 2005, the total annual cost of diabetes was $10.6 billion AUD, adjusted to $14.6 billion AUD in 2010 to account for inflation (Lee et al., 2013). Moreover, approximately $2.7 billion AUD of total healthcare expenditure in 2015-2016 was spent on diabetes (AIHW, 2020).

Characterised by chronically elevated blood glucose (hyperglycaemia), DM is a complex clinical syndrome that can be divided into multiple types, but two of those types are more common: Type 1 (T1DM) and Type 2 (T2DM) (Zimmet et al., 2016, Tan et al., 2019a). T1DM is an autoimmune disease caused by the body destroying insulin-secreting β-cells, whereas T2DM is marked by insulin resistance and subsequent reduction in plasma insulin levels (Tuomi et al., 2014, Tan et al., 2019a). Treatment of DM differs depending on the type of diabetes. Exogenous insulin administration is used in T1DM intervention (Katsarou et al., 2017). Lifestyle intervention is the most efficacious; however, several pharmacological agents have been developed to treat hyperglycaemia in T2DM (Table 1.1) (Artasensi et al., 2020). DM patients can also develop complications involving other organ systems such as the kidneys (nephropathy), eyes (retinopathy), nervous system (neuropathy), cardiovascular dysfunction, and diabetic foot complications including ulcers (Tan et al., 2019a, Ahmad, 2016).

Table 1.1: Broad classes of anti-hyperglycaemic agents used in T2DM and their mechanisms of action. Modified from Artasensi et al. (2020).

Drug Type	**Example**	**Mechanism of Action**
Insulin or Insulin analogues	Glargine	Restoration of glycaemic control through administration of exogenous insulin.
Biguanides	Metformin	Inhibits hepatic gluconeogenesis
Sulphonylureas	Gliclazide	Stimulates pancreatic insulin production
Thiazolidinediones	Pioglitazone	Improves peripheral sensitivity to insulin
Incretin-Based Drugs *GLP1a **DPP4i	 Semaglutide Vildagliptin	 Postprandial glucose-mediated insulin release

*Glucagon-like peptide-1 agonists

**Dipeptidyl peptidase-4 (DPP4) inhibitors

1.2 Diabetic Foot Ulcers and their Relationship to other diabetic complications

DFU is a chronic, recurrent, and costly complication for diabetic patients. DFU shows an increased prevalence in male (4.5%) compared to female (3.5%) patients and in T2DM (6.4%) than T1DM (5.5%) patients (Zhang et al., 2017). The global prevalence is 6.3%; however, the prevalence of DFU in Australia has been estimated to be 5.4% which is low when compared to other developed nations such as the United States of America (13%) or Belgium (16.6%) (Zhang et al., 2017, Ahmed et al., 2021). Of concern is the higher risk of developing diabetic foot complications, such as an ulcer or amputation, in Aboriginal and Torres Strait Islander Australians compared to Non-Indigenous Australians; which can be three to six times more likely in Aboriginal and Torres Strait Islander Australians (West et al., 2017, Steffen and O'Rourke, 1998, O'Rourke et al., 2013, Ewald et al., 2001). Diabetic foot disease, which incorporates DFU, has been estimated to cost annually $1.6 billion dollars in Australia (Lazzarini et al., 2018). Moreover, DFU history is associated with more

complications compared to patients with no-ulcer history (Zhang et al., 2017, Iversen et al., 2009). The risk of DFU development in DM patients ranges from 15-34%, with a reoccurrence rate of 40% within one year and 65% within five years (Reiber, 1996, Armstrong et al., 2017). Amputation rates of the lower extremities for DFU patients can vary from 3.6% to 47.7% (Moulik et al., 2003, Costa et al., 2017, Bondor et al., 2016, Jeffcoate and Harding, 2003). Additionally, all-cause mortality is increased 1.8-2.5 times in DFU compared to non-ulcerated DM; an effect that may be due to an increase in cardiovascular disease burden (Brownrigg et al., 2012, Saluja et al., 2020). Diabetic neuropathy and peripheral arterial disease (PAD), combined with foot injury, increased weight bearing, and deformities, can initiate the formation of DFU (Primadhi and Herman, 2021, Bandyk, 2018). Diabetic peripheral neuropathy leads to anatomical changes in the foot, such as hammer toe deformities or Charcot's neuropathic osteoarthropathy (also known as rocker bottom foot), through loss of nervous function with subsequent muscular atrophy and dysfunction (Rogers et al., 2011, Bandyk, 2018, Holmes and Hastings, 2021, Primadhi and Herman, 2021). In addition, high pressure zones on the bottom of the foot and callus formation are precipitated by this widespread nervous dysfunction (Boulton et al., 2004). Improper skincare and trauma can lead to infection, which can infiltrate deep foot structures, resulting in amputation of the affected limb (Bandyk, 2018). DFU is currently managed using surgical debridement, offloading, appropriate wound dressing, assessment of underlying vasculature, controlling blood sugar levels, and infection control (Everett and Mathioudakis, 2018). Considering the rates of DFU reoccurrence and the increased risk of death associated with DFU, suggests that the current treatment methods are ineffective. These observations indicate a better understanding of DFU pathology is needed to treat the condition effectively.

1.3 Growth Factors and their role in wound healing

DFU can be defined as ulcers or abrasions that involve epithelial loss, sometimes incorporating deeper layers of tissues, muscle and bone (Zubair and Ahmad, 2019). Wound healing, a complex process that is initiated in response to tissue trauma, consists of cellular and molecular pathways involved in haemostasis, inflammation, proliferation, and

remodelling (Jimi et al., 2020, Rodrigues et al., 2019). Abnormalities in haemostasis, inflammation, cellular proliferation, and remodelling have been observed in diabetic patients (Fattah et al., 2004, Li et al., 2021, Tsalamandris et al., 2019, Garcia et al., 2010, Loots et al., 1999, Usui et al., 2008, McLennan et al., 2006). Moreover, associated cellular and molecular processes are altered in wound healing in DM, one of which is levels of growth factors (Zubair and Ahmad, 2019, Brem and Tomic-Canic, 2007). Wound healing involves growth factors from several different pathways and levels of said growth factors, such as TGF-β, are increased in acute wound healing (Barrientos et al., 2008, Ramirez et al., 2014). In chronic wounds, such as DFU, the levels of the growth factors are decreased (Barrientos et al., 2008, Zubair and Ahmad, 2019). Dysregulation of growth factor levels has been seen in T1DM and T2DM, with and without diabetic complications (Ambinathan et al., 2021, Zhang et al., 2020a). Growth factors offer a potential therapeutic target for DFU pathology. Therapies used in the treatment of DFU, such as hyperbaric oxygen, and negative pressure wound therapy have been shown to improve expression of growth factors and their receptors *in vitro* and *in vivo* (Huang et al., 2020, Yang et al., 2017, Dhamodharan et al., 2019). Topical application of growth factors has also seen success in treating DFU, accelerating healing and lowering amputation rates (Gilligan et al., 2015, Park et al., 2018, Sridharan and Sivaramakrishnan, 2018). Growth factors also participate in a majority of the wound resolution processes that are altered in chronic wounds (Barrientos et al., 2008); therefore the targeting growth factors in DFU resolution is a viable concept.

1.4 Transforming growth factor-beta (TGF-β) family of growth factors in diabetic complications.

The transforming growth factor-β (TGF-β) family of growth factors participate in the onset of diabetic complications including DFU. Composed of over 40 members, signalling by the TGF-β family contributes to cell proliferation, recognition, apoptosis, differentiation, and specification in mature and embryonic tissues (Brown and Schneyer, 2010, Shi and Massagué, 2003). A simplified model of the signalling pathways involved is outlined in Figure 1.1. Members of the TGF-β family include the bone morphogenetic proteins (BMPs), activins, myostatin, growth and differentiation factors, glial-derived neurotrophic factor,

anti-müllerian hormone (also known as müllerian inhibitory substance), lefty, and nodal (Brown and Schneyer, 2021, Worby et al., 1996, Cate et al., 1986, Chen and Shen, 2004). At the level of the pancreas, this superfamily is involved in growth, cell specialisation, and development (Oliver-Krasinski and Stoffers, 2008). Furthermore, these growth factors have a physiological role in glucose homeostasis including regulation of fat, insulin sensitivity and signalling, and glucose tolerance (Brown and Schneyer, 2021, Brown and Schneyer, 2010).

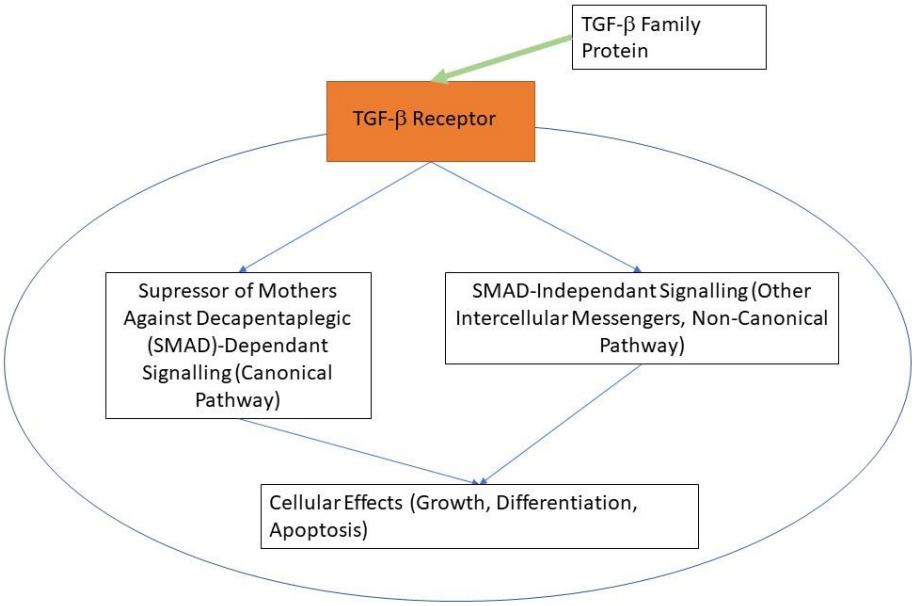

Figure 1.1: A simplified model of the TGF-β signalling pathways. TGF-β or TGF-β group members bind to Type 1 and Type 2 receptors with certain members, such as BMP-2,-10, Inhibin A and Myostatin B, interacting with the BMP receptors and Type 2 Activin receptor (Sorensen and van Berlo, 2020). Two intracellular pathways are activated in response to receptor-ligand binding, the canonical and non-canonical pathways (Finnson et al., 2020). SMADs 2 and 3 are the principal signal transducers in the canonical pathway (Sorensen and van Berlo, 2020). Signalling via the non-canonical pathway involves SMADs 1 and 5, in addition to other secondary messenger pathways such as ERK, JNK, p38, PI3K-Akt, and JAK2/STAT3 (Finnson et al., 2020). These other secondary messengers can further interact with SMAD signalling to either promote or diminish its effects (Finnson et al., 2020).

Medication used in the treatment of DM has also been observed to interact with the TGF-β family, with DPP-4 inhibitor/metformin combination having the lowest basal level of active TGF-β in DM patients (Pscherer et al., 2013). Exenatide, a glucagon-like peptide-1 (GLP-1) mimetic, was shown to affect TGF-β1 secretion in humans (Zhang et al., 2012).

Members of the TGF-β family also contribute to the development of diabetic complications such as fibrosis, retinopathy, nephropathy, and neuropathy; both experimentally and in patients (Tuleta and Frangogiannis, 2021, Bonfiglio et al., 2020, Perez-Gomez et al., 2021, Hussain et al., 2016, Ybarra et al., 2010, Iwano et al., 1996, Perera et al., 2020, Herman-Edelstein et al., 2011, John and Yadla, 2019, Bian et al., 2019, Chung et al., 2021). Other members of this growth factor family, such as BMP-7, can be beneficial in such conditions. BMP-7 plays a role in the development of insulin secreting cells and insulin sensitivity (Casana et al., 2021, Ghani et al., 2020, Cun et al., 2021, Chattopadhyay et al., 2017). In addition to possessing anti-inflammatory and anti-fibrotic properties, BMP-7 can antagonise TGF-β (Kim et al., 2020, Aluganti Narasimhulu and Singla, 2020). Human studies show that there is a loss of BMP-7 in diabetic nephropathy patients (Korbut et al., 2023, John and Yadla, 2019, Wong et al., 2013). Several DM mediated pathologies including nephropathy, bone degeneration, sarcopenia, and cardiomyopathy respond favourably to BMP-7 treatment (Peng et al., 2022, Yu et al., 2023, Liu et al., 2019, Narasimhulu and Singla, 2023, Ouyang et al., 2022, Xiao et al., 2022, Tate et al., 2021, Han et al., 2022). Currently, there is no known effect of BMP-7 in DFU.

The literature shows that TGF-β is involved in DFU pathology. Serum TGF-β1 levels were increased in a South Indian population of DFU patients compared to non-ulcerated T2DM patients, this difference was more pronounced with DFU patients with chronic kidney disease (Smina et al., 2019). DFU-derived fibroblasts responded abnormally to TGF-β stimulation *in vitro*, failing to produce fibronectin, an essential protein involved in wound healing (Maione et al., 2016). Conversely, enhanced levels of TGF-β1 and the type 1 TGF-β receptor (TGF-βR1) were increased in keratinocytes isolated from DFU margins (Galkowska et al., 2006). Earlier work found a difference in isoform levels, with TGF-β1 being expressed less in DFU than TGF-β2 and TGF-β3 (Jude et al., 2002). However, TGF-β2 and TGF-β3 levels were increased in DFU compared to controls, but TGF-β1 level were decreased in DFU (Jude et al., 2002). Expression of TGF-βR1 and the type 2 receptor (TGF-βR2) were reduced in DFU compared to normal skin (Jude et al., 2002, Jude et al., 1999). Unhealed DFU expressed lower levels of TGF-β in wound exudate compared to healed DFU (Liu et al., 2009). DFU treatments, such as continuous oxygen diffusion, negative pressure wound therapy, maggot debridement therapy and ozone therapy, have been shown to increase TGF-β gene, mRNA,

and protein expression in DFU patients (Lavery et al., 2020, Yang et al., 2017, Karam et al., 2018, Zhang et al., 2014, Zhang et al., 2022a). Experimental therapies that ameliorate DFU, such angiotensin II inhibition, flavonoids, plant alkaloids, cold atmospheric plasma, and argon, have been shown to enhance TGF-β signalling in rodent models (El-Salamouni et al., 2021, Mao et al., 2021, Sun et al., 2020, Li et al., 2020, Kandhare et al., 2014, He et al., 2020, Ning et al., 2019, Tan et al., 2019b, Li et al., 2018). The literature indicates a role for the TGF-β family in diabetic pathologies, including DFU, particularly a substantial role for TGF-β. As previously mentioned, DFU is a secondary pathology to PAD, diabetic neuropathy, and impaired wound healing. The TGF-β family has been shown to participate in these primary pathologies.

1.5 TGF-β family in Peripheral Arterial Disease

PAD is a common vascular comorbidity in DFU. Approximately 50% of DFU patients are comorbid with PAD, which difficult to diagnose due to the presence of other comorbidities (Morbach et al., 2012, Prompers et al., 2007, Fejfarová et al., 2021). Additionally, comorbid PAD and DM results in prolonged hospitalisation, critical lower limb ischaemia, and increased cardiovascular and cerebrovascular diseases (extensively reviewed by Thiruvoipati et al. (2015). As PAD is DFU aggravating pathology, there could be a role for the TGF-β family in PAD. Serum levels of TGF-β1 did not differ between patients with PAD and patients with normal vasculature, however, serum TGF-β was associated with 40% increased risk of PAD development (Agarwal et al., 2015, McDermott et al., 2005). Moreover, plasma TGF-β levels were increased in PAD patients (Corrêa et al., 2011) suggesting that more recent studies have developed better detection methods. Short and long term increases in serum levels of TGF-β1, post intervention, was observed in PAD patients who developed restenosis (Wildgruber et al., 2007). At the cellular level, restenotic PAD plaques showed increased levels of TGF-β and associated signalling molecules (Krishnan et al., 2016, Edlin et al., 2009). Additionally, TGF-β1 and TGF-β receptor type 2 levels were increased in the skeletal and vascular smooth muscle of PAD patients (Ismaeel et al., 2022, Ha et al., 2016). TGF-β1 levels in the vascular smooth muscle was correlated to collagen deposition in the gastrocnemius of PAD patients (Ha et al., 2016). Endothelial cell derived Platelet-Derived Growth Factor

(PDGF) and Connective Tissue Growth Factor (CTGF) increased TGF-β1 and TGF-β signalling in smooth muscle cells (Ismaeel et al., 2022). This research shows that TGF-β and associated signalling molecules participate in PAD pathology particularly restenotic plaques. It is unknown if this increase in TGF-β signalling is observed DFU with PAD comorbidity or if it an attempt to re-establish vascular homeostasis using pro-angiogenic growth factors such as PDGF and CTGF.

1.6 TGF-β family in Diabetic Neuropathy and associated complications

Diabetic neuropathy (DN) presents a problem for DFU prevention. The prevalence of DN has been estimated to be anywhere from 7-50%, depending on the number of years since diabetes has been diagnosed (Yagihashi et al., 2011, Yang et al., 2020a). However, if subclinical cases are considered, the prevalence of DN could exceed 90% (Yagihashi et al., 2011). Asymptomatic presentation occurs in nearly 50% of diabetic neuropathy cases, contributing to pedal injuries through failing to implement appropriate footcare (Draznin et al., 2022). Furthermore, the anatomical changes observed in DFU are driven by neuropathy associated muscle atrophy (Bandyk, 2018). TGF-β has a complex role in nerve function and development (Skundric and Lisak, 2003). Recently, decreased serum TGF-β levels were reported in an Emirati cohort of DN patients (Mussa et al., 2021). However, total serum TGF-β1 levels were higher in Spanish patients with diabetic neuropathy (Ybarra et al., 2010). It is unknown if this inconsistency is due to cohort differences or laboratory protocols. The role of TGF-β in animal models of diabetes is more consistent with elevated levels of TGF-β and decreased levels of SMAD-7, an inhibitor of TGF-β signalling, in nervous tissues being reported in several studies (Anjaneyulu et al., 2008, El-Sawaf et al., 2021, Calabrese et al., 2014, Cho et al., 2014). In addition, TGF-β1 and TGF-β2 isoforms were also shown to exacerbate neuronal injury under high glucose conditions (Anjaneyulu et al., 2008). All TGF-β isoforms inhibited neurite outgrowth, however, this effect was greater with TGF-β3 (Anjaneyulu et al., 2008). Additionally, TGF-β1 has been shown to induce muscular atrophy through the generation of reactive oxygen species (ROS) (Abrigo et al., 2016). Ameliorated muscular atrophy observed in treated type 1 diabetic mice was partially due to suppression of TGF-β1 signalling (Guo et al., 2019). Human studies have been inconsistent

around the levels of TGF-β family members in DN. Nervous tissues isolated from animal models have shown increases in TGF-β and decreased negative regulators of TGF-β signalling. Moreover, these animal models have partially revealed the mechanisms of how TGF-β can affect nervous tissue and subsequent muscle atrophy.

1.7 Summary

Diabetes and its associated complications are medical issues of global concern, due to the number of patients and associated costs. Treatment resistance, increased co-morbidity and mortality, and high rates of reoccurrence indicate that DFU is a diabetic complication that requires better understanding and potential therapies. Growth factors and their signalling can become dysfunctional in diabetes, which makes them a suitable target for investigation. One such growth factor family is TGF-β, which consists of over 40 members. Pancreatic growth and differentiation in addition to insulin signalling and function are driven in part by this family of growth factors. Furthermore, changes in TGF-β family signalling has been observed in diabetes or diabetic complications. Other members of the TGF-β family have not been studied in DFU, however, there is a body of work that has investigated the titular growth factor in DFU. Figure 1.2 summarises the role of TGF-β in DFU precipitating pathologies. Circulating levels of TGF-β are elevated in DFU patients, with TGF-β signalling being altered in DFU compared to controls. Conversely, response to treatment was consistently associated with increased TGF-β, indicating that signalling by the said growth factor is required for DFU recovery. It is currently unknown if other members of the TGF-β family participate in DFU pathology or recovery.

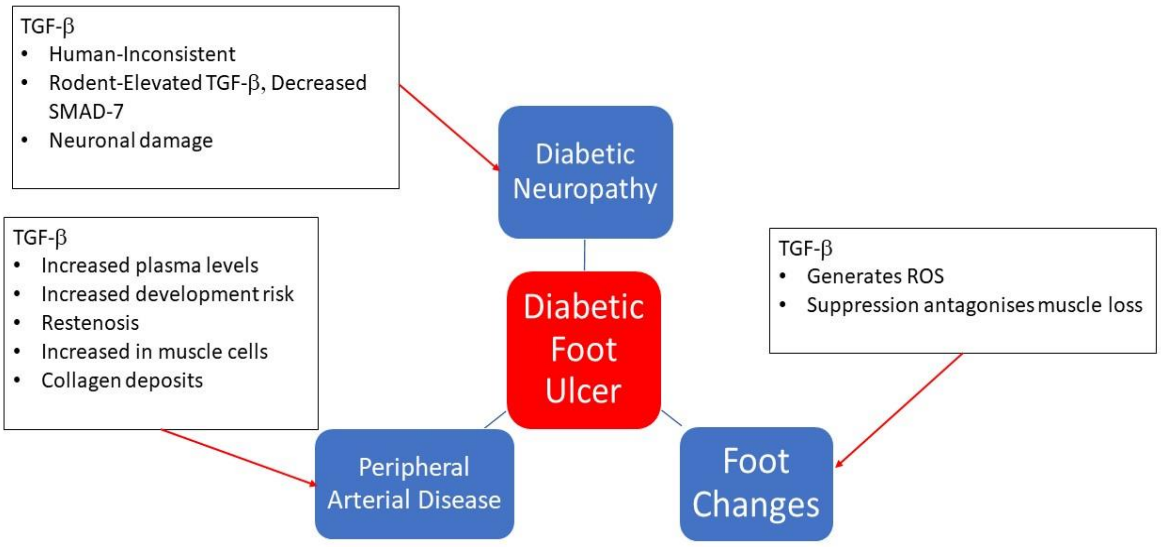

Figure 1.2: The role of TGF-β in DFU causal pathologies. Animal model of diabetic nerve damage show increased levels of TGF-β which further damages nerves. PAD studies show increased levels of TGF-β in circulation and in tissues surrounding affected vessels. This increased TGF-β increases the risk of PAD development and increased collagen deposition in the gastrocnemius. TGF-β is a ROS generator which drives muscle wastage, an end effect of DN and partial cause of foot deformities in diabetes. Partial suppression of TGF-β leads to reduced muscle atrophy in diabetic animal models.

1.8 Thesis Questions

In a clinical setting, preventing DFU formation by treating the underlying DM and associated complications would be ideal. The current treatment regime for DFU does manage the condition, however, the high rates of reoccurrence and associated mortality indicate a better understanding of the underlying pathophysiology is required. Growth factor signalling, along with other cellular processes, are disturbed in the diabetic state. The function of the TGF-β superfamily of growth factors, proteins which regulate insulin signalling and pancreas function, are altered in several diabetic complications including DFU. The titular growth factor has been studied in DFU and its precipitating pathologies, revealing that TGF-β signalling drives the formation of DFU and possible recovery of DFU. It is

currently unknown if other members of the TGF-β family, such as BMP-7, are changed in response to DFU.

From the evidence above, it could be hypothesised that there is a negative change in TGF-β family signalling observed in DFU which is corrected with intervention. The following work aims to determine the levels of TGF-β1 and BMP-7, a natural antagonist of TGF-β1 and member of the TGF-β growth factor family, in the serum of DFU patients. Moreover, by measuring the changes in these growth factors in recovered and active DFU, a possible role for these growth factors in DFU recovery may be elucidated. A secondary aim of this work is to investigate the relationship between TGF-β1, BMP-7, and various biochemical and physiological measures associated with DFU and DM. Additionally, relationships between ulcer ratio, HbA1c ratio and growth factors will be investigated to determine if the is a correlation between growth factors and ulcer healing or disease response.

CHAPTER 2: SYSTEMATIC REVIEW

2.1 Abstract

Background: The TGF-β family of growth factors has been shown to participate in diabetic pathology including diabetic foot ulcer (DFU). However, there is no research to determine if the TGF-β growth factor family has value in DFU prognostics or as a therapeutic target in human DFU.

Objective: The aim of this systematic review was to review the current literature to examine the role of the TGF-β family in DFU, both as a prognostic or therapeutic marker.

Method: Databases (PubMed, Medline, Scopus, and Web of Science) were searched for studies that investigated TGF-β family members in human DFU. Sixteen records were included for study. Due to the overall heterogeneity of the study data, no meta-analysis was performed.

Results: Biochemical studies showed a perturbation in TGF-β1, -β2, and -β3 signalling in DFU patients. Growth and Differentiation Factor-15 (GDF-15) was increased in DFU patients compared to controls. One population study found increased circulating TGF-β1 in DFU patients compared to DM patients. Therapeutic intervention studies revealed an increase in TGF-β1 in response to interventions compared to standard care.

Conclusion: These studies indicate a strong role for TGF-β family signalling in DFU, particularly in pathology formation and recovery. However, the small sample sizes and cohort ethnicity potentially limit the application of data obtained from the trials. Larger, ethnically heterogenous, trials are needed to determine the applicability of the TGF-β family in DFU therapy and management.

2.2 Introduction

DFU is one of the top 10 causes of disability globally, especially when combined with other diabetes mellitus (DM) related lower extremity complications, such as diabetic neuropathy and amputation (Zhang et al., 2020b). The global prevalence of DFU is 6.3%, with DFU being more common in male patients and Type 2 DM (Zhang et al., 2017). The risk of developing DFU has been estimated to be between 15%-34% for patients, with a reoccurence rate of

40% within one year and 65% within five years (Reiber, 1996, Armstrong et al., 2017). DFU is co-morbid with more medical complications, and is more likely to cause death than non-ulcerated DM (Zhang et al., 2017, Brownrigg et al., 2012, Saluja et al., 2020, Iversen et al., 2009). Currently, treatment of DFU involves a multifactorial approach of wound debridement, wound off-loading, correct wound dressing, vascular assessment, diabetes control, and controlling infection where required (Everett and Mathioudakis, 2018). DFU is a life threatening and recurrent pathology, and given the rates of DFU reoccurrence with current treatment regimes, a better understanding of the cellular mechanisms involved with DFU are required.

Haemostasis, inflammation, proliferation, and remodelling are the key pathways involved in wound healing; deviation in these molecular pathways are observed in DM (Jimi et al., 2020, Rodrigues et al., 2019, Zubair and Ahmad, 2019). Growth factors, compounds that control the growth and differentiation of cells, participate in wound healing by interacting with cellular receptors; perturbation of growth factor function has been reported in DM (Stone et al., 2022, Steed, 1997, Ambinathan et al., 2021). Wound healing involves growth factors from several different pathways and levels of said growth factors are increased in acute wound healing (Barrientos et al., 2008). Conversely, the levels of the growth factors are decreased in chronic wounds, such as DFU (Barrientos et al., 2008, Zubair and Ahmad, 2019). Key aspects of wound healing are regulated by all three isoforms of transforming growth factor-β (TGF-β1,2,3) (Ramirez et al., 2014, Barrientos et al., 2008). Conversely, changes in signalling by TGF-β and its associated family of growth factors has been implicated in a number of diabetic complications (Tuleta and Frangogiannis, 2021, Bonfiglio et al., 2020, Perez-Gomez et al., 2021, Hussain et al., 2016, Ybarra et al., 2010, Iwano et al., 1996, Perera et al., 2020, Herman-Edelstein et al., 2011, John and Yadla, 2019, Bian et al., 2019, Chung et al., 2021). TGF-β has been studied to some extent in DFU pathology, revealing that serum TGF-β is increased; however wound exudate levels of TGF-β were decreased in unhealed DFU (Smina et al., 2019, Liu et al., 2009). Furthermore changes in both TGF-β protein and receptor expression, in addition to changes in molecular signalling, have also been observed in DFU (Galkowska et al., 2006, Jude et al., 2002, Jude et al., 1999).

However, therapies targeting DFU increase TGF-β gene, mRNA, and protein levels (Lavery et al., 2020, Yang et al., 2017, Karam et al., 2018, Zhang et al., 2014).

DFU is a chronic and recurrent health problem that can cause disability and increased mortality in diabetic patients, indicating that the underlying wound pathology is poorly understood. Wound healing is partially driven by growth factor effects across an number of molecular pathways, which are altered in the diabetic state. Wound healing and recovery is managed by TGF-β isoforms. TGF-β levels and signalling are altered in DFU patients, and TGF-β levels are increased by therapies that address DFU. This points to TGF-β, or the family of growth factors, being a protein of interest as either a therapeutic target or risk biomarker. This systematic review aims to look at available human studies to determine the value of TGF-β and the associated family of growth factors as a target in DFU intervention.

2.3 Methods

2.3.1 Eligibility

Inclusion criteria was for therapeutic studies of human DFU studies where TGF-β was measured at least twice. Other studies were included that compared DFU to a control or another group and where TGF-β was measured at least twice. Exclusion criteria were Non-English language, animal studies, human studies where TGF-β was only measured once.

2.3.2 Information Sources and Search Strategy

Four databases (Medline, Pubmed, Scopus, and Web of Science) were searched to source literature for this review. Databases were accessed from 28/2/2023 to the 17/3/2023. The search terms used were TGF-beta, TGF-beta1, TGF-beta2, TGF-beta3, Transforming Growth Factor-Beta, BMP, Bone Morphogenetic Protein, Activin, Activin A, Activin B, MSTN, Myostatin, Nodal, Anti-Mullerian Hormone, Lefty, and DFU or diabetic foot ulcer.

2.3.3 Selection Process

Individual database results were downloaded into EndNote (version 9.3.3) where duplicates were removed. These duplicate-free results were then combined, and subsequent duplicates were deleted. Titles and abstracts from this new list were then scrutinised according to the eligibility criteria mentioned in section 2.1. This process is outlined in Figure 2.3.1. This method was completed by one author independently and studies generated were scrutinised by two other authors.

2.3.4 Data Collection

A table outlining key study characteristics was used to extract data by one author. These key characteristics were: Country, Study Year, Study Category, Study Type, Study Duration, Sample Size, Intervention, Control/s, TGF-β measured/method, and Endpoints. Study category classifies the selected studies into broad categories: biochemical study, population study, or therapeutic intervention. Study design describes if the studies fit into a cross sectional, cohort, case-control, or randomised control trial. The category of TGF-β family measured/ method describes what kind of TGF-β was measured (mRNA, gene, or Protein) and how the TGF-β levels were measured. This table was then sent to two other authors to verify that the extracted data was correct.

2.3.5 Study Risk of Bias Assessment

The Appraisal tool for Cross-Sectional Studies (AXIS) tool was used to assess the risk of bias within the selected studies (Downes et al., 2016). A table was developed from the AXIS tool to record the answers to the AXIS questionnaire (Table 2.2). The included studies were assessed according to this table by one author and verified by two other authors.

2.3.6 Synthesis Methodology

The Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) checklist was used in the synthesis of this systematic review (Page et al., 2021). A qualitative

assessment of the included studies is mentioned in section 2.4 and is tabulated in the Results section (Table 2.1).

2.4 Results

2.4.1 Study Selection

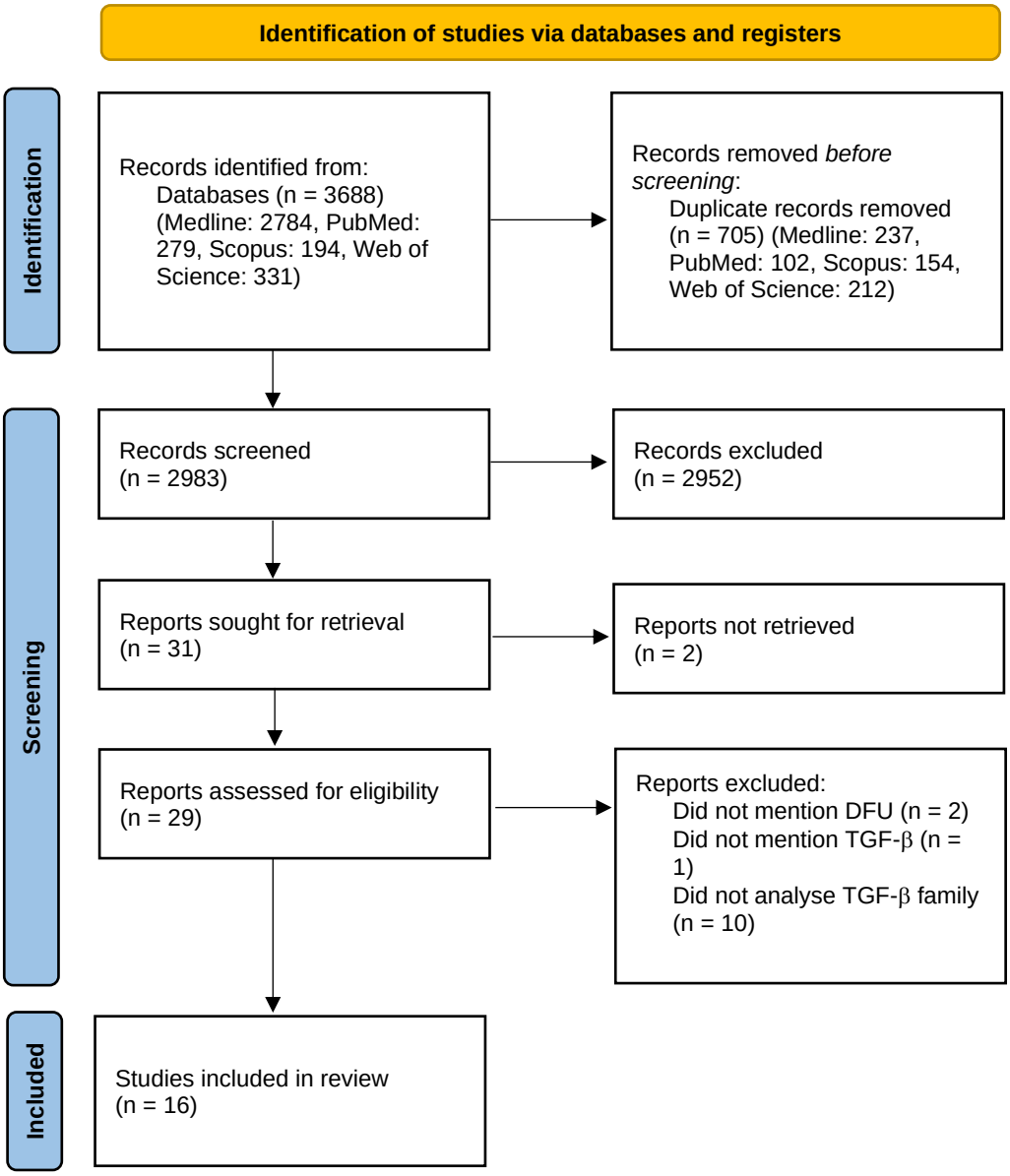

Figure 2.1: PRISMA Flowchart of the process used to isolate the studies used in this review. Adapted from Page et al. (2021).

2.4.2 Study Characteristics

16 trials were included in the final analysis, and their key characteristics are described in Table 2.1. Seven out of the 16 studies were therapeutic intervention trials where TGF-β was analysed as a growth factor of interest, three of these studies were conducted in China. The other studies coming from Mexico, Egypt, Turkey, and the United States of America. One population study involved TGF-β, this study investigated the development of kidney disease pre- and post- DFU onset in India. The remaining nine studies were laboratory studies that investigated TGF-β in DFU. Three of these laboratory studies came from the United Kingdom, two from England and one from Wales, two studies were from Poland, one study was from the United States of America, one study was from Turkey, two studies originated from China and one study was of Australian origin.

2.4.3 Risk of Bias

Risk of bias assessment on the included was done by using the AXIS tool and the results were collected in Table 2.2 (Downes et al., 2016). A lack of sample size justification was observed across all of the included studies. Additionally, study limitations were not discussed in seven out of the 16 studies. Conflicts of interest or funding sources were not reported in two studies. Basic patient data was not listed in one study.

2.4.4 Synthesis of Results

Biochemical Investigations

Nine of the 16 studies investigated changes in TGF-β biochemistry in relation to DFU. Two studies investigated changes in TGF-β isoform and TGF-β receptor expression in DFU skin compared to diabetic and normal skin (Jude et al., 2002, Jude et al., 1999). Immunohistochemistry (IHC) and immunofluorescence (IF) found that there was a decreased expression of TGF-β1 protein in DFU skin sections when compared diabetic and normal samples, this decreased expression was significant when compared to TGF-β2 and TGF-β3 expression in DFU ($P < 0.001$) (Jude et al., 2002, Jude et al., 1999). Western blot

studies confirmed this decreased expression of TGF-β1 (Jude et al., 2002, Jude et al., 1999). Additionally, IF revealed an increased TGF-β2 and TGF-β3 protein expression in DFU that was significant when compared to normal and diabetic skin ($P < 0.05$) (Jude et al., 2002). The increased expression of TGF-β2 and TGF-β3 was also observed with IHC and Western blots (Jude et al., 2002). IHC showed decreased expression of TGF-β receptor type I and the type II in DFU skin (Jude et al., 2002). A significant decrease in IHC staining for both TGF-β1 and TGF-βRI was observed in both the suprabasal and basal keratinocytes of DFU patients ($p < 0.05$ compared to non-diabetic controls) (Galkowska et al., 2006). Vascular endothelium expressed decreased TGF-β1 intensity but increased TGF-βRI intensity in DFU samples ($p < 0.05$ compared to non-diabetic controls) (Galkowska et al., 2006). Another study found an increased expression of TGF-β1 mRNA in the serum and dorsalis pedis arteries of DFU patients compared to controls ($P < 0.01$)(Zhang et al., 2016). Conversely, decreased TGF-β1 mRNA was observed in the ulcerated muscles compared to controls ($p < 0.01$) (Zhang et al., 2016). The expression of TGF-β1 protein followed the same trend in DFU patients compared to controls ($p < 0.05$ for serum samples, $p < 0.01$ for arterial and muscle samples) (Zhang et al., 2016). Work by Xu et al. (2019) found an increase in relative expression of TGF-β mRNA in DFU compared to control, but this was not significant; there were no significant changes in TGF-β expression with Western blot analysis. Wound exudate concentrations of TGF-β1 protein were significantly ($P < 0.01$) decreased on clinic presentation in DFU patients who did not recover after 12 weeks compared to patients who healed in the same time period (Liu et al., 2009). Serum concentration of TGF-β1, -β2, and -β3 were observed to be significantly lower in DFU patients compared to non-ulcerated controls ($p = 0.005$, 0.035, and 0.012 for β1, β2, and β3 isoforms, respectively) (Kupczyk et al., 2021). Growth differentiation factor-15 (GDF-15), a member of the TGF-β family, was significantly ($p < 0.001$) elevated in DFU patients compared to DM or healthy controls (Sendur et al., 2022). Moreover, increasing ulcer severity was associated with increased serum GDF-15 ($p = 0.006$) (Sendur et al., 2022). Expression of the TGF-β1 gene was increased in T-cells isolated from patients where DFU that failed to heal compared to responsive ulcers (Theocharidis et al., 2022). These studies point to a change in the expression of TGF-β in DFU, particularly TGF-β1. There has been a consistent decrease in the expression of TGF-β1 protein and mRNA in DFU which may be localised to the ulcer area. Furthermore, protein expression of TGF-β2

and TGF-β3 is increased and the type I and II receptors are decreased in DFU. One study has shown that GDF-15, a member of the TGF-β family, was increased in DFU and was associated with increased ulcer severity. There appears to be evidence that TGF-β1 may be predictive for DFU recovery or required as a part of said recovery. Additionally, new evidence suggests that other members of the TGF-β family may participate in DFU pathology.

Population Studies

One study investigated TGF-β in larger populations of DFU patients. An ambispective study was conducted on a South Indian population of Type 2 DM (T2DM), diabetic kidney disease (CKD), DFU, and DFU with co-morbid diabetic kidney disease (CKDDFU) (Smina et al., 2019). Serum concentrations of TGF-β1 were increased in DFU, CKD, and CKDDFU when compared to T2DM patients ($p < 0.001$ for CKD, DFU, CKDDFU compared to T2DM, $p < 0.001$ for CKDDFU versus CKD, $P<0.001$ for CKDDFU compared to DFU) (Smina et al., 2019). This study shows that there is a change in TGF-β signalling that is observable in some populations.

Therapeutic Intervention

The remaining six studies addressed therapeutic intervention in DFU. Two of these studies investigated pharmaceuticals and the other studies investigated post operative care methods (Gasca-Lozano et al., 2017, Li et al., 2015, Zhang et al., 2014, Lavery et al., 2020, Karam et al., 2018). Pirfenidone (PFD), an anti-fibrogenic drug and extracellular matrix modulator, and modified diallyl disulfide oxide (M-DDO), a compound with germicidal properties, were tested in DFU (Gasca-Lozano et al., 2017). These agents was compared against ketanserin (KTS), a serotonin antagonist of the 5-hydroxytryptamine receptor type 2 (Gasca-Lozano et al., 2017). Mean relative ulcer volume, a marker of DFU healing, in addition to ulcer count was improved with the PFD+M-DDO compared to KTS (Gasca-Lozano et al., 2017). Gene expression of TGF-β1 and TGF-β3 was increased two months post treatment for both groups, however, gene expression was higher in PFD+M-DDO ($p < 0.05$ compared to baseline, $p < 0.05$ compared to two month KTS group value) (Gasca-Lozano et

al., 2017). Autologous platelet-rich gel was tested in another trial and both mRNA and IHC density for TGF-β1 increased in response to treatment (P = 0.004 for the mRNA, p = 0.0134 for protein) (Li et al., 2015). Advanced moist wound therapy acted as an control for negative pressure wound therapy in DFU treatment (Karam et al., 2018). Negative pressure wound therapy significantly (p = 0.0001) increased the mRNA of TGF-β1 at day 10 post treatment; an effect that was not seen with the advanced moist wound care (Karam et al., 2018). A significant (P < 0.001) improvement in the average dressing changes to 100% granulation tissue was also observed with negative pressure wound therapy (Karam et al., 2018). Negative pressure wound therapy significantly improved fibronectin and TGF-β1 mRNA and protein expression in DFU granulation tissue compared to control patients (p < 0.01 for both fibronectin and TGF-β1) (Yang et al., 2017). Continuous diffusion of oxygen, where oxygen is continously delivered through the dressing, and ozone therapy was trialled in DFU patients (Lavery et al., 2020, Zhang et al., 2014). TGF-β mRNA increased post treatment and remained elevated upto 3 weeks post treatment, however, there was no difference in mRNA expression between patients who responded to continous diffusion of oxygen therapy and non reponders (Lavery et al., 2020). Ozone therapy increased the protein levels of TGF-β in wound exudate and in tissue samples post treatment, which was more pronounced than the control treatment (Zhang et al., 2014). An increase in TGF-β mRNA and protein is consistently reported across all of the above mentioned studies. However, only a few of the studies used current standards of DFU treatment as a control which can obscure the value of the TGF-β as a therapeutic target.

Table 2.1: Characteristics of the studies included in the systematic review.

Reference	Country	Study Category	Study Design	Study Duration	Sample Size	Intervention	Control/s	TGF-β family measured/method	Endpoints
Jude et al. (1999)	England	Biochemical Investigation	Cross Sectional	N/A	58	N/A	Healthy skin, Diabetic, skin	Protein, Immunohistochemistry, Western blot	Expression of nitric oxide synthases, arginase, and TGF-β in DFU
Jude et al. (2002)	England	Biochemical Investigation	Cross Sectional	N/A	32	N/A	Normal skin, Diabetic skin, Chronic Venous Ulcer	Protein, Immunohistochemistry, Immunofluorescence Western blot	TGF-β expression in DFU and Chronic Venous Ulcer
Zhang et al. (2016)	China	Biochemical Investigation	Cross Sectional	N/A	41	N/A	Trauma Amputation	mRNA, Protein, RT-qPCR, Western blot	Expression of TGF-β1 and regulatory pathways in DFU
Xu et al. (2019)	China	Biochemical Investigation	Cross-Sectional	N/A	6	N/A	Trauma debridement	mRNA, Protein, RT-qPCR, Western blot	Long, non-coding RNAs and their regulation of the immune system in DFU.
Liu et al. (2009)	Australia	Biochemical Investigation	Cohort	3 months	56	N/A	Healed ulcers	Protein, ELISA	TGF-β1, MMP-9, MMP-2, and TIMP-1 in wound fluid from DFU
(Galkowska et al., 2006)	Poland	Biochemical Investigation	Cross Sectional	N/A	17	N/A	Non-diabetic orthopedic patients	Protein, IHC	TGF-β1 in DFU affected cells and tissues
(Kupczyk et al., 2021)	Poland	Biochemical Investigation	Cross Sectional	N/A	38	N/A	Healthy controls	Protein, ELISA	Cytokine and growth factor concentrations in serum
(Sendur et al., 2022)	Turkey	Biochemical Investigation	Cross Sectional	N/A	54	N/A	Healthy Controls, DM patients	Protein, ELISA	Relationship between Serum GDF-15 and DFU severity
(Theocharidis et al., 2022)	United States of America	Biochemical Investigation	Cross Sectional	3 Months	27	Surgery	Non-DM controls, DM patients	RNA, scRNASeq	Molecular differences of DFU healers versus non-healers

Table 2.1: Characteristics of the studies included in the systematic review continued.

Reference	Country	Study Category	Study Design	Study Duration	Sample Size	Intervention	Control/s	TGF-β family measured/method	Endpoints
Smina et al. (2019)	India	Population Study	Cohort	N/A	428	N/A	Type 2 Diabetes	Protein, ELISA	Levels of TGF-β1, CCN2 and CCN3 in DFU, Diabetics with chronic kidney disease, and DFU patients with chronic kidney disease
Gasca-Lozano et al. (2017)	Mexico	Therapeutic Intervention	Randomized, controlled trial	6 months	37	Ketanserin, Pirfenidone + modified diallyl disulfide oxide (PFD+M-DDO)	PFD+M-DDO	mRNA, RT-PCR	Relative Ulcer Volume, Wound Healing, wound healing histopathology, molecular assessment of genes involved, safety, tolerability
Karam et al. (2018)	Egypt	Therapeutic Intervention	Cohort	Not Stated	40	Advanced moist wound care, Negative pressure wound therapy	Advanced Moist Wound Care	mRNA, RT-qPCR	100% granulation tissue, Levels of mRNA for wound healing proteins

Table 2.1: Characteristics of the studies included in the systematic review continued.

Reference	Country	Study Category	Study Design	Study Duration	Sample Size	Intervention	Control/s	TGF-β family measured/method	Endpoints
Lavery et al. (2020)	United States of America	Therapeutic Intervention	Cross-Sectional	3 weeks	23	Continuous Diffusion of Oxygen	Non-healers	mRNA, qPCR	Percent wound area reduction (50% considered healed), mRNA of growth factors and cytokines.
Li et al. (2015)	China	Therapeutic Intervention	Cohort	12 weeks	25	Autologous Platelet-Rich Gel	Pre intervention	mRNA, Protein, IHC, q RT-PCR	Wound healing, mRNA and protein levels of signalling molecules involved
Zhang et al. (2014)	China	Therapeutic Intervention	Cohort	20 Days	50	Oxygen-Ozone Therapy	Surgical Debridement and wound care.	Protein, IHC, ELISA	Wound Healing, Levels of growth factors associated with healing
(Yang et al., 2017)	China	Therapeutic Intervention	Cohort	7 days	40	Negative Pressure Wound Therapy	Advanced Moist Wound Therapy	Protein, IHC, Western Blot, mRNA, RT-PCR	NPWT induced fibronectin production and expression of TGF-β1 in granulation tissue

Table 2.2: Critical assessment answers used to determine study bias. Adapted from Downes et al. (2016).

Assessment Criteria	(Jude et al., 1999)	(Jude et al., 2002)	(Zhang et al., 2016)	(Xu et al., 2019)	(Liu et al., 2009)	(Galkowska et al., 2006)	(Kupczyk et al., 2021)	(Sendur et al., 2022)	(Theocharidis et al., 2022)
Aims/Objectives	Y	Y	Y	Y	Y	Y	Y	Y	Y
Study Design	Y	Y	Y	Y	Y	Y	Y	Y	Y
Sample Size Justification	Not Given	Not Given	Not Given	Not Given	Not Given	Not Given	Not Given	Not Given	Not Given
Target population	Y	Y	Y	Y	Y	Y	Y	Y	Y
Sample frame	Y	Y	Y	Y	Y	Y	Y	Y	Y
Selection Process	Not given	Not given	Y	Y	Y	Y	Y	Y	Y
Non-responders	N/A	N/A	N/A	N/A	Y	N/A	N/A	N/A	Y
Risk Factors and Outcomes	Y	Y	Y	Y	Y	Y	Y	Y	Y
Risk factor Measurement	Y	Y	Y	Y	Y	Y	Y	Y	Y
Statistical Significance	Y	Y	Y	Y	Y	Y	Y	Y	Y
Methodology	Y	Y	Y	Y	Y	Y	Y	Y	Y
Basic Data description	Y	Y	Minimal	Minimal	Y	Y	Minimal	Y	Y
Response Rate	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Non-responders described	N/A	N/A	N/A	N/A	Y	N/A	N/A	N/A	Y
Results	N/A	N/A	N/A	Partial	Y	N/A	Y	N/A	Y
Results for all analyses	Y	Y	Y	Y	Y	Y	Y	Y	Y
Discussion and Conclusion	Y	Y	Y	Y	Y	Y	Y	Y	Y
Limitations	N	N	N	Y	N	N	Y	Y	Y
Conflict of Interest/Funding	N	Y	N	Y	Y	Y	Y	Y	Y
Ethics	Y	Y	Y	Y	Y	Y	Y	Y	Y

Table 2.2: Critical assessment answers used to determine study bias continued. Adapted from Downes et al. (2016).

Assessment Criteria	(Smina et al., 2019)	(Gasca-Lozano et al., 2017)	(Karam et al., 2018)	(Lavery et al., 2020)	(Li et al., 2015)	(Zhang et al., 2014)	(Yang et al., 2017)
Aims/Objectives	Y	Y	Y	Y	Y	Y	Y
Study Design	Y	Y	Y	Y	Y	Y	Y
Sample Size Justification	Not Given	Not Given	Not Given	Not Given	Not Given	Not Given	Not Given
Target population	Y	Y	Y	Y	N	N	N
Sample frame	Y	Y	N	Y	Y	Y	Y
Selection Process	Y	Y	Y	Y	Y	Y	Y
Non-responders	N/A	Y	N/A	Y	Y	N/A	N/A
Risk Factors and Outcomes	Y	Y	Y	Y	Y	Y	Y
Risk factor Measurement	Y	Y	Y	Y	Y	Y	Y
Statistical Significance	Y	Y	Y	Y	Y	Y	Y
Methodology	Y	Y	Y	Y	Y	Y	Y
Basic Data description	Y	Y	Y	Minimal	N	Y	Y
Response Rate	N/A	N	N/A	N	N	Y	N/A
Non-responders described	N/A	Y	N/A	Y	Y	N/A	N/A
Results	N/A	Y	N/A	N	Y	N/A	Y
Results for all analyses	Y	Y	Y	Y	Y	Y	Y
Discussion and Conclusion	Y	Y	Y	Y	Y	Y	Y
Limitations	Y	N	Y	Y	Y	N	N
Conflict of interest/Funding	Y	Y	Y	Y	Y	Y	Y
Ethics	Y	Y	Y	Y	Y	Y	Y

2.5 Discussion

This review revealed that whilst there is little evidence of involvement for other members of the TGF-β family in DFU, there is a role for TGF-β in DFU particularly for recovery. Generally, TGF-β signalling is increased in response to therapeutic intervention for DFU (Gasca-Lozano et al., 2017, Karam et al., 2018, Li et al., 2015, Lavery et al., 2020, Zhang et al., 2014). The most consistent response is seen with TGF-β1. Decreases in TGF-β1 protein and mRNA are reported by a number of biochemical studies; therapeutic interventions resulted in an increase in TGF-β1 (Jude et al., 2002, Jude et al., 1999, Liu et al., 2009, Zhang et al., 2016, Karam et al., 2018, Li et al., 2015, Gasca-Lozano et al., 2017). Changes in isoform expression was also observed, TGF-β2 and TGF-β3 proteins was increased in DFU and the mRNA for TGF-β3 was increased in response to intervention (Jude et al., 2002, Gasca-Lozano et al., 2017). However, expression of the type I and type II TGF-β receptor was decreased in DFU (Jude et al., 2002). The selected studies did investigate TGF-β in DFU, in either a therapeutic or pathological scope, and found an overall negative change in TGF-β function in relation to DFU related to possible loss of receptors.

The studies analysed in this review show a dysfunction in TGF-β signalling which offers an attractive, yet problematic, clinical target. A number of growth factors including recombinant human epidermal growth factor (rhEGF) and platelet-rich plasma, a source of multiple growth factors, have shown efficacy and little side effects in the treatment of DFU according to a number of meta-analysis (Zhao et al., 2020, Yang et al., 2020b, Bui et al., 2019, Sridharan and Sivaramakrishnan, 2018, del Pino-Sedeño et al., 2019). The TGF-β isoforms are key mediators in wound healing, however, the TGF-β1 and TGF-β2 isoforms also exhibit pathological properties in diabetes (Ramirez et al., 2014, Barrientos et al., 2008, Du et al., 2017, Popescu et al., 2018, Wang et al., 2021, Conedera et al., 2021). The reviewed studies largely investigated TGF-β1 with only a few looking at the other two isoforms or receptor expression, future works should also probe the levels of TGF-β2, TGF-β3, or the TGF receptors to determine their role in DFU. Moreover, attempts to increase TGF-β1 through pharmaceutical interventions or exogenous TGF-β1 should focus on the ulcer area to reduce systemic complications. Conversely, the increase in TGF-β1 reported by some could be used as a prognostic marker for DFU development; assuming an increase in serum

TGF-β1 is consistent across populations and non-invasive sampling can be developed to quantify TGF-β1 (Smina et al., 2019, Xu et al., 2019, Zhang et al., 2016).

There are limitations in the currently reviewed studies. Firstly, several studies use homogenous, ethnic populations. Genetics vary between patient cohorts, as such, the changes in growth factor signalling observed in these studies may not apply to the wider DFU population (Huang et al., 2015). However, given the prevalence of DFU in these countries, these results may be more applicable to these populations (Zhang et al., 2017). Larger studies that incorporate multiple ethnicities may address whether these growth factor alterations are applicable to the DFU population as a whole.

Cohort size and cohort selection was also a limitation for a number of the included studies. Inaccurate and underpowered conclusions may be drawn from small cohort sizes, leading to either false positives or false negatives (Downes et al., 2016). Some of the studies also selected patients presenting to a hospital or clinic; this introduces a selection bias which can make interpreting the overall results more difficult. Larger cohort sizes and randomised selection may lead to more consistent outcomes in future work.

2.6 Conclusion

This review has found there is a change in TGF-β function in DFU, both at a pathological level and in response to intervention. Signalling by TGF-β, particularly TGF-β1, was decreased in DFU patients through a loss of gene, mRNA, and protein expression of TGF-β1 and corresponding receptors. This effect was antagonised with therapeutic interventions. Conversely, expression of the other TGF-β isoforms was increased in DFU; an effect that needs to be investigated further. Interpretation of these results should be done with caution. The overall heterogenous nature of the studies and small number of included studies reduces the applicability of the results. Furthermore, the selection of ethnic cohorts and the size and selection of cohort potentially interferes with the interpretation of the results from the current studies. Larger studies that incorporate multiple nationalities may prove useful in determining if TGF-β participates DFU in the broader population or if it only applies to select ethnicities.

CHAPTER 3: METHODOLOGY

3.1 Patient samples

Twenty matched serum samples (10 patients sampled at zero and 12 weeks) were selected from the Galvus Anti-Inflammatory Effects in Diabetic Foot Ulcer (GIED) study to conduct the current study. The GIED study was a prospective, randomized, double blind, placebo controlled, single center study to determine the wound healing effects of Galvus (Vildagliptin) (Vangaveti et al., 2022). The study was conducted in accordance with CPMP/GCP/135/95 (Note for Guidance on Good Clinical Practice), CPMP/GCP/135/95 (Note for Guidance on Good Clinical Practice annotated with Therapeutic Goods Administration comments [DSEB, July 2000]), The NHMRC National Statement on Ethical Conduct in Human Research (2007), and the Declaration of Helsinki. Written, informed consent of all participants was obtained prior to the start of the study.

Patients were randomised into either placebo plus standard wound care or Vildagliptin (100mg/day) plus standard wound care by a hospital trial pharmacist to ensure a split of insulin users (80%) to non-insulin users (20%). The study lasted 12 weeks, with clinic visits at 0, 6, and 12 weeks and podiatry visits at 0, 2, 4, 6, 8, 10, and 12 weeks. Blood samples (collected at 0,6, and 12 weeks) and laboratory parameters were collected during clinic visits. Laboratory parameters collected were: Total Protein, Albumin, Globulin, Fibrinogen, Urea/Creatinine, Estimated glomerular filtration rate (eGFR), White blood cell count (WBC), Neutrophils, Haematocrit, Haemoglobin (Hb), Platelets, Cholesterol, Triglycerides, High density lipoprotein (HDL), Low density lipoprotein (LDL) cholesterol, Very low density lipoprotein (VLDL) cholesterol, Urine Creatinine, Urine Albumin, and Glycated haemoglobin (HbA1c). Ulcer measurements were collected at podiatry appointments, with blood samples being stored for further analysis.

3.1.1 Inclusion Criteria

Patients were included if they met the following criteria:

- T2DM patients ≥ 18 years on diet only intervention or any diabetic medication were included in the study.
- A HbA1c ≥ 7.0% within 12 weeks prior to, or on the day of, study inclusion.

- An existing foot ulcer of grade A1 (University of Texas Wound Classification System) on day of inclusion. In the case of multiple ulcers, the ulcer with the largest wound bed was used for study.

3.1.2 Exclusion Criteria

Patients were excluded if:

- T1DM was present
- Current foot ulcer due to non-diabetic pathophysiology
- Major surgery prior to day of enrolment (≤ 6 months) or planned surgery prior to study completion.
- Sensitivity to Vildagliptin or contraindications against Vildagliptin
- Lactic acidosis history
- Current treatment with GLP-1 analogues or DPP4 inhibitors
- Pathologies that can inhibit wound healing or alter serum immune markers, chronic inflammatory diseases, or active malignancies in the ulcer area.
- Acute infections on the day of enrolment and baseline measurements except diabetic foot ulcer infections.
- Medications that could alter the serum concentration of immune markers including systemic corticosteroids, immunosuppressants, growth factor products, and chemotherapeutic agents.
- Hyperbaric or normothermic oxygen therapy treatment.
- Treatment with skin or dermal substitutes within 30 days prior to enrolment.
- Enzymatic debridement.
- Active Charcot's foot
- Renal impairment (eGFR < 30mL/min)
- Child-bearing potential, pregnancy or lactation
- Participation in any other clinical trial
- Inability to comply with the current study protocol
- Current or new warnings, precautions, or contraindications relevant to the patient at the time of study enrolment as listed in the current Galvus product information.

3.2 Ethics Approval

This work was approved under Human Research Ethics Committee (HREC) number: HREC/13/QTHS/65, Project ID: 19925 and James Cook University Approval: C14.

3.3 ELISA

This study used sandwich enzyme linked immunosorbent assays (ELISA)s which works by "sandwiching" the antigen of interest between two layers of antibodies (Alhajj and Farhana, 2022). Optimisation of ELISAs and subsequent analysis was done according to manufacturer's instructions. A brief synopsis is provided below:

3.3.1 TGF-β1 (Invitrogen: #BMS249-4)

1. External standards were prepared by serial dilution of a 4 ng/mL TGF-β1 standard, generating standard values between 2 ng/mL to 31 pg/mL.
2. Serum samples were diluted 1:10 with 1X assay buffer. 1M HCl was added to the sample and incubated at room temperature for one hour.
3. Incubated samples were neutralised with 1M NaOH.
4. The microwell plate was washed twice with wash buffer.
5. External standards were added to the plate, with 1X assay buffer acting as a zero standard.
6. 40 μL of sample was added to sample wells along with 60 μL of assay buffer.
7. The plate was incubated at room temperature for 2 hours using a microplate shaker.
8. The plate was emptied and washed multiple times.
9. Prepared Biotin-conjugate was added to each well, and the plate was incubated, with shaking, for a further hour at room temperature.
10. Step eight was repeated.
11. Prepared Streptavidin-Horseradish Peroxidase (HRP) was added to each well and incubated, with shaking, at room temperature for one hour.
12. Step eight was repeated.

13. Tetramethyl-benzidene(TMB)-Substrate solution was added to each well and the plate was incubated, with shaking for approximately 30 mins at room temperature in the dark.
14. Stop solution was added to stop the reaction when the most concentrated standard had reached a dark blue colouration.
15. Results were read on a spectrophotometer (BMG POLARstar Omega) at 450nm.

3.3.2 BMP-7 (Invitrogen: EHBMP7)

1. External standards were prepared by serial dilution of a 50 ng/mL BMP-7 standard, generating standard values between 6000 pg/mL to 8.23 pg/mL.
2. Serum samples were diluted 1:2 with assay diluent A.
3. External standards were added to the plate, with assay diluent A acted as a zero standard.
4. 100 μL of sample was added to sample wells.
5. The plate was incubated at room temperature for 2.5 hours using a microplate shaker.
6. The plate was emptied and washed multiple times
7. Prepared Biotin-conjugate was added to each well, and the plate was incubated, with shaking, for a further hour at room temperature.
8. Step six was repeated.
9. Prepared Streptavidin-HRP was added to each well and incubated, with shaking, at room temperature for 45 minutes.
10. Step six was repeated.
11. TMB-Substrate solution was added to each well and the plate was incubated, with shaking for approximately 30 mins at room temperature in the dark.
12. Stop solution was added to stop the reaction when the most concentrated standard had reached a dark blue colouration.
13. Results were read on a spectrophotometer (BMG POLARstar Omega) at 450nm.

3.4 Software and Data Management

Standards and samples were run in duplicate to obtain an average serum value for the growth factors of interest. ELISA absorbance values were obtained using OMEGA software (BMG, Ver. 5.50 R4) then exported to MARS software (BMG, Ver. 3.32). From the MARS software, the absorbance values were then exported in Excel (Microsoft, Ver. 2202, Build 14931.20764) which were the copied into Prism (GraphPad, Ver. 13). Prism was used to determine the standard curves for each ELISA. Patient values were determined from the standard curve or extrapolated from the curve using Prism (GraphPad, Ver. 13). Statistical comparisons between ELISA levels and study parameters was calculated using SPSS (IBM, Ver. 27). Paired Student's *t* test was used to compare ELISA results. Regression analysis was used to compare Ulcer and HbA1c ratios were compared to ELISA values. The ELISA results were compared to nominal data using analysis of covariance (ANCOVA) with Bonferroni correction. A $p < 0.05$ was determined to be statistically significant. Statistical power was calculated using G*Power (RRID:SCR_013726, Ver. 3.1.9.4).

CHAPTER 4: RESULTS

4.1 Patient Data

Table 4.1 shows the demographic and clinical data for the patient samples used in this study. The demographic data shows that of the 10 T2DM patients sampled, nine were Caucasians and the majority were male patients. Three co-morbidities were common in the patients: neuropathy, dyslipidaemia and hypertension. Four patients experienced complete ulcer healing, however, the surface areas of the ulcers decreased from an average of 197 mm^2 to 49 mm^2.

Table 4.1: Demographic and health data for patient samples. M:Male, F: Female, ATSI: Aboriginal and Torres Strait Islander.

Demographic Data	***n***
Total number	10
Gender M:F	8:2
Ethnicity ATSI: Non-ATSI	1:9
Average Age (Range)	65.7 years (43-80 years)
Average Weight (Range)	96.6 kg (62-135 kg)
Diabetes status and Interventions	
Type 2 Diabetes	10
Treatment Placebo: Vildagliptin	5:5
Average Years with Diabetes (Range)	19.3 years (3-26 years)
Treatment with Insulin	6
Treatment with Oral Antihyperglycaemics	6
Cotreatment with Insulin and Oral Antihyperglycaemics	4

Table 4.1: Demographic and health data for patient samples continued.

Risk Factors and Comorbidities	***n***
Current Alcohol	4
Current Smoking	1
Diabetic Retinopathy	3
Diabetic Nephropathy/Chronic Kidney Disease	3
Diabetic Neuropathy	7
Microvascular Complications	7
Dyslipidaemia	7
Coronary Arterial Disease	3
Peripheral Vascular Disease	5
Peripheral Vascular Disease Surgery	3
Non-Traumatic Amputation	3
Hypertension	7
Ulcer status	
Complete Ulcer Healing	4
Average Initial Surface Area (Range)	197.6 mm^2 (8-750 mm^2)
Average Final Surface Area (Range)	49 mm^2 (0-300 mm^2)

4.2 ELISA Results

The changes in serum TGF-$\beta 1$ and BMP-7 in DFU patients over the 12 week trial period are shown in Figures 4.1 and 4.2, respectively. An increase in serum TGF-$\beta 1$ was observed, with an average initial value of 7686 pg/mL and increasing to 11226 pg/mL by the end of the study. Additionally, BMP-7 also increased from 42.37 pg/mL, initially, to 49.23pg/mL by the end of the trial. The improvement in BMP-7 levels was significant ($p < 0.01$) according to a paired Student's *t* test.

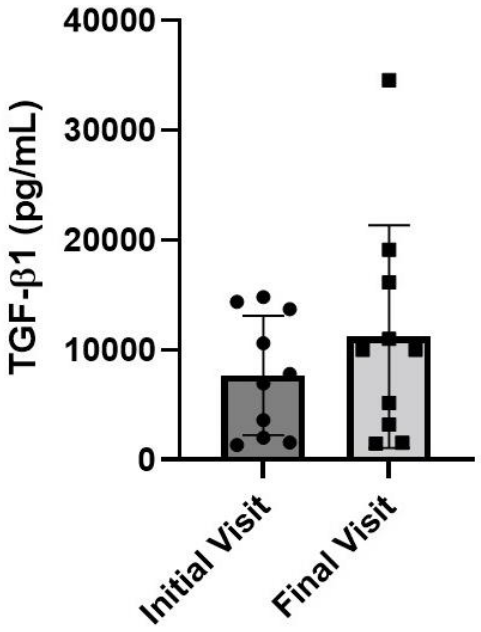

Figure 4.1: Serum TGF-β1 levels at the initial visit (Dark Grey) and final visit (Light Grey) as measured by sandwich ELISA. *n* = 10 per visit.

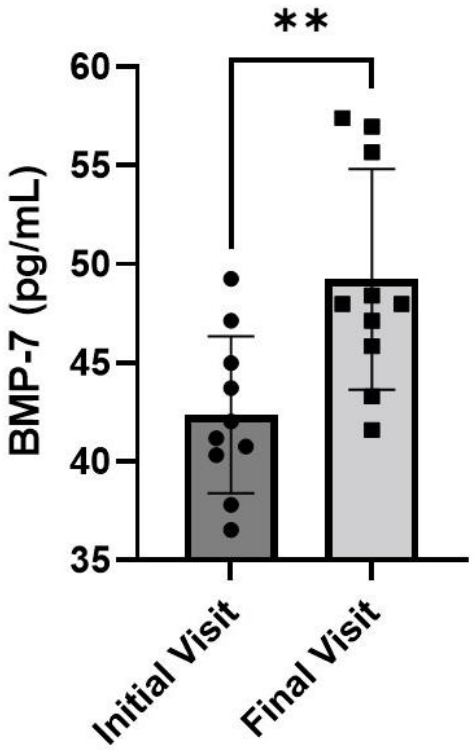

Figure 4.2: Serum BMP-7 levels at the initial visit (Dark Grey) and final visit (Light Grey) as measured by sandwich ELISA. **: $p < 0.01$ (Student's *t* test). *n* = 10 per visit.

4.3 Analysis of Covariance (ANCOVA) Results

Table 4.2 shows the results of ANCOVA test, with Bonferroni correction to test for significance, between TGF-β1 values and nominal data categories. No effect was seen at week 12 for TGF-β1 between control and treatment groups (p = 0.202). Moreover, no relationship was seen between TGF-β1 at week 12 and ulcer healing (p = 0.771). Significant (p < 0.05) increases in serum TGF-β1 at week 12 were observed DFU patients without neuropathy, microvascular complications, dyslipidaemia or hypertension (Figures 4.3-4.6).

Table 4.2: ANCOVA results for TGF-β1. A significant effect was only observed with neuropathy, microvascular complications, dyslipidaemia, and hypertension; all other factors had moderate to no effect on 12 week TGF-β1 levels.*: p < 0.05 with Bonferroni comparison. OHG: Oral Anti-hyperglycaemics, PVD: Peripheral vascular disease.

Dependent variable	**Covariate**	**Fixed Factor**	**Significance**
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Treatment Group	0.202
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Gender	0.215
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	ATSI	0.956
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Alcohol Status	0.677
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Smoking Status	0.294
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Insulin Treatment	0.951
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	OHG	0.677
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Insulin OHG Co-treatment	0.435
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Retinopathy	0.477
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Kidney Disease	0.379
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Neuropathy	0.043*
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Microvascular complications	0.043*
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Dyslipidemia	0.043*
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Coronary Arterial Disease	0.811
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Peripheral Vascular Disease	0.520
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	PVD Surgery	0.309
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Amputation	0.477
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Hypertension	0.043*
TGF-β1 (12 Weeks)	TGF-β1 (0 Weeks)	Ulcer Healing	0.771

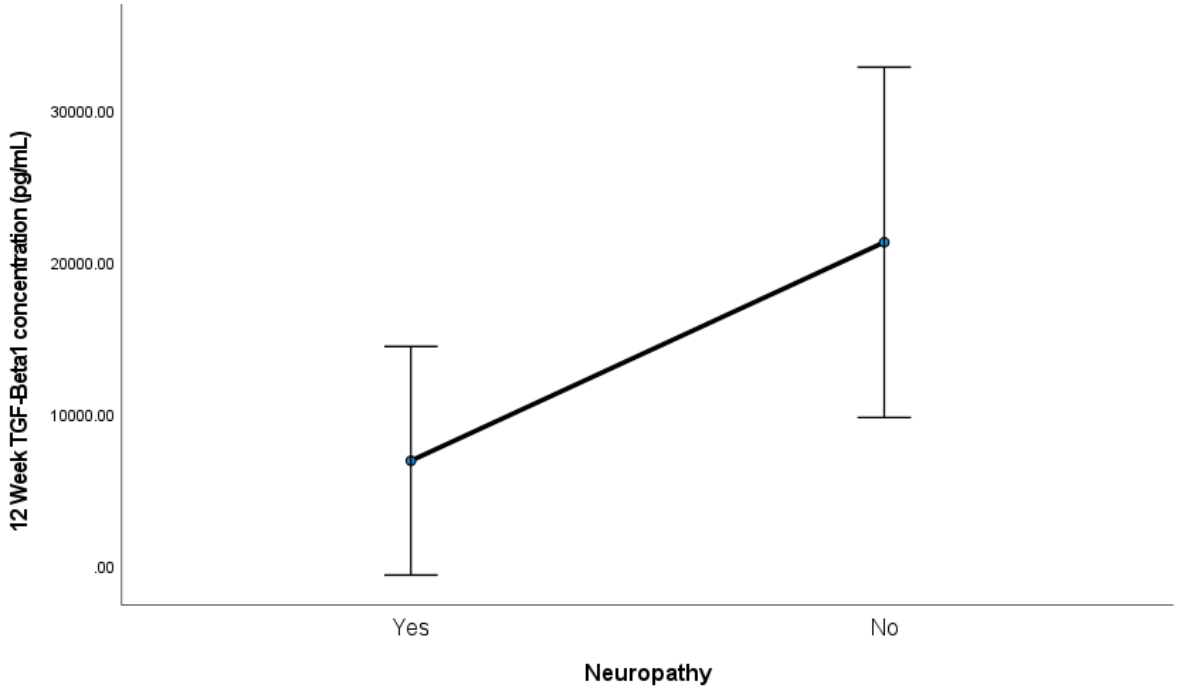

Figure 4.3: ANCOVA graph showing that 12 week TGF-β1 values were higher in DFU patients without diabetic neuropathy compared to those with DFU.

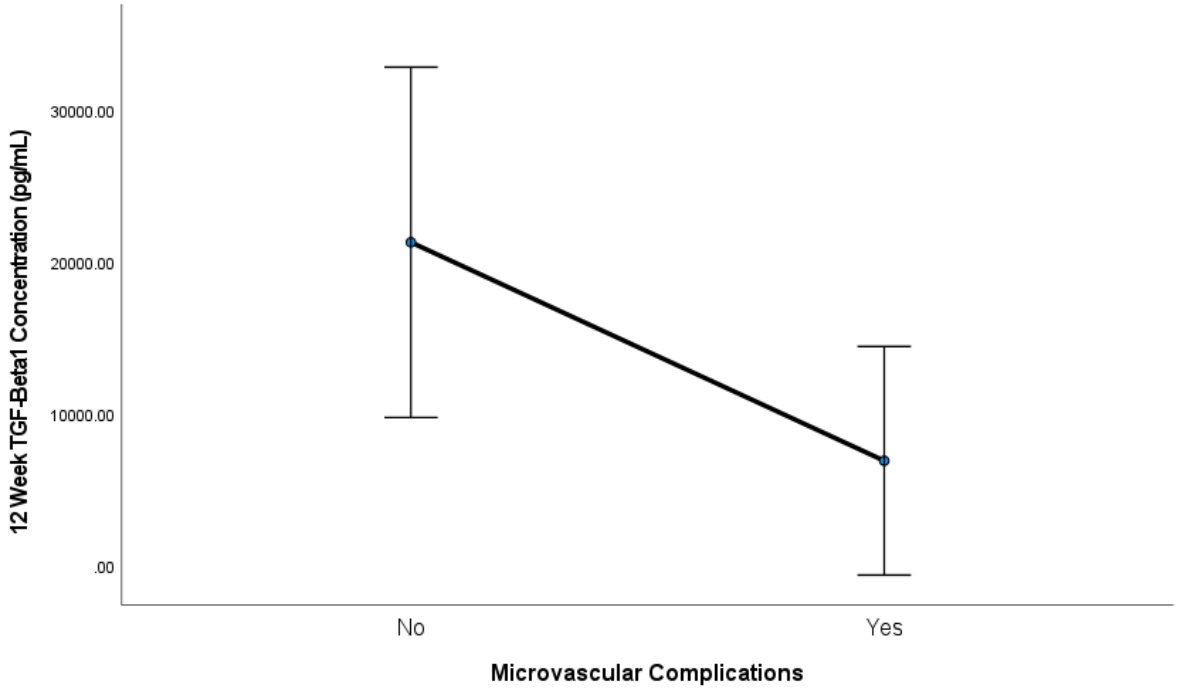

Figure 4.4: ANCOVA graph indicating higher 12 week TGF-β1 values in patients without microvascular complications compared to those with complications.

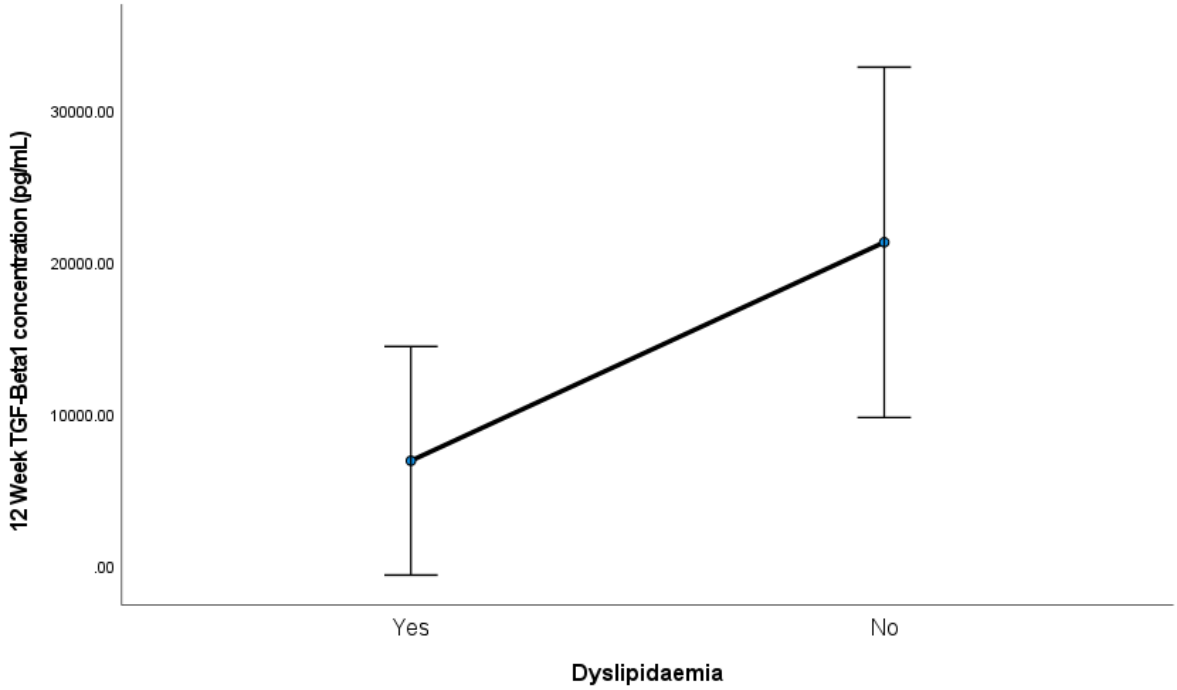

Figure 4.5: ANCOVA analysis indicating that the presence of dyslipidaemia effects 12 week TGF-β1 values in DFU patients.

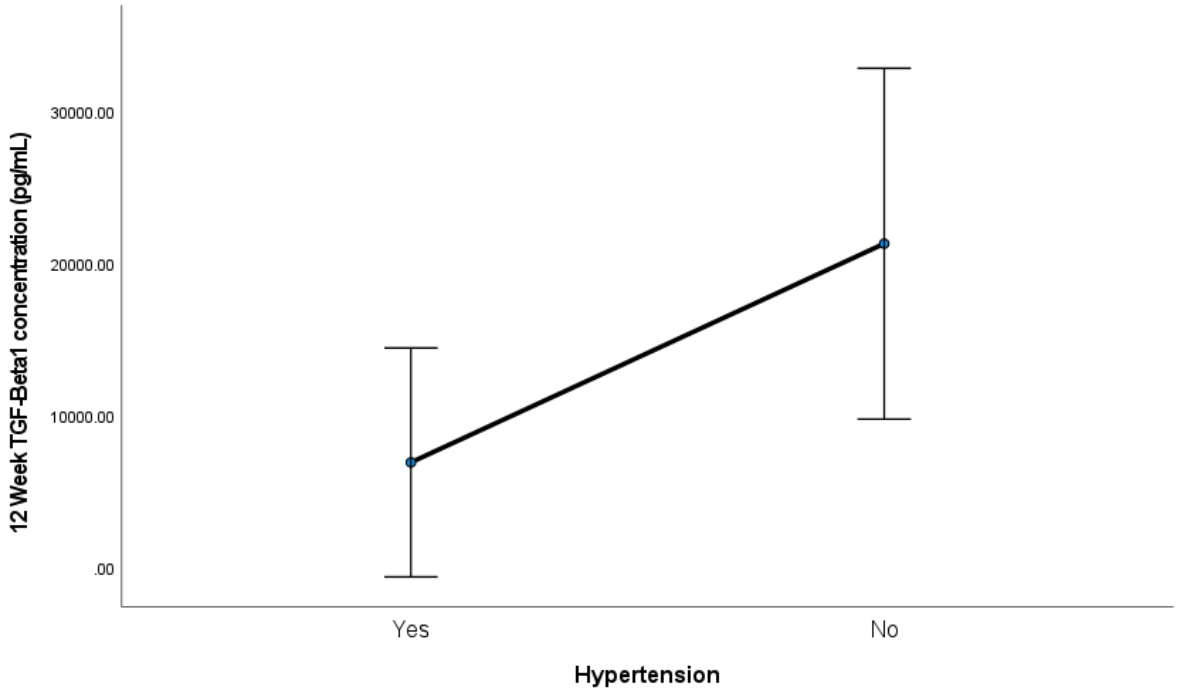

Figure 4.6: The absence of hypertension leads to increases in 12 week serum TGF-β1 values compared to DFU patients with hypertension according to ANCOVA analysis.

The results of the ANCOVA analysis for BMP-7 is shown in Table 4.3. No significant relationship was observed between BMP-7 and treatment group or ulcer healing (p = 0.881 and 0.282, respectively). A significant (p < 0.05) relationship was observed between insulin treatment and BMP-7 levels; an effect not observed with oral antihyperglycemics or co-treatment (p = 0.918 for oral antihyperglycaemics and p = 0.231 for co-treatment). Additionally, the relationship between alcohol consumption and BMP-7 approached significance (p = 0.082).

Table 4.3: ANCOVA results for BMP-7. A significant effect was only observed with insulin treatment, all other factors had moderate to no effect on 12 week BMP-7 values. *: p < 0.05 with Bonferroni comparison.

Dependent variable	**Covariate**	**Fixed Factor**	**Significance**
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Treatment Group	0.881
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Gender	0.997
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	ATSI	0.415
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Alcohol Status	0.082
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Smoking Status	0.343
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Insulin Treatment	0.032*
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	OHG	0.918
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Insulin OHG Co-treatment	0.231
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Retinopathy	0.710
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Kidney Disease	0.077
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Neuropathy	0.915
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Microvascular complications	0.915
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Dyslipidaemia	0.915
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Coronary Arterial Disease	0.794
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Peripheral Vascular Disease	0.992
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	PVD Surgery	0.470
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Amputation	0.710
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Hypertension	0.915
BMP-7 (12 Weeks)	BMP-7 (0 Weeks)	Ulcer Healing	0.282

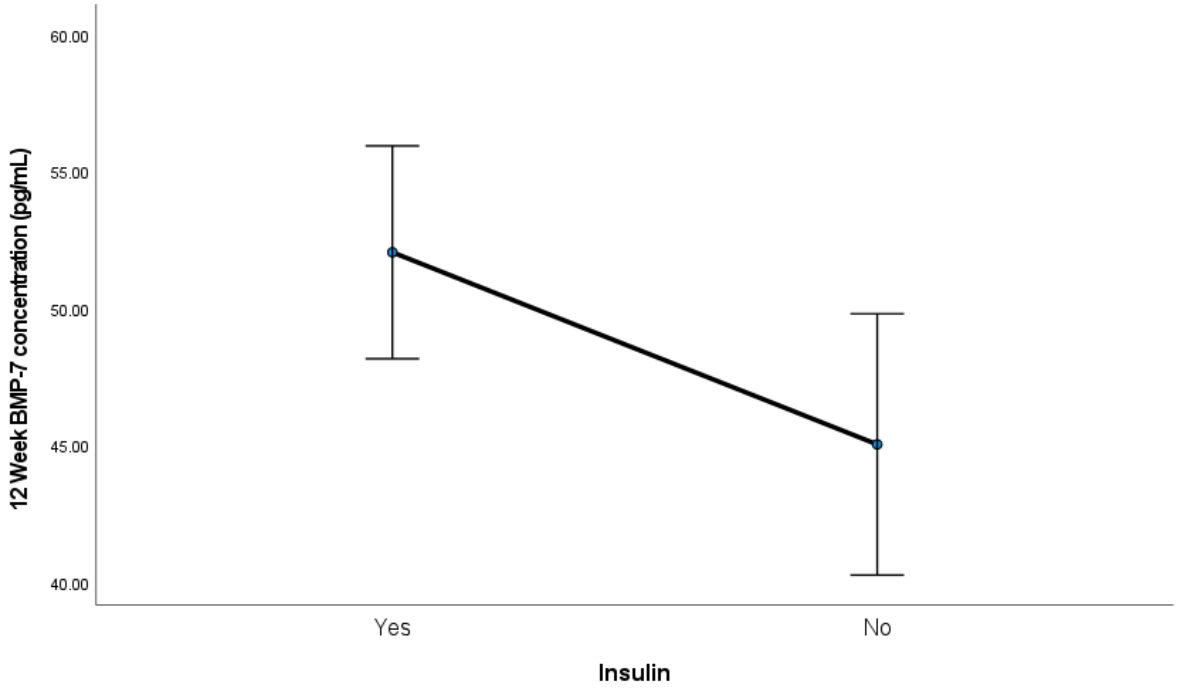

Figure 4.7: Insulin treatment improved 12 week BMP-7 levels compared to those receiving oral anti-hyperglycaemics alone or combination therapy with both agents according to ANCOVA analysis.

4.4 Spearman's Correlation Analysis

Tables 4.4 and 4.5 show correlation values between intake values for the serum growth factors and intake values for other variables. No correlation was observed between growth factor concentrations and ulcer surface area (SA) or HbA1c values at either time point. A significant, negative correlation ($p < 0.05$) was observed between intake TGF-β1 and globulins. A negative correlation was observed between intake interleukin (IL)-6 levels and TGF-β1 that was non-significant ($p = 0.071$). Furthermore, a significant ($p < 0.05$) positive correlation occurred between intake BMP-7 levels and serum albumin. A negative correlation was also observed between intake IL-6 and BMP-7 that was also significant ($p < 0.05$). Tables 4.6 and 4.7 show the correlations between 12 week serum growth factor levels and 12 week values for other variables. A significant ($p < 0.05$), positive relationship was observed between 12 week TGF-β1 values and corresponding hypoxia inducible factor (HIF)-α values. A negative correlation between 12 week TGF-β1 values and 12 week white blood cell count (WBC) was non significant ($p = 0.09$). Two significant ($p < 0.05$) correlations were

observed for 12 week BMP-7 values; a positive relationship with 12 week globulin values, and a negative relationship with 12 week WBC. A negative relationship between 12 week BMP-7 and 12 week neutrophil was close to significance ($p < 0.052$).

Table 4.4: Correlation data for intake TGF-β1. A relationship was observed between initial globulin values and intake TGF-β1, no relationship was observed with other data categories. *: $p < 0.05$ Spearman's rho (2-tail). n.s.: non significant. BP: Blood Pressure, CRP: C-reactive protein, TNF-α: Tumour necrosis factor-α

Variable	**Correlation Coefficient**	**Significance (2-Tailed)**
Age	-0.073	n.s.
Years with Diabetes	-0.160	n.s.
Height	-0.146	n.s.
Weight	-0.365	n.s.
Systolic BP	0.226	n.s.
Diastolic BP	-0.166	n.s.
Ulcer SA	-0.134	n.s.
Total Protein	-0.561	n.s.
Albumin	0.598	n.s.
Globulin	-0.716	0.02*
Urea/Creatinine	0.268	n.s.
eGFR	0.472	n.s.
WBC	0.357	n.s.
Neutrophils	0.452	n.s.
Haematocrit	-0.507	n.s.
Hb	-0.228	n.s.
Platelets	0.357	n.s.
Cholesterol	0.273	n.s.
Triglyceride	-0.103	n.s.

Table 4.4: Correlation data for intake TGF-β1 continued. *: $p < 0.05$ Spearman's rho (2-tail). n.s.: non significant.

Variable	**Correlation Coefficient**	**Significance (2-Tailed)**
HDL	0.355	n.s.
Total/HDL ratio	0.150	n.s.
LDL Chol	0.370	n.s.
VLDL Chol	-0.091	n.s.
Urine Creatinine	-0.233	n.s.
Urine Albumin	-0.033	n.s.
Urine Albumin/Creatinine ratio	0.050	n.s.
HbA1c	-0.317	n.s.
IL-6	-0.667	0.071
IL-1β	-0.273	n.s.
TNF-α	-0.418	n.s.
Adiponectin	-0.491	n.s.
CRP	-0.261	n.s.
HIF-α	-0.048	n.s.
BMP-7	0.115	n.s.

Table 4.5: Correlation data for intake BMP-7. Relationships were observed between initial albumin and initial IL-6 values and intake BMP-7, no relationship was observed with other data categories. *: $p < 0.05$ Spearman's rho (2-tail). n.s.: non significant.

Variable	**Correlation Coefficient**	**Significance (2-Tailed)**
Age	-0.182	n.s.
Years with Diabetes	-0.068	n.s.
Height	0.480	n.s.
Weight	0.413	n.s.
Systolic BP	0.506	n.s.
Diastolic BP	0.320	n.s.
Ulcer SA	0.298	n.s.
Total Protein	0.439	n.s.
Albumin	0.762	0.010*
Globulin	-0.606	n.s.
Urea/Creatinine	0.201	n.s.
eGFR	0.032	n.s.
WBC	0.548	n.s.
Neutrophils	0.310	n.s.
Haematocrit	0.127	n.s.
Hb	-0.012	n.s.
Platelets	0.214	n.s.
Cholesterol	0.515	n.s.

Table 4.5: Correlation data for intake BMP-7 continued. *: $p < 0.05$ Spearman's rho (2-tail). n.s.: non significant.

Variable	**Correlation Coefficient**	**Significance (2-Tailed)**
Triglyceride	-0.426	n.s.
HDL	0.440	n.s.
Total/HDL ratio	0.275	n.s.
LDL Chol	0.503	n.s.
VLDL Chol	-0.415	n.s.
Urine Creatinine	0.367	n.s.
Urine Albumin	-0.200	n.s.
Urine Albumin/Creatinine ratio	-0.200	n.s.
HbA1c	-0.171	n.s.
IL-6	-0.786	0.021*
IL-1β	0.176	n.s.
TNF-α	-0.479	n.s.
Adiponectin	0.236	n.s.
CRP	-0.042	n.s.
HIF-α	0.619	n.s.

Table 4.6: Correlation data for 12 Week TGF-β1. A strong relationship was observed between HIF-α and TGF-β1 at 12 weeks, no significant relationships were observed with other data categories. *: $p < 0.05$ Spearman's rho (2-tail). n.s.: non significant.

Variable	**Correlation Coefficient**	**Significance (2-Tailed)**
Ulcer SA	-0.131	n.s.
Total Protein	0.228	n.s.
Albumin	-0.253	n.s.
Globulin	0.299	n.s.
Urea/Creatinine	0.299	n.s.
eGFR	-0.409	n.s.
WBC	-0.685	0.09
Neutrophils	-0.571	n.s.
Haematocrit	0.036	n.s.
Hb	0.072	n.s.
Platelets	-0.071	n.s.
Cholesterol	-0.018	n.s.
Triglyceride	-0.108	n.s.
HDL	0.074	n.s.
Total/HDL ratio	-0.090	n.s.
LDL Chol	-0.288	n.s.
VLDL Chol	-0.075	n.s.
Urine Creatinine	0.257	n.s.
Urine Albumin	0.314	n.s.

Table 4.6: Correlation data for 12 Week TGF-β1 continued. *: $p < 0.05$ Spearman's rho (2-tail). n.s.: non significant.

Variable	**Correlation Coefficient**	**Significance (2-Tailed)**
Urine Albumin/Creatinine ratio	0.486	n.s.
HbA1c	-0.024	n.s.
IL-6	-0.467	n.s.
IL-1β	0.467	n.s.
TNF-α	0.127	n.s.
Adiponectin	0.309	n.s.
CRP	0.236	n.s.
HIF-α	0.886	0.019
BMP-7	0.207	n.s.

Table 4.7: Correlation data for 12 week BMP-7. Relationships were observed between Globulin, WBC and BMP-7 at 12 weeks, with a strong relationship between neutrophils and BMP-7. No other interactions were observed with data categories. *: $p < 0.05$ Spearman's rho (2-tail). n.s.: non significant.

Variable	**Correlation Coefficient**	**Significance (2-Tailed)**
Ulcer SA	-0.041	n.s.
Total Protein	0.060	n.s.
Albumin	-0.566	n.s.
Globulin	0.731	0.04
Urea/Creatinine	-0.263	n.s.
eGFR	0.136	n.s.
WBC	-0.847	0.016
Neutrophils	-0.750	0.052
Haematocrit	0.143	n.s.
Hb	0.126	n.s.
Platelets	-0.321	n.s.
Cholesterol	0.162	n.s.
Triglyceride	-0.306	n.s.
HDL	-0.111	n.s.
Total/HDL ratio	0.180	n.s.
LDL Chol	0.270	n.s.
VLDL Chol	-0.112	n.s.
Urine Creatinine	-0.486	n.s.
Urine Albumin	-0.371	n.s.

Table 4.7: Correlation data for 12 week BMP-7 continued. *: $p < 0.05$ Spearman's rho (2-tail). n.s.: non significant.

Variable	Correlation Coefficient	Significance (2-Tailed)
Urine Albumin/Creatinine ratio	-0.314	n.s.
HbA1c	0.055	n.s.
IL-6	-0.050	n.s.
IL-1β	0.176	n.s.
TNF-α	-0.201	n.s.
Adiponectin	0.280	n.s.
CRP	-0.316	n.s.
HIF-α	-0.086	n.s.

4.5 Regression Analysis

Regression analysis was performed on the ulcer and HbA1c ratios and ratios of TGF-β1 and BMP-7. Additional regression analysis was conducted on the ulcer ratios, HbA1c and intake or 12 week TGF-β1 and BMP-7 values. Tables 4.8 and 4.9 showed no significant interactions between any of the measured ratios. Further regression analysis showed no interactions between intake growth factors and either ulcer or HbA1c ratios (Tables 4.8 and 4.9). The same analysis found no relationship between final growth factor concentrations and either of the ratios used (Table 4.8 and 4.9).

Table 4.8: Regression analysis comparing the ulcer ratio to growth factors ratios, intake or final concentrations. No interactions were observed. n.s: non significant. Ratios were calculated as the 12 week value for the growth factor divided by the intake value for the same growth factor.

Ratio/Value	Pearson Correlation	Significance
TGF-β1 ratio	-0.085	n.s
BMP-7 ratio	-0.104	n.s
Intake TGF-β1	0.154	n.s
Intake BMP-7	0.324	n.s.
12 week TGF-β1	-0.249	n.s
12 week BMP-7	0.126	n.s

Table 4.9: Regression analysis comparing the Hb1Ac ratio to growth factors ratios, intake or final concentrations. No interactions were observed. n.s: non significant.

Ratio	**Pearson Correlation**	**Significance**
TGF-β1 ratio	-0.293	n.s
BMP-7 ratio	-0.227	n.s
Intake TGF-β1	0.198	n.s
Intake BMP-7	0.476	n.s
12 week TGF-β1	0.029	n.s
12 week BMP-7	-0.319	n.s

CHAPTER 5: DISCUSSION

5.1 General Discussion

This work tested the levels of TGF-β1 and BMP-7 in the serum of DFU patients who participated in the GIED trial, and compared the levels to other physiological and laboratory parameters. This study found an increase in the serum concentrations of both TGF-β1 and BMP-7 at the end of 12 weeks. A significant effect was observed for BMP-7. Further analysis found a significant interaction between 12 week TGF-β1 values and diabetic neuropathy, microvascular complications, dyslipidaemia, and hypertension. The same analysis found a significant relationship between insulin treatment and 12 week BMP-7 values. Correlation analysis found further relationships. Intake TGF-β1 had a negative correlation with intake globulin concentrations. BMP-7 levels at week zero were positively correlated with albumin levels and negatively correlated to IL-6. 12 week HIF-α values were positively correlated to TGF-β1 values from the same time point. BMP-7 levels at the 12 week timepoint were positively correlated 12 week globulin values and negatively correlated with 12 week WBC.

Regression analysis was conducted between the growth factor ratios and the ulcer and HbA1c ratios. The latter two ratios are used as surrogate markers for ulcer and disease improvement, respectively. These tables show that there is no relationship between any of the ratios selected. This is consistent with other analysis conducted in this study which did not find any relationship between ulcer recovery, HbA1c and TGF-β1 or BMP-7. However, this result is contrary to the literature. Correlations between HbA1c and TGF-β1 have been reported by others (Gao et al., 2022, Shaker and Sadik, 2013). *In vivo* work has shown no effect for BMP-7 on HbA1c (Tate et al., 2021). Liu et al. (2009) found that patients with non-responsive ulcers initially presented with lower levels of circulating TGF-β1. This conflict with the established literature may be explained by the small sample size which may have resulted in a false negative (see Section 5.3).

5.1.1 TGF-β1

We also found a non-significant increase in the levels of TGF-β in DFU, an effect that is consistent with the literature. Human studies have shown that TGF-β signalling is increased, in response to intervention, in DFU (Zhang et al., 2022a, Karam et al., 2018, Lavery et al., 2020, Yang et al., 2017, Zhang et al., 2014). Circulating TGF-β1 is elevated in DFU patients however, at the ulcer bed, TGF-β1 signalling is decreased (Zhang et al., 2016, Smina et al., 2019, Jude et al., 2002, Jude et al., 1999). Furthermore, patients with non-healing ulcers present with lower levels of TGF-β1 in the wound fluid compared to patients with responsive ulcers (Liu et al., 2009). Maggot debridement treatment resulted in an increase in tissue TGF-β and an increase in T_{reg} cells (Zhang et al., 2022a). Whether or not this increase in serum TGF-β1 is related to changes in regulatory T-cells (T_{reg}) cell activity is unknown, which may be a point of further study.

ANCOVA analysis revealed significant increases in 12-week TGF-β1 in patients without diabetic neuropathy, microvascular complications, dyslipidaemia, or hypertension. Diabetic neuropathy was placed under the category of microvascular complications, which comprised the bulk of that data category, explaining why there was an effect observed with diabetic neuropathy or microvascular complications and 12-week TGF-β1 as opposed to diabetic retinopathy or diabetic nephropathy and 12-week TGF-β1 (Figures 4.3 and 4.4, Appendix 2 Figures A11 and A12). TGF-β signalling is dysfunctional in diabetic neuropathy, additionally, hyperlipidaemia has been shown to activate TGF-β signalling (Mussa et al., 2021, Hussain et al., 2016, Alvarado-Vázquez et al., 2019, Ybarra et al., 2010, Su et al., 2020, Wang et al., 2021, Mühlfeld et al., 2004, Li et al., 2016). Hypertension can also upregulate TGF-β signalling through activity of the Renin-Angiotensin-Aldosterone system, increasing TGF-β through transcriptional and post transcriptional mechanisms (reviewed by Matsuki et al. (2014)). The ANCOVA result for diabetic neuropathy adds to the contradictory role of TGF-β signalling. This relationship suggests that enhanced TGF-β signalling required for DFU recovery may be attenuated in patients with co-morbid diabetic neuropathy. The ANCOVA results for dyslipidaemia and hypertension are logical in terms of the literature, suggesting that diminished TGF-β production might occur in DFU patients with dyslipidaemia or

hypertension. These results suggest that uncomplicated DFU produces more TGF-β1 in response to intervention compared to DFU with co-morbidities.

Correlation analysis showed a significant, negative relationship between control TGF-β and globulin levels. Globulin is a fraction of the serum that contains hundreds of proteins, this fraction is divided into four categories: α_1, α_2, β and γ (Busher, 1990). Globulins, particularly immunoglobulins, are decreased in diabetic patients and depressed α_2 globulin levels were associated with duration of diabetes (Sulaiman et al., 2022, Guo et al., 2016, Mazer et al., 2011, McMillan, 1970). Additionally, peripheral globulin levels were observed to be a risk factor for the progression of DFU to minor or major amputation (Jiang et al., 2015). TGF-β has a modulating effect on globulins; inhibiting immunoglobulin production in some cases in addition to regulating cellular response to select immunoglobulins (Letterio and Roberts, 1998, Cazac and Roes, 2000, Stavnezer, 1995). The inhibitory effects of TGF-β on immunoglobulin production may explain the negative correlation between control TGF-β and control globulins.

A significant positive relationship was observed between 12 week TGF-β1 and 12 week HIF-α. Nerve regeneration via vascular endothelial growth factor A (VEGF-A), as well as enhanced production of the TGF-β3 gene, is driven by HIF-α in damaged nerve tissue(Cho et al., 2015). Additionally, HIF-α is a regulator of TGF-β under hypoxic conditions (Distler et al., 2007). Hyperglycaemic conditions decreased HIF-α activation *in vitro* and diabetic tissues failed to produce VEGF in response to hypoxic challenge (Thangarajah et al., 2009). Serum levels of HIF-α and VEGF levels are decreased in DFU patients and rodents (Lin et al., 2019). Conversely, TGF-β stimulates VEGF production *in vitro*, promoting angiogenesis (Kaminska et al., 2005, Jin et al., 2018, Cho et al., 2015). This positive relationship between 12 week values for TGF-β1 and HIF-α in this study suggests improved angiogenesis and possible nerve regeneration in DFU.

5.1.2 BMP-7

We have found serum levels of BMP-7 significantly ($p < 0.01$) increased over the 12-week period. The increase in serum BMP-7 in DFU has not been reported previously. Increases in

BMP-7 in response to treatment have been reported in an animal model of hypertrophic scarring (Zhang et al., 2022b). BMP-7 is known to have beneficial effects in bone repair, but also possess beneficial actions in soft tissue repair leading to reduced scarring (Mathavan et al., 2020, Guo et al., 2017, Kowtharapu et al., 2018, Kabuto et al., 2015). BMP-7 has been shown to promote the conversion of naïve T-cells to T_{reg} cells *in vitro* and *in vivo* (Sconocchia et al., 2021). Considering the reduction in ulcer surface area, the increases in BMP-7 may be an attempt to correct wound healing, possibly through the effects of T_{reg} cells at the ulcer bed.

ANCOVA analysis found that 12-week BMP-7 levels were significantly increased in patients who were receiving insulin treatment compared to patients who were not using insulin. This relationship was not observed in patients using oral anti-hyperglycaemics or in patients receiving co-treatment (Appendix 2, Figures 23,24). This result is partially confirmed by the available literature. The TGF-β family of growth factors signal using two pathways, the canonical and non-canonical pathways (Finnson et al., 2020). SMAD 2/3 is the main intracellular messenger of the canonical pathway whereas SMAD 1/5 or other intracellular messenger pathways, such as the phosphatidylinositol 3-kinase (PI3K) /protein kinase B (Akt) pathway, are the signalling molecules used in the non-canonical pathway (Finnson et al., 2020, Cecchi et al., 2016). Insulin signals through the PI3K-Akt pathway to coordinate nutrients with cellular processes such as proliferation and growth (Hopkins et al., 2020). One human study found positive correlations between serum BMP-7 and fasting insulin levels and HOMA2-%B, a measure of insulin secretion, in healthy patients (Zeng et al., 2011). Furthermore, in murine models of diabetes, BMP-7 was found to improve glucose uptake and insulin tolerance (Chattopadhyay et al., 2017). Co-administration of BMP-7 with insulin improved glucose uptake *in vitro*, an effect which was lost when BMP-7 was administered alone (Chattopadhyay et al., 2017). *In vitro* work from the same group showed activation of the PI3K-PDK-Akt pathway and increase in GLUT4 expression on the cell membrane (Chattopadhyay et al., 2017). Medication has been shown to affect BMP-7 production. Liraglutide, a glucagon-like peptide-1 analogue, improved the expression of BMP-7 in the kidneys of T1DM rodents (Ramzy et al., 2019). Enalapril, an ACE inhibitor, has been reported to improve renal BMP-7 expression in T1DM rodents compared to controls (Wang et al.,

2003). Pitavastatin, a lipid controlling medication, has a similar positive effect on BMP-7 function in models of diabetic kidney damage, although the positive effects on BMP-7 were from maintenance of levels as opposed to increased production (Ohigashi et al., 2017, Ohigashi et al., 2016). The current results reveal that exogenous insulin could improve BMP-7 production; a similar effect seen in DM rodents treated with liraglutide or enalapril. Mechanistically, the upregulation observed in this work could occur through insulin mediated PI3K-Akt signalling, a different intracellular pathway, or through a different mechanism entirely.

Correlation analysis revealed significant relationships between serum BMP-7 levels and physiological measurements. Control BMP-7 values were significantly correlated to control albumin and IL-6. Albumin has been associated with insulin resistance in human studies and insulin has been shown to increase the production of albumin *in vivo* and *in vitro* (Bae et al., 2013, Ishizaka et al., 2007, Lloyd et al., 1987, Peavy et al., 1985). Moreover, albumin can undergo glycation to become an advanced glycation end products (AGEs); glycated albumin can then act as an auto antigen or through the receptor of AGE (RAGE) to drive oxidative stress and inflammation (Bhat et al., 2017). Albumin has been shown to be a pro-inflammatory compound in kidney disease, stimulating immune cells infiltration through the production of chemokines (Lim et al., 2014). Control albumin was positively correlated to control BMP-7 levels. Anti-inflammatory and anti-oxidant effects have been reported for BMP-7 with diabetic and albumin-mediated inflammatory responses being antagonised by BMP-7 in non-human models (Lim et al., 2014, Aluganti Narasimhulu and Singla, 2020, Aluganti Narasimhulu and Singla, 2021, Elmadbouh and Singla, 2021, Li et al., 2014). This observed relationship between BMP-7 and albumin in this study may be an attempt by the body, at the time of admission, to control the inflammatory response required for wound healing.

Spearson's correlation showed a negative relationship between control IL-6 and control BMP-7. A pro-inflammatory cytokine, IL-6 and its associated signalling is a driver of chronic inflammation and is associated with a higher risk of T2DM (Bowker et al., 2020). Diabetic

patients have increased circulating IL-6, an effect that is compounded with active cardiac issues or hypertension (Lukic et al., 2014, Souza et al., 2008, Pradhan et al., 2001, Kreiner et al., 2022). Positive and negative effects on energy metabolism are seen with IL-6. Improved glycaemic control and anti-inflammatory effect with IL-6 being reported by several studies and medication that inhibits IL-6 signalling improves insulin resistance (Qu et al., 2014, Kreiner et al., 2022, Schultz et al., 2010). Conversely, IL-6 enhanced the breakdown of insulin degrading enzyme (IDE) and anti-IL-6 treatment resulted in weight gain (Kurauti et al., 2017, Patsalos et al., 2020). BMP-7 inhibited IL-6 expression in diabetic complications such as cardiomyopathy, tubulopathy and myopathy; possibly through inhibition of TNF-α (Elmadbouh and Singla, 2021, Aluganti Narasimhulu and Singla, 2021, Li et al., 2014, Gould et al., 2002). The chronic nature of DFU would suggest an inflammatory, and possible hyperglycaemic, profile for IL-6. This negative correlation indicates two scenarios, either there is an attempt to decrease circulating IL-6 in DFU with BMP-7 or the chronic inflammation leads to increased IL-6 which could down regulate BMP-7.

Significant and nearly significant (p = 0.052) negative correlations were seen between 12 week BMP-7 and 12 week WBC and 12 week neutrophils values, respectively. BMP-7 has an overall anti-inflammatory effect on immune cells, however, BMP-7 signalling been observed to increase inflammatory immune cells in other conditions such as psoriasis (Sconocchia et al., 2021, Sconocchia and Sconocchia, 2021, Rocher and Singla, 2013, Rocher et al., 2012). Furthermore, BMP-7 has been shown to act as a chemoattractant and increase monocyte migration and adhesion *in vitro* (Sovershaev et al., 2016, Postlethwaite et al., 1994). Conversely, in diabetic cardiomyopathy and atherosclerosis models, BMP-7 inhibited infiltrating monocytes and inflammatory macrophages in cardiac and arterial tissue (Elmadbouh and Singla, 2021, Singla et al., 2016). BMP-7 has also been observed to inhibit monocyte-mediated TGF-$\beta 1$ synthesis *in vitro* (Zhang et al., 2005). Additionally, in a rodent model of ischaemic acute renal failure, BMP-7 treatment reduced neutrophil numbers and activity in the kidney (Vukicevic et al., 1998). The inverse relationship observed between WBC, neutrophils, and BMP-7 could be explained by the anti-inflammatory effects of BMP-7 in immune cells. Whilst BMP-7 can modify the profile of macrophages and T-cells; other cells, such as B cells, can undergo apoptosis when exposed to BMP-7 (Bollum et al., 2017, Sconocchia and Sconocchia, 2021). This, combined with the inhibitory effects of BMP-7 on

neutrophil function, suggests that elevated BMP-7 reduced circulating WBCs. It is to be determined if this reduction is an attempt to improve wound healing by signalling the proliferation stage of wound healing or is pathological; aggravating DFU pathology through a loss of WBC function and signalling.

Additionally, a positive correlation was observed between 12-week globulin and 12 week BMP-7 values. The positive relationship between 12 week BMP-7 and 12 week globulin is contrary to the literature as BMP-7 has been shown to inhibit immunoglobulin production in B cells *in vitro* (Huse et al., 2011). However, it should be noted that *in vitro* studies provide minimal insight to disease pathology and can lack translational ability to the human condition (Ribitsch et al., 2020, Balon and Wiatrak, 2021). A possible explanation for this is non-immunoglobulins such as α_2-macroglobulin. This globulin binds several cytokines and growth factors, including TGF-β, and inhibits neutrophil excreted proteases (Vandooren and Itoh, 2021, Gonias et al., 2000, Huang et al., 1988). α_2-macroglobulin acts as a carrier protein for TGF-β, forming a latent complex in serum and enhancing hepatic clearance of TGF-β1 (O'Connor-McCourt and Wakefield, 1987, Huang et al., 1988, LaMarre et al., 1991). Conversely, TGF-β activity in some assays is inhibited by this protein complex but is enhanced in others, the inhibitory effects were attributed to the complexing of TGF-β therefore preventing receptor substrate interaction (Webb et al., 1998, Stouffer et al., 1993). Furthermore, the liver is the main site of α_2-macroglobulin production (Talamini et al., 1998), which may not be subject to BMP-7 inhibition. The positive relationship between BMP-7 and globulins may represent an increase in other globulin fractions as opposed to a net increase in globulins, possibly through hepatic production. Additionally, if the production of α_2-macroglobulin is associated with BMP-7 levels, this may represent a new mechanism by which TGF-β is antagonised by BMP-7.

5.2 Strengths

This is the first study to investigate BMP-7 in DFU. BMP-7 is a member of the TGF-β family of growth factors that has anti-inflammatory properties and is associated with bone and cartilage development (Cecchi et al., 2016, Aluganti Narasimhulu and Singla, 2020, Sconocchia and Sconocchia, 2021). Animal models of DM and associated conditions such as

diabetic myopathy, diabetic cardiomyopathy, and diabetic kidney disease, show improvement with BMP-7 treatment (Narasimhulu and Singla, 2023, Aluganti Narasimhulu and Singla, 2021, Xiao et al., 2022, Peng et al., 2022, Tate et al., 2021, Chattopadhyay et al., 2017). Given the results observed in this trial, and the effects of BMP-7 in DM *in vivo*, there is the potential for BMP-7 to a therapeutic target in DFU therapy.

One of the major strengths of this study is the use of human samples. Several *in vitro* and *in vivo* models exist for DFU however, these models are unable to predict what can occur in human patients and are limited in interpretative ability (van der Worp et al., 2010, Rai et al., 2022, Sanapalli et al., 2021, Phang et al., 2021). Additionally, *in vivo* work suffers from reproducibility issues which can reduce confidence in data obtained from such studies (Landi et al., 2021). Whilst the study size is small (see chapters 5.3 and 6.2), these results are an end product in the human condition, allowing for more direct interpretation of results and further application.

The fact that this study investigates serum concentrations of growth factors is another potential strength. Genes have a complex interaction with the environment and, in some cases, only contribute an increased risk of disease development (Virolainen et al., 2023). When combined with the high number of errors in transcription and translation (Carey, 2015), measuring the circulating growth factors give the best result for a small study size. This is because ELISA measures the end product of the aforementioned biological processes, giving a direct measure of changes in the growth factors and potential signalling in DFU.

5.3 Limitations

This study is limited by the small sample size used for analysis. A small sample size creates issues surrounding false negatives and true, observable effects (Button et al., 2013). However, overall limited sample size combined with external factors such as sample availability inhibits the ability to improve the *n* value of this study. Potential ways to address this issue are discussed in detail in the Future Directions section (Chapter 6.2). The major way to correct this underpowered study would be to add more patient samples exposed to the same treatment conditions. A different option would be to include DM patients to broaden the number of samples obtained, however, this would increase the number of

groups used and tend to elucidate the effects of TGF-β family signalling in DM. *In vivo* and *in vitro* studies could be used to reinforce the results of this study; the limits of either type of model are the lack of translatability and availability of specialist knowledge to use said models.

Sample integrity is another potential issue associated with this study. The exact effect of repeated freeze-thaw cycles on TGF-β activity is unknown, with some studies reporting decreased activity and others showing no change (Grainger et al., 1995, Scholman et al., 2018, Ueland et al., 2011, Simpson et al., 2020). Decreases in TGF-β activity ranging from 11% to 90% have been reported for three cycles (Grainger et al., 1995, Ueland et al., 2011); considering the age of the samples, the samples may have been exposed to at least four freeze-thaw cycles which may have altered TGF-β levels. At the time of this study, there is a lack of information regarding the thermal stability of BMP-7, however, there could be an unknown effect of repeated freeze-thaw cycling on BMP-7 also. This could be easily addressed by testing the levels of both growth factors using fresh serum and comparing it to the levels found in this study.

One weakness of this study was the detection range of the ELISAs that were used. Section 3.2 shows the detection range of the ELISAs which ranged from nanograms/millilitre to picograms/millilitre. ELISAs have been designed to detect analytes in the femtograms/millilitre range and, given that these analysed values for BMP-7 were extrapolated from the control curve, the use of a more sensitive ELISA could have given a more accurate values for BMP-7. Additionally, the observation that BMP-7 decreases with age in animal models of diabetes and low levels of circulating BMP-7 in healthy controls (Chattopadhyay et al., 2017, Ali et al., 2021), indicates the use of a more sensitive ELISA if possible.

CHAPTER 6: CONCLUSION AND FUTURE DIRECTIONS

6.1 Conclusion

Diabetic foot ulcers are a costly and potentially lethal pathology caused by DM. DM patients are at a high risk of developing DFU, even with current treatment regimes. Current best practice for DFU is mostly supportive and involves managing causal or aggravating pathologies, such as infection; even with this management, DFU patients are at a greater risk of dying compared to DM patients without foot ulcers. The underlying pathology of DFU is complex with several molecular processes being disturbed, including growth factor signalling. The TGF-β family of growth factors is involved in key aspects of cellular function including growth, differentiation, and apoptosis. In energy metabolism, the same family participates in pancreatic growth and function, in addition to insulin signalling and glucose metabolism. Only TGF-β has been studied in DFU with human studies showing disrupted TGF-β signalling. However, intervention has been shown to consistently improve circulating levels of the growth factor.

This study analysed the circulating levels of TGF-β and BMP-7, the latter being a member of the same family and potential antagonist of TGF-β, in DFU patients participating in the vildagliptin trial for DFU (GIED trial). Analysis found serum levels of both growth factors had increased over the 12 weeks in the patient samples used. However, only the BMP-7 levels had significantly increased over the trial period. No relationship was observed between these increased growth factors and treatment group. Additionally, no interaction was observed between circulating levels of the measured growth factors and ulcer surface area or HbA1c. ANCOVA analysis found that there were significantly higher levels of TGF-β in patients without DN, microvascular complications, dyslipidaemia or hypertension at the end of the trial period. The same analysis found that patients treated with insulin monotherapy had significantly increased BMP-7 at the end of 12 weeks, an effect that was absent in patients receiving oral anti-hyperglycaemics or co-treatment with insulin. No correlation was observed between levels of either growth factor at either zero or 12 week time points. For the intake values, there was a negative correlation between TGF-β and globulin values and BMP-7 and IL-6 with a positive correlation between BMP-7 and Albumin. A positive

correlation was observed between 12 week TGF-β and HIF-α. For 12 week BMP-7, a positive correlation with globulins was observed and a negative correlation with WBC occurred.

This is the first study to report the levels of BMP-7 in DFU and the possible relationship to biochemical parameters associated with the condition. The small sample size limits the interpretation of results and corresponding effects. The results are consistent with literature that shows an increase in TGF-β in response to intervention in DFU. An anti-inflammatory and wound resolution effect for intervention is suggested given the relationships between TGF-β, co-morbidities, and biochemical factors. Furthermore, the relationships observed with BMP-7 reinforce this anti-inflammatory profile for intervention. This is the first time BMP-7 has been investigated in DFU, revealing possible changes in antiflammatory signalling mediated by the TGF-β family. Whilst this study is small, the results suggest that the TGF-β family are a potential new target for DFU therapy.

6.2 Future Directions

As previously mentioned, the overall small sample size for this study hampers the interpretation and validity of the results (Button et al., 2013). There are a few ways to address the shortcomings associated with this study. *A priori* power analysis suggests a much larger sample size (*n* = 132) (Appendix 4) which could be addressed by including more DFU patients in the analysis either as a separate study or from previous studies. A more accurate treatment effect and ability to apply the results to a general population would occur by including more patients (Biau et al., 2008); however, it may be difficult to obtain this many patient samples when external factors such as attrition are considered. Moreover, the addition of samples from different studies may confound interpretation of any potential results through new variables such as treatment group or age. The addition of DM patients in different stages of the disease would also allow for potential tracking of changes in TGF-β family signalling over the progression of DM into DFU.

Considering the difficulty of obtaining human samples, *in vivo* models could be considered. Both T1DM and T2DM have been modelled in rodents to using diabetogenic compounds or high fat feeding respectively (Rai et al., 2022). DFU can be investigated by performing

surgical incisions on the dorsal aspect of the foot of these models (Rai et al., 2022). Samples would have to be pooled given the limited quantity of tissues that could be harvested from individual animals. Additionally, wound physiology in rodents is not the same as in the human (Sanapalli et al., 2021, Phang et al., 2021), which can limit the interpretation of results. Porcine models of wound healing in DM are also an option as pig skin is very similar to human skin (Sanapalli et al., 2021). However, the high costs associated with porcine work and placement of wounds can also limit the potential use of the said model (Sanapalli et al., 2021). These models could be used to study the TGF-β family in DFU and test for other relevant parameters, such as the effect of certain anti-diabetic medications of TGF-β signalling in DFU. Animal models of DFU would be best served to augment the results of the study conducted in this thesis.

In vitro work could also serve to validate the results obtained in this study. Several wound healing models exist that involve either cell culture or organ culture (Neves et al., 2023, Phang et al., 2021). Culture conditions could be easily modified to replicate a high glucose environment that would be observed in DM (Phang et al., 2021). This could be used to investigate the response of the TGF-β family to DM by damaging the different *in vitro* models to simulate damage. Again, these models are hampered by cost and specialist knowledge required to develop and maintain such models.

There are over 40 members of the TGF-β growth factor family and only the titular growth factor has been investigated in DFU. The use of a semi-quantitative ELISAs could allow for the detection of several members of the TGF-β family from a smaller amount of serum. Those results could then dictate the use of quantitative ELISAs for more accurate determination of proteins. Analysis of wound histology and exudate would also be recommended as few studies have looked at TGF-β signalling at the site of DFU. This could elucidate further mechanisms surrounding TGF-β signalling in DFU, potentially uncovering new therapeutic targets.

CHAPTER 7: REFERENCES

ABRIGO, J., RIVERA, J. C., SIMON, F., CABRERA, D. & CABELLO-VERRUGIO, C. 2016. Transforming growth factor type beta (TGF-β) requires reactive oxygen species to induce skeletal muscle atrophy. *Cellular Signalling,* 28**,** 366-376.

AGARWAL, I., ARNOLD, A., GLAZER, N. L., BARASCH, E., DJOUSSE, L., FITZPATRICK, A. L., GOTTDIENER, J. S., IX, J. H., JENSEN, R. A., KIZER, J. R., RIMM, E. B., SISCOVICK, D. S., TRACY, R. P., WONG, T. Y. & MUKAMAL, K. J. 2015. Fibrosis-related biomarkers and large and small vessel disease: The Cardiovascular Health Study. *Atherosclerosis,* 239**,** 539-546.

AHMAD, J. 2016. The diabetic foot. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews,* 10**,** 48-60.

AHMED, M. U., TANNOUS, W. K., AGHO, K. E., HENSHAW, F., TURNER, D. & SIMMONS, D. 2021. Prevalence and Burden of Diabetes-Related Foot Disease in New South Wales, Australia: Evidence from the 45 and Up Study Survey Data Linked with Health Services Data. *International Journal of Environmental Research and Public Health,* 18.

AIHW. 2020. *Diabetes* [Online]. Canberra. Available: https://www.aihw.gov.au/getmedia/5020b399-3e7e-4762-852e-21d99769c8be/diabetes.pdf.aspx?inline=true [Accessed 3/05 2021].

ALHAJJ, M. & FARHANA, A. 2022. Enzyme Linked Immunosorbent Assay. *StatPearls.* Treasure Island (FL): StatPearls Publishing

Copyright © 2022, StatPearls Publishing LLC.

ALI, A. A., MUKHTAR, M. M., SHAHEEN, S. & MOHAMED, A. O. 2021. Assessment of plasma BMP-2, BMP-7, BMP-10, vitamin D, and TGF β 1 in simple fractures among Sudanese patients. *PLOS ONE,* 16**,** e0247472.

ALUGANTI NARASIMHULU, C. & SINGLA, D. K. 2020. The Role of Bone Morphogenetic Protein 7 (BMP-7) in Inflammation in Heart Diseases. *Cells,* 9**,** 280.

ALUGANTI NARASIMHULU, C. & SINGLA, D. K. 2021. Amelioration of diabetes-induced inflammation mediated pyroptosis, sarcopenia, and adverse muscle remodelling by bone morphogenetic protein-7. *Journal of Cachexia, Sarcopenia and Muscle,* 12**,** 403-420.

ALVARADO-VÁZQUEZ, P. A., GROSICK, R. L., MORACHO-VILRRIALES, C., WARD, E., THREATT, T. & ROMERO-SANDOVAL, E. A. 2019. Cytokine production capabilities of human primary monocyte-derived macrophages from patients with diabetes mellitus type 2 with and without diabetic peripheral neuropathy. *Journal of Pain Research,* 12**,** 69-81.

AMBINATHAN, J. P. N., SRIDHAR, V. S., LYTVYN, Y., LOVBLOM, L. E., LIU, H., BJORNSTAD, P., PERKINS, B. A., LOVSHIN, J. A. & CHERNEY, D. Z. I. 2021. Relationships between inflammation, hemodynamic function and RAAS in longstanding type 1 diabetes and diabetic kidney disease. *Journal of Diabetes and its Complications,* 35**,** 107880.

ANJANEYULU, M., BERENT-SPILLSON, A., INOUE, T., CHOI, J., CHERIAN, K. & RUSSELL, J. W. 2008. Transforming growth factor-β induces cellular injury in experimental diabetic neuropathy. *Experimental Neurology,* 211**,** 469-479.

ARMSTRONG, D. G., BOULTON ANDREW, J. M. & BUS, S. A. 2017. Diabetic Foot Ulcers and Their Recurrence. *The New England Journal of Medicine,* 376**,** 2367-2375.

ARTASENSI, A., PEDRETTI, A., VISTOLI, G. & FUMAGALLI, L. 2020. Type 2 Diabetes Mellitus: A Review of Multi-Target Drugs. *Molecules,* 25.

BAE, J. C., SEO, S. H., HUR, K. Y., KIM, J. H., LEE, M. S., LEE, M. K., LEE, W. Y., RHEE, E. J. & OH, K. W. 2013. Association between Serum Albumin, Insulin Resistance, and Incident Diabetes in Nondiabetic Subjects. *Endocrinology and Metabolism (Seoul),* 28**,** 26-32.

BALON, K. & WIATRAK, B. 2021. PC12 and THP-1 Cell Lines as Neuronal and Microglia Model in Neurobiological Research. *Applied Sciences,* 11**,** 3729.

BANDYK, D. F. 2018. The diabetic foot: Pathophysiology, evaluation, and treatment. *Seminars in Vascular Surgery,* 31**,** 43-48.

BARRIENTOS, S., STOJADINOVIC, O., GOLINKO, M. S., BREM, H. & TOMIC-CANIC, M. 2008. PERSPECTIVE ARTICLE: Growth factors and cytokines in wound healing. *Wound Repair and Regeneration,* 16**,** 585-601.

BHAT, S., JAGADEESHAPRASAD, M. G., VENKATASUBRAMANI, V. & KULKARNI, M. J. 2017. Abundance matters: role of albumin in diabetes, a proteomics perspective. *Expert Review of Proteomics,* 14**,** 677-689.

BIAN, X., GRIFFIN, T. P., ZHU, X., ISLAM, M. N., CONLEY, S. M., EIRIN, A., TANG, H., O'SHEA, P. M., PALMER, A. K., MCCOY, R. G., HERRMANN, S. M., MEHTA, R. A., WOOLLARD, J. R., RULE, A. D., KIRKLAND, J. L., TCHKONIA, T., TEXTOR, S. C., GRIFFIN, M. D., LERMAN, L. O. & HICKSON, L. J. 2019. Senescence marker activin A is increased in human diabetic kidney disease: association with kidney function and potential implications for therapy. *BMJ Open Diabetes Research & Care,* 7**,** e000720.

BIAU, D. J., KERNÉIS, S. & PORCHER, R. 2008. Statistics in Brief: The Importance of Sample Size in the Planning and Interpretation of Medical Research. *Clinical Orthopaedics and Related Research,* 466**,** 2282-2288.

BOLLUM, L. K., HUSE, K., OKSVOLD, M. P., BAI, B., HILDEN, V. I., FORFANG, L., YOON, S. O., WÄLCHLI, S., SMELAND, E. B. & MYKLEBUST, J. H. 2017. BMP-7 induces apoptosis in human germinal center B cells and is influenced by TGF-β receptor type I ALK5. *PLOS ONE,* 12**,** e0177188.

BONDOR, C. I., VERESIU, I. A., FLOREA, B., VINIK, E. J., VINIK, A. I. & GAVAN, N. A. 2016. Epidemiology of Diabetic Foot Ulcers and Amputations in Romania: Results of a Cross-Sectional Quality of Life Questionnaire Based Survey. *Journal of Diabetes Research,* 2016**,** 5439521.

BONFIGLIO, V., PLATANIA, C. B. M., LAZZARA, F., CONTI, F., PIZZO, C., REIBALDI, M., RUSSO, A., FALLICO, M., ORTISI, E., PIGNATELLI, F., LONGO, A., AVITABILE, T., DRAGO, F. & BUCOLO, C. 2020. TGF-β Serum Levels in Diabetic Retinopathy Patients and the Role of Anti-VEGF Therapy. *International Journal of Molecular Sciences,* 21**,** 9558.

BOULTON, A. J. M., KIRSNER, R. S. & VILEIKYTE, L. 2004. Neuropathic Diabetic Foot Ulcers. *New England Journal of Medicine,* 351**,** 48-55.

BOWKER, N., SHAH, R. L., SHARP, S. J., LUAN, J. A., STEWART, I. D., WHEELER, E., FERREIRA, M. A. R., BARAS, A., WAREHAM, N. J., LANGENBERG, C. & LOTTA, L. A. 2020. Meta-analysis investigating the role of interleukin-6 mediated inflammation in type 2 diabetes. *eBioMedicine,* 61.

BREM, H. & TOMIC-CANIC, M. 2007. Cellular and molecular basis of wound healing in diabetes. *The Journal of Clinical Investigation,* 117**,** 1219-1222.

BROWN, M. L. & SCHNEYER, A. 2021. A Decade Later: Revisiting the TGFβ Family's Role in Diabetes. *Trends in Endocrinology & Metabolism,* 32**,** 36-47.

BROWN, M. L. & SCHNEYER, A. L. 2010. Emerging roles for the TGFβ family in pancreatic β-cell homeostasis. *Trends in Endocrinology & Metabolism,* 21**,** 441-448.

BROWNRIGG, J. R. W., DAVEY, J., HOLT, P. J., DAVIS, W. A., THOMPSON, M. M., RAY, K. K. & HINCHLIFFE, R. J. 2012. The association of ulceration of the foot with cardiovascular and all-cause mortality in patients with diabetes: a meta-analysis. *Diabetologia,* 55**,** 2906-2912.

BUI, T. Q., BUI, Q. V. P., NÉMETH, D., HEGYI, P., SZAKÁCS, Z., RUMBUS, Z., TÓTH, B., EMRI, G., PÁRNICZKY, A., SARLÓS, P. & VARGA, O. 2019. Epidermal Growth Factor is Effective in the Treatment of Diabetic Foot Ulcers: Meta-Analysis and Systematic Review. *International Journal of Environmental Research and Public Health,* 16**,** 2584.

BUSHER, J. T. 1990. Serum Albumin and Globulin. *In:* WALKER, H. K., HALL, W. D. & HURST, J. W. (eds.) *Clinical Methods: The History, Physical, and Laboratory Examinations,* Butterworths, Boston, pp 497-499.

BUTTON, K. S., IOANNIDIS, J. P. A., MOKRYSZ, C., NOSEK, B. A., FLINT, J., ROBINSON, E. S. J. & MUNAFÒ, M. R. 2013. Power failure: why small sample size undermines the reliability of neuroscience. *Nature Reviews Neuroscience,* 14**,** 365-376.

CALABRESE, D., GIATTI, S., ROMANO, S., PORRETTA-SERAPIGLIA, C., BIANCHI, R., MILANESE, M., BONANNO, G., CARUSO, D., VIVIANI, B., GARDONI, F., GARCIA-SEGURA, L. M. & MELCANGI, R. C. 2014. Diabetic neuropathic pain: a role for testosterone metabolites. *Journal of Endocrinology,* 221**,** 1-13.

CAREY, L. B. 2015. RNA polymerase errors cause splicing defects and can be regulated by differential expression of RNA polymerase subunits. *eLife,* 4**,** e09945.

CASANA, E., JIMENEZ, V., SACRISTAN, V., MUÑOZ, S., JAMBRINA, C., RODÓ, J., GARCIA, M., MALLOL, C., LEÓN, X., FRANCKHAUSER, S. & BOSCH, F. 2021. BMP7 overexpression in adipose tissue induces white adipogenesis and improves insulin sensitivity in ob/ob mice. *International Journal of Obesity,* 45**,** 449-460.

CATE, R. L., MATTALIANO, R. J., HESSION, C., TIZARD, R., FARBER, N. M., CHEUNG, A., NINFA, E. G., FREY, A. Z., GASH, D. J., CHOW, E. P., FISHER, R. A., BERTONIS, J. M., TORRES, G., WALLNER, B. P., RAMACHANDRAN, K. L., RAGIN, R. C., MANGANARO, T. F., MACLAUGHLIN, D. T. & DONAHOE, P. K. 1986. Isolation of the bovine and human genes for müllerian inhibiting substance and expression of the human gene in animal cells. *Cell,* 45**,** 685-698.

CAZAC, B. B. & ROES, J. 2000. TGF-β Receptor Controls B Cell Responsiveness and Induction of IgA In Vivo. *Immunity,* 13**,** 443-451.

CECCHI, S., BENNET, S. J. & ARORA, M. 2016. Bone morphogenetic protein-7: Review of signalling and efficacy in fracture healing. *Journal of Orthopaedic Translation,* 4**,** 28-34.

CHATTOPADHYAY, T., SINGH, R. R., GUPTA, S. & SUROLIA, A. 2017. Bone morphogenetic protein-7 (BMP-7) augments insulin sensitivity in mice with type II diabetes mellitus by potentiating PI3K/AKT pathway. *BioFactors,* 43**,** 195-209.

CHEN, C. & SHEN, M. M. 2004. Two Modes by which Lefty Proteins Inhibit Nodal Signaling. *Current Biology,* 14**,** 618-624.

CHO, N. H., SHAW, J. E., KARURANGA, S., HUANG, Y., DA ROCHA FERNANDES, J. D., OHLROGGE, A. W. & MALANDA, B. 2018. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice,* 138**,** 271-281.

CHO, Y., SHIN, J. E., EWAN, E. E., OH, Y. M., PITA-THOMAS, W. & CAVALLI, V. 2015. Activating Injury-Responsive Genes with Hypoxia Enhances Axon Regeneration through Neuronal HIF-1 α . *Neuron,* 88**,** 720-34.

CHO, Y. R., LIM, J. H., KIM, M. Y., KIM, T. W., HONG, B. Y., KIM, Y.-S., CHANG, Y. S., KIM, H. W. & PARK, C. W. 2014. Therapeutic Effects of Fenofibrate on Diabetic Peripheral Neuropathy by Improving Endothelial and Neural Survival in db/db Mice. *PLOS ONE,* 9**,** e83204.

CHUNG, J. O., CHUNG, M. Y., PARK, S.-Y., CHO, D. H. & CHUNG, D. J. 2021. Relationship between plasma growth differentiation factor-15 level and estimated glomerular filtration rate in type 2 diabetes patients with and without albuminuria. *Journal of Diabetes and its Complications,* 35**,** 107849.

CONEDERA, F. M., QUINTELA POUSA, A. M., PRESBY, D. M., MERCADER, N., ENZMANN, V. & TSCHOPP, M. 2021. Diverse Signaling by TGF β Isoforms in Response to Focal Injury is Associated with Either Retinal Regeneration or Reactive Gliosis. *Cellular and Molecular Neurobiology,* 41**,** 43-62.

CORRÊA, C. R., DIAS-MELICIO, L. A., CALVI, S. A., LASTÓRIA, S. & SOARES, A. M. V. C. 2011. Activation of monocytes and cytokine production in patients with peripheral atherosclerosis obliterans. *Journal of Inflammation,* 8**,** 23.

COSTA, R. H. R., CARDOSO, N. A., PROCÓPIO, R. J., NAVARRO, T. P., DARDIK, A. & DE LOIOLA CISNEROS, L. 2017. Diabetic foot ulcer carries high amputation and mortality rates, particularly in the presence of advanced age, peripheral artery disease and anemia. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews,* 11**,** S583-S587.

CUN, L. G., GHANI, M. W., YI, Z., JIANG, W., YE, L., BIN, L., BIRMANI, M. W., LILONG, A. & MEI, X. 2021. Different combinations of GABA, BMP7, and Activin A induced the in vitro

differentiation of rat pancreatic ductal stem cells into insulin-secreting islet-like cell clusters. *Life Sciences,* 267**,** 118451.

DEL PINO-SEDEÑO, T., TRUJILLO-MARTÍN, M. M., ANDIA, I., ARAGÓN-SÁNCHEZ, J., HERRERA-RAMOS, E., IRUZUBIETA BARRAGÁN, F. J. & SERRANO-AGUILAR, P. 2019. Platelet-rich plasma for the treatment of diabetic foot ulcers: A meta-analysis. *Wound Repair and Regeneration,* 27**,** 170-182.

DHAMODHARAN, U., KARAN, A., SIREESH, D., VAISHNAVI, A., SOMASUNDAR, A., RAJESH, K. & RAMKUMAR, K. M. 2019. Tissue-specific role of Nrf2 in the treatment of diabetic foot ulcers during hyperbaric oxygen therapy. *Free Radical Biology and Medicine,* 138**,** 53-62.

DISTLER, J. H. W., JÜNGEL, A., PILECKYTE, M., ZWERINA, J., MICHEL, B. A., GAY, R. E., KOWAL-BIELECKA, O., MATUCCI-CERINIC, M., SCHETT, G., MARTI, H. H., GAY, S. & DISTLER, O. 2007. Hypoxia-induced increase in the production of extracellular matrix proteins in systemic sclerosis. *Arthritis & Rheumatism,* 56**,** 4203-4215.

DOWNES, M. J., BRENNAN, M. L., WILLIAMS, H. C. & DEAN, R. S. 2016. Development of a critical appraisal tool to assess the quality of cross-sectional studies (AXIS). *BMJ Open,* 6**,** e011458.

DRAZNIN, B., ARODA, V. R., BAKRIS, G., BENSON, G., BROWN, F. M., FREEMAN, R., GREEN, J., HUANG, E., ISAACS, D., KAHAN, S., LEON, J., LYONS, S. K., PETERS, A. L., PRAHALAD, P., REUSCH, J. E. B., YOUNG-HYMAN, D., DAS, S. & KOSIBOROD, M. 2022. 12. Retinopathy, Neuropathy, and Foot Care: Standards of Medical Care in Diabetes-2022. *Diabetes Care,* 45**,** S185-s194.

DU, L., HAO, M., LI, C., WU, W., WANG, W., MA, Z., YANG, T., ZHANG, N., ISAAC, A. T., ZHU, X., SUN, Y., LU, Q. & YIN, X. 2017. Quercetin inhibited epithelial mesenchymal transition in diabetic rats, high-glucose-cultured lens, and SRA01/04 cells through transforming growth factor-β 2/phosphoinositide 3-kinase/Akt pathway. *Molecular and Cellular Endocrinology,* 452**,** 44-56.

EDLIN, R. S., TSAI, S., YAMANOUCHI, D., WANG, C., LIU, B. & KENT, K. C. 2009. Characterization of primary and restenotic atherosclerotic plaque from the superficial femoral artery: Potential role of Smad3 in regulation of SMC proliferation. *Journal of Vascular Surgery,* 49**,** 1289-1295.

EL-SALAMOUNI, N. S., GOWAYED, M. A., SEIFFEIN, N. L., ABDEL- MONEIM, R. A., KAMEL, M. A. & LABIB, G. S. 2021. Valsartan solid lipid nanoparticles integrated hydrogel: A challenging repurposed use in the treatment of diabetic foot ulcer, in-vitro/in-vivo experimental study. *International Journal of Pharmaceutics,* 592**,** 120091.

EL-SAWAF, E. S., SALEH, S., ABDALLAH, D. M., AHMED, K. A. & EL-ABHAR, H. S. 2021. Vitamin D and rosuvastatin obliterate peripheral neuropathy in a type-2 diabetes model through modulating Notch1, Wnt-10 α, TGF-β and NRF-1 crosstalk. *Life Sciences,* 279**,** 119697.

ELMADBOUH, I. & SINGLA, D. K. 2021. BMP-7 Attenuates Inflammation-Induced Pyroptosis and Improves Cardiac Repair in Diabetic Cardiomyopathy. *Cells,* 10**,** 2640.

EVERETT, E. & MATHIOUDAKIS, N. 2018. Update on management of diabetic foot ulcers. *Annals of the New York Academy of Sciences,* 1411**,** 153-165.

EWALD, D., PATEL, M. & HALL, G. 2001. HOSPITAL SEPARATIONS INDICATE INCREASING NEED FOR PREVENTION OF DIABETIC FOOT COMPLICATIONS IN CENTRAL AUSTRALIA. *Australian Journal of Rural Health,* 9**,** 275-279.

FATTAH, M. A., SHAHEEN, M. H. & MAHFOUZ, M. H. 2004. Disturbances of Haemostasis in Diabetes Mellitus. *Disease Markers,* 19**,** 797458.

FEJFAROVÁ, V., MATUŠKA, J., JUDE, E., PIŤHOVÁ, P., FLEKAČ, M., ROZTOČIL, K., WOSKOVÁ, V., DUBSKÝ, M., JIRKOVSKÁ, A., BÉM, R., HUSÁKOVÁ, J. & LÁNSKÁ, V. 2021. Stimulation TcPO2 Testing Improves Diagnosis of Peripheral Arterial Disease in Patients With Diabetic Foot. *Frontiers in Endocrinology,* 12.

FINNSON, K. W., ALMADANI, Y. & PHILIP, A. 2020. Non-canonical (non-SMAD2/3) TGF-β signaling in fibrosis: Mechanisms and targets. *Seminars in Cell & Developmental Biology,* 101**,** 115-122.

GALKOWSKA, H., WOJEWODZKA, U. & OLSZEWSKI, W. L. 2006. Chemokines, cytokines, and growth factors in keratinocytes and dermal endothelial cells in the margin of chronic diabetic foot ulcers. *Wound Repair and Regeneration,* 14**,** 558-565.

GAO, C., LIN, X., FAN, F., LIU, X., WAN, H., YUAN, T., ZHAO, X. & LUO, Y. 2022. Status of higher TGF-β1 and TGF-β2 levels in the aqueous humour of patients with diabetes and cataracts. *BMC Ophthalmology,* 22, 156.

GARCIA, C., FEVE, B., PERRE, P., HALIMI, S., BAIZRI, H., BORDIER, L., GUIU, G., DUPUY, O., BAUDUCEAU, B. & MAYAUDON, H. 2010. Diabetes and inflammation: Fundamental aspects and clinical implications. *Diabetes & Metabolism,* 36**,** 327-338.

GASCA-LOZANO, L. E., LUCANO-LANDEROS, S., RUIZ-MERCADO, H., SALAZAR-MONTES, A., SANDOVAL-RODRIGUEZ, A., GARCIA-BANUELOS, J., SANTOS-GARCIA, A., DAVILA-RODRIGUEZ, J. R., NAVARRO-PARTIDA, J., BOJORQUEZ-SEPULVEDA, H., CASTANEDA-GOMEZ, J., DOMINGUEZ-ROSALES, J., RUIZ-ARCOS, M. A., SANCHEZ-PARADA, M. G. & ARMENDARIZ-BORUNDA, J. 2017. Pirfenidone Accelerates Wound Healing in Chronic Diabetic Foot Ulcers: A Randomized, Double-Blind Controlled Trial. *Journal of Diabetes Research,* 2017.

GHANI, M. W., BIN, L., JIE, Y., YI, Z., JIANG, W., YE, L., CUN, L. G., BIRMANI, M. W., ZHUANGZHI, Z., LILONG, A. & MEI, X. 2020. Differentiation of rat pancreatic duct stem cells into insulin-secreting islet-like cell clusters through BMP7 inducement. *Tissue Cell,* 67**,** 101439.

GILLIGAN, A. M., WAYCASTER, C. R. & MOTLEY, T. A. 2015. Cost-effectiveness of becaplermin gel on wound healing of diabetic foot ulcers. *Wound Repair and Regeneration,* 23**,** 353-360.

GONIAS, S. L., CARMICHAEL, A., METTENBURG, J. M., ROADCAP, D. W., IRVIN, W. P. & WEBB, D. J. 2000. Identical or Overlapping Sequences in the Primary Structure of Human α₂-Macroglobulin Are Responsible for the Binding of Nerve Growth Factor-β, Platelet-derived Growth Factor-BB, and Transforming Growth Factor-β *. *Journal of Biological Chemistry,* 275**,** 5826-5831.

GOULD, S. E., DAY, M., JONES, S. S. & DORAI, H. 2002. BMP-7 regulates chemokine, cytokine, and hemodynamic gene expression in proximal tubule cells¹. *Kidney International,* 61**,** 51-60.

GRAINGER, D. J., MOSEDALE, D. E., METCALFE, J. C., WEISSBERG, P. L. & KEMP, P. R. 1995. Active and acid-activatable TGF-β in human sera, platelets and plasma. *Clinica Chimica Acta,* 235**,** 11-31.

GUO, J., LIN, Q., SHAO, Y., RONG, L. & ZHANG, D. 2017. BMP-7 suppresses excessive scar formation by activating the BMP-7/Smad1/5/8 signaling pathway. *Mol Med Rep,* 16**,** 1957-1963.

GUO, S., CHEN, Q., SUN, Y. & CHEN, J. 2019. Nicotinamide protects against skeletal muscle atrophy in streptozotocin-induced diabetic mice. *Archives of Physiology and Biochemistry,* 125**,** 470-477.

GUO, X., MENG, G., LIU, F., ZHANG, Q., LIU, L., WU, H., DU, H., SHI, H., XIA, Y., LIU, X., LI, C., BAO, X., SU, Q., GU, Y., FANG, L., YU, F., YANG, H., YU, B., SUN, S., WANG, X., ZHOU, M., JIA, Q., CHEN, X., HUANG, G., SONG, K. & NIU, K. 2016. Serum levels of immunoglobulins in an adult population and their relationship with type 2 diabetes. *Diabetes Research and Clinical Practice,* 115**,** 76-82.

HA, D. M., CARPENTER, L. C., KOUTAKIS, P., SWANSON, S. A., ZHU, Z., HANNA, M., DESPIEGELAERE, H. K., PIPINOS, I. I. & CASALE, G. P. 2016. Transforming growth factor-beta 1 produced by vascular smooth muscle cells predicts fibrosis in the gastrocnemius of patients with peripheral artery disease. *Journal of Translational Medicine,* 14**,** 39.

HAN, J., ZHENG, Q., CHENG, Y., LIU, Y., BAI, Y., YAN, B., GUO, S., YU, J., LI, X. & WANG, C. 2022. Toll-like receptor 9 (TLR9) gene deletion-mediated fracture healing in type II diabetic osteoporosis associates with inhibition of the nuclear factor-kappa B (NF-κB) signaling pathway. *Bioengineered,* 13**,** 13689-13702.

HE, R., LI, Q., SHEN, W., WANG, T., LU, H., LU, J., LU, F., LUO, M., ZHANG, J., GAO, H., WANG, D., XING, W., JIA, W. & LIU, F. 2020. The efficacy and safety of cold atmospheric plasma as a

novel therapy for diabetic wound in vitro and in vivo. *International Wound Journal,* 17**,** 851-863.

HERMAN-EDELSTEIN, M., THOMAS, M. C., THALLAS-BONKE, V., SALEEM, M., COOPER, M. E. & KANTHARIDIS, P. 2011. Dedifferentiation of Immortalized Human Podocytes in Response to Transforming Growth Factor-β :A Model for Diabetic Podocytopathy. *Diabetes,* 60**,** 1779-1788.

HOLMES, C. J. & HASTINGS, M. K. 2021. The Application of Exercise Training for Diabetic Peripheral Neuropathy. *Journal of Clinical Medicine,* 10.

HOPKINS, B. D., GONCALVES, M. D. & CANTLEY, L. C. 2020. Insulin–PI3K signalling: an evolutionarily insulated metabolic driver of cancer. *Nature Reviews Endocrinology,* 16**,** 276-283.

HUANG, S. S., O'GRADY, P. & HUANG, J. S. 1988. Human transforming growth factor beta.alpha 2-macroglobulin complex is a latent form of transforming growth factor beta. *Journal of Biological Chemistry,* 263**,** 1535-1541.

HUANG, T., SHU, Y. & CAI, Y.-D. 2015. Genetic differences among ethnic groups. *BMC Genomics,* 16**,** 1093.

HUANG, X., LIANG, P., JIANG, B., ZHANG, P., YU, W., DUAN, M., GUO, L., CUI, X., HUANG, M. & HUANG, X. 2020. Hyperbaric oxygen potentiates diabetic wound healing by promoting fibroblast cell proliferation and endothelial cell angiogenesis. *Life Sciences,* 259**,** 118246.

HUSE, K., BAKKEBØ, M., OKSVOLD, M. P., FORFANG, L., HILDEN, V. I., STOKKE, T., SMELAND, E. B. & MYKLEBUST, J. H. 2011. Bone morphogenetic proteins inhibit CD40L/IL-21-induced Ig production in human Bcells: Differential effects of BMP-6 and BMP-7. *European Journal of Immunology,* 41**,** 3135-3145.

HUSSAIN, G., RIZVI, S. A. A., SINGHAL, S., ZUBAIR, M. & AHMAD, J. 2016. Serum levels of TGF-β 1 in patients of diabetic peripheral neuropathy and its correlation with nerve conduction velocity in type 2 diabetes mellitus. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews,* 10**,** S135-S139.

ISHIZAKA, N., ISHIZAKA, Y., NAGAI, R., TODA, E.-I., HASHIMOTO, H. & YAMAKADO, M. 2007. Association between serum albumin, carotid atherosclerosis, and metabolic syndrome in Japanese individuals. *Atherosclerosis,* 193**,** 373-379.

ISMAEEL, A., MISERLIS, D., PAPOUTSI, E., HAYNATZKI, G., BOHANNON, W. T., SMITH, R. S., EIDSON, J. L., CASALE, G. P., PIPINOS, I. I. & KOUTAKIS, P. 2022. Endothelial cell-derived pro-fibrotic factors increase TGF-β 1 expression by smooth muscle cells in response to cycles of hypoxia-hyperoxia. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1868**,** 166278.

IVERSEN, M. M., TELL, G. S., RIISE, T., HANESTAD, B. R., ØSTBYE, T., GRAUE, M. & MIDTHJELL, K. 2009. History of foot ulcer increases mortality among individuals with diabetes: ten-year follow-up of the Nord-Trøndelag Health Study, Norway. *Diabetes Care,* 32**,** 2193-2199.

IWANO, M., KUBO, A., NISHINO, T., SATO, H., NISHIOKA, H., AKAI, Y., KURIOKA, H., FUJII, Y., KANAUCHI, M., SHIIKI, H. & DOHI, K. 1996. Quantification of glomerular TGF-β 1 mRNA in patients with diabetes mellitus. *Kidney International,* 49**,** 1120-1126.

JEFFCOATE, W. J. & HARDING, K. G. 2003. Diabetic foot ulcers. *The Lancet,* 361**,** 1545-1551.

JIANG, Y., RAN, X., JIA, L., YANG, C., WANG, P., MA, J., CHEN, B., YU, Y., FENG, B., CHEN, L., YIN, H., CHENG, Z., YAN, Z., YANG, Y., LIU, F. & XU, Z. 2015. Epidemiology of Type 2 Diabetic Foot Problems and Predictive Factors for Amputation in China. *The International Journal of Lower Extremity Wounds,* 14**,** 19-27.

JIMI, S., JAGUPAROV, A., NURKESH, A., SULTANKULOV, B. & SAPAROV, A. 2020. Sequential Delivery of Cryogel Released Growth Factors and Cytokines Accelerates Wound Healing and Improves Tissue Regeneration. *Frontiers in Bioengineering and Biotechnology,* 8.

JIN, X., AIMAITI, Y., CHEN, Z., WANG, W. & LI, D. 2018. Hepatic stellate cells promote angiogenesis via the TGF-β 1-Jagged1/VEGFA axis. *Experimental Cell Research,* 373**,** 34-43.

JOHN, P. & YADLA, M. 2019. Noninvasive method of differentiating diabetic nephropathy and nondiabetic renal disease using serum bone morphogenetic protein-7 and transforming

growth factor-beta 1 levels in patients with type-2 diabetes mellitus. *Saudi Journal of Kidney Diseases and Transplantation,* 30**,** 1300-1309.

JUDE, E. B., BLAKYTNY, R., BULMER, J., BOULTON, A. J. M. & FERGUSON, M. W. J. 2002. Transforming growth factor-beta 1, 2, 3 and receptor type I and II in diabetic foot ulcers. *Diabetic Medicine,* 19**,** 440-447.

JUDE, E. B., BOULTON, A. J. M., FERGUSON, M. W. J. & APPLETON, I. 1999. The role of nitric oxide synthase isoforms and arginase in the pathogenesis of diabetic foot ulcers: possible modulatory effects by transforming growth factor beta 1. *Diabetologia,* 42**,** 748-757.

KABUTO, Y., MORIHARA, T., SUKENARI, T., KIDA, Y., ODA, R., ARAI, Y., SAWADA, K., MATSUDA, K., KAWATA, M., TABATA, Y., FUJIWARA, H. & KUBO, T. 2015. Stimulation of Rotator Cuff Repair by Sustained Release of Bone Morphogenetic Protein-7 Using a Gelatin Hydrogel Sheet. *Tissue Engineering Part A,* 21**,** 2025-33.

KAMINSKA, B., WESOLOWSKA, A. & DANILKIEWICZ, M. 2005. TGF beta signalling and its role in tumour pathogenesis. *Acta Biochimica Polonica,* 52**,** 329-337.

KANDHARE, A. D., GHOSH, P. & BODHANKAR, S. L. 2014. Naringin, a flavanone glycoside, promotes angiogenesis and inhibits endothelial apoptosis through modulation of inflammatory and growth factor expression in diabetic foot ulcer in rats. *Chemico-Biological Interactions,* 219**,** 101-112.

KARAM, R. A., REZK, N. A., ABDEL RAHMAN, T. M. & AL SAEED, M. 2018. Effect of negative pressure wound therapy on molecular markers in diabetic foot ulcers. *Gene,* 667**,** 56-61.

KATSAROU, A., GUDBJÖRNSDOTTIR, S., RAWSHANI, A., DABELEA, D., BONIFACIO, E., ANDERSON, B. J., JACOBSEN, L. M., SCHATZ, D. A. & LERNMARK, Å. 2017. Type 1 diabetes mellitus. *Nature Reviews Disease Primers,* 3**,** 17016.

KIM, S., JEONG, C.-H., SONG, S. H., UM, J. E., KIM, H. S., YUN, J. S., HAN, D., CHO, E. S., NAM, B. Y., YOOK, J. I., KU, M., YANG, J., KIM, M.-D., KIM, N. H. & YOO, T.-H. 2020. Micellized Protein Transduction Domain-Bone Morphogenetic Protein-7 Efficiently Blocks Renal Fibrosis Via Inhibition of Transforming Growth Factor-Beta–Mediated Epithelial–Mesenchymal Transition. *Frontiers in Pharmacology,* 11.

KORBUT, A. I., ROMANOV, V. V. & KLIMONTOV, V. V. 2023. Urinary Markers of Tubular Injury and Renal Fibrosis in Patients with Type 2 Diabetes and Different Phenotypes of Chronic Kidney Disease. *Life (Basel),* 13.

KOWTHARAPU, B. S., PRAKASAM, R. K., MURÍN, R., KOCZAN, D., STAHNKE, T., WREE, A., JÜNEMANN, A. G. M. & STACHS, O. 2018. Role of Bone Morphogenetic Protein 7 (BMP7) in the Modulation of Corneal Stromal and Epithelial Cell Functions. *International Journal of Molecular Sciences,* 19**,** 1415.

KREINER, F. F., KRAAIJENHOF, J. M., VON HERRATH, M., HOVINGH, G. K. K. & VON SCHOLTEN, B. J. 2022. Interleukin 6 in diabetes, chronic kidney disease, and cardiovascular disease: mechanisms and therapeutic perspectives. *Expert Review of Clinical Immunology,* 18**,** 377-389.

KRISHNAN, P., PURUSHOTHAMAN, K. R., PURUSHOTHAMAN, M., TURNBULL, I. C., TARRICONE, A., VASQUEZ, M., JAIN, S., BABER, U., LASCANO, R. A., KINI, A. S., SHARMA, S. K. & MORENO, P. R. 2016. Enhanced neointimal fibroblast, myofibroblast content and altered extracellular matrix composition: Implications in the progression of human peripheral artery restenosis. *Atherosclerosis,* 251**,** 226-233.

KUPCZYK, D., BILSKI, R., PRZEWIEZLIKOWSKI, A., STUDZINSKA, R. & WOZNIAK, A. 2021. Concentration of proinflammatory cytokines in patients with ulcers as a complication of type 2 diabetes mellitus. *Postepy Dermatologii I Alergologii,* 38**,** 767-772.

KURAUTI, M. A., COSTA-JÚNIOR, J. M., FERREIRA, S. M., SANTOS, G. J., SPONTON, C. H. G., CARNEIRO, E. M., TELLES, G. D., CHACON-MIKAHIL, M. P. T., CAVAGLIERI, C. R., REZENDE, L. F. & BOSCHERO, A. C. 2017. Interleukin-6 increases the expression and activity of insulin-degrading enzyme. *Scientific Reports,* 7**,** 46750.

LAMARRE, J., HAYES, M. A., WOLLENBERG, G. K., HUSSAINI, I., HALL, S. W. & GONIAS, S. L. 1991. An alpha 2-macroglobulin receptor-dependent mechanism for the plasma clearance of transforming growth factor-beta 1 in mice. *The Journal of Clinical Investigation,* 87**,** 39-44.

LANDI, M., EVERITT, J. & BERRIDGE, B. 2021. Bioethical, Reproducibility, and Translational Challenges of Animal Models. *ILAR Journal,* 62**,** 60-65.

LAVERY, L. A., KILLEEN, A. L., FARRAR, D., AKGUL, Y., CRISOLOGO, P. A., MALONE, M. & DAVIS, K. E. 2020. The effect of continuous diffusion of oxygen treatment on cytokines, perfusion, bacterial load, and healing in patients with diabetic foot ulcers. *International Wound Journal,* 17**,** 1986-1995.

LAZZARINI, P. A., VAN NETTEN, J. J., FITRIDGE, R. A., GRIFFITHS, I., KINNEAR, E. M., MALONE, M., PERRIN, B. M., PRENTICE, J. & WRAIGHT, P. R. 2018. Pathway to ending avoidable diabetes-related amputations in Australia. *Medical Journal of Australia,* 209**,** 288-290.

LEE, C. M. Y., COLAGIURI, R., MAGLIANO, D. J., CAMERON, A. J., SHAW, J., ZIMMET, P. & COLAGIURI, S. 2013. The cost of diabetes in adults in Australia. *Diabetes Research and Clinical Practice,* 99**,** 385-390.

LETTERIO, J. J. & ROBERTS, A. B. 1998. Regulation of immune responses by TGF-beta. *Annual Review of Immunology,* 16**,** 137-61.

LI, J., CHOU, H., LI, L., LI, H. & CUI, Z. 2020. Wound healing activity of neferine in experimental diabetic rats through the inhibition of inflammatory cytokines and nrf-2 pathway. *Artificial Cells, Nanomedicine, and Biotechnology,* 48**,** 96-106.

LI, L., CHEN, D., WANG, C., LIU, G. & RAN, X. 2015. The Effect of Autologous Platelet-Rich Gel on the Dynamic Changes of the Matrix Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinase-2 Expression in the Diabetic Chronic Refractory Cutaneous Ulcers. *Journal of Diabetes Research,* 2015**,** 954701.

LI, RUI X., YIU, WAI H., WU, HAO J., WONG, DICKSON W. L., CHAN, LORETTA Y. Y., LIN, M., LEUNG, JOSEPH C. K., LAI, KAR N. & TANG, SYDNEY C. W. 2014. BMP7 reduces inflammation and oxidative stress in diabetic tubulopathy. *Clinical Science,* 128**,** 269-280.

LI, W., FANG, Q., ZHONG, P., CHEN, L., WANG, L., ZHANG, Y., WANG, J., LI, X., WANG, Y., WANG, J. & LIANG, G. 2016. EGFR Inhibition Blocks Palmitic Acid-induced inflammation in cardiomyocytes and Prevents Hyperlipidemia-induced Cardiac Injury in Mice. *Scientific Reports,* 6**,** 24580.

LI, W., KANDHARE, A. D., MUKHERJEE, A. A. & BODHANKAR, S. L. 2018. Hesperidin, a plant flavonoid accelerated the cutaneous wound healing in streptozotocin-induced diabetic rats: Role of TGF-ß/Smads and Ang-1/Tie-2 signaling pathways. *EXCLI journal,* 17**,** 399-419.

LI, X., WEBER, N. C., COHN, D. M., HOLLMANN, M. W., DEVRIES, J. H., HERMANIDES, J. & PRECKEL, B. 2021. Effects of Hyperglycemia and Diabetes Mellitus on Coagulation and Hemostasis. *Journal of Clinical Medicine,* 10**,** 2419.

LIM, A. I., CHAN, L. Y. Y., TANG, S. C. W., YIU, W. H., LI, R., LAI, K. N. & LEUNG, J. C. K. 2014. BMP-7 represses albumin-induced chemokine synthesis in kidney tubular epithelial cells through destabilization of NF- κ B-inducing kinase. *Immunology & Cell Biology,* 92**,** 427-435.

LIN, C. J., LAN, Y. M., OU, M. Q., JI, L. Q. & LIN, S. D. 2019. Expression of miR-217 and HIF-1 α /VEGF pathway in patients with diabetic foot ulcer and its effect on angiogenesis of diabetic foot ulcer rats. *Journal of Endocrinological Investigation,* 42**,** 1307-1317.

LIN, X., XU, Y., PAN, X., XU, J., DING, Y., SUN, X., SONG, X., REN, Y. & SHAN, P.-F. 2020. Global, regional, and national burden and trend of diabetes in 195 countries and territories: an analysis from 1990 to 2025. *Scientific Reports,* 10**,** 14790.

LIU, L., WANG, Y., YAN, R., LIANG, L., ZHOU, X., LIU, H., ZHANG, X., MAO, Y., PENG, W., XIAO, Y., ZHANG, F., LIU, L., SHI, M. & GUO, B. 2019. BMP-7 inhibits renal fibrosis in diabetic nephropathy via miR-21 downregulation. *Life Sciences,* 238**,** 116957.

LIU, Y., MIN, D., BOLTON, T., NUÉE, V., TWIGG, S. M., YUE, D. K. & MCLENNAN, S. V. 2009. Increased matrix metalloproteinase-9 predicts poor wound healing in diabetic foot ulcers. *Diabetes Care,* 32**,** 117-119.

LLOYD, C. E., KALINYAK, J. E., HUTSON, S. M. & JEFFERSON, L. S. 1987. Stimulation of albumin gene transcription by insulin in primary cultures of rat hepatocytes. *American Journal of Physiology-Cell Physiology,* 252**,** C205-C214.

LOOTS, M. A. M., LAMME, E. N., MEKKES, J. R., BOS, J. D. & MIDDELKOOP, E. 1999. Cultured fibroblasts from chronic diabetic wounds on the lower extremity (non-insulin-dependent diabetes mellitus) show disturbed proliferation. *Archives of Dermatological Research,* 291**,** 93-99.

LUKIC, L., LALIC, N. M., RAJKOVIC, N., JOTIC, A., LALIC, K., MILICIC, T., SEFEROVIC, J. P., MACESIC, M. & GAJOVIC, J. S. 2014. Hypertension in Obese Type 2 Diabetes Patients is Associated with Increases in Insulin Resistance and IL-6 Cytokine Levels: Potential Targets for an Efficient Preventive Intervention. *International Journal of Environmental Research and Public Health,* 11**,** 3586-3598.

MAIONE, A. G., SMITH, A., KASHPUR, O., YANEZ, V., KNIGHT, E., MOONEY, D. J., VEVES, A., TOMIC-CANIC, M. & GARLICK, J. A. 2016. Altered ECM deposition by diabetic foot ulcer-derived fibroblasts implicates fibronectin in chronic wound repair. *Wound Repair and Regeneration,* 24**,** 630-643.

MAO, X., LI, Z., LI, B. & WANG, H. 2021. Baicalin regulates mRNA expression of VEGF-c, Ang-1/Tie2, TGF-β and Smad2/3 to inhibit wound healing in streptozotocin-induced diabetic foot ulcer rats. *Journal of Biochemical and Molecular Toxicology,* 35**,** e22893.

MATHAVAN, N., RAINA, D. B., TAGIL, M. & ISAKSSON, H. 2020. Longitudinal in vivo monitoring of callus remodeling in BMP-7- and Zoledronate-treated fractures. *Journal of Orthopaedic Research,* 38**,** 1905-1913.

MATSUKI, K., HATHAWAY, C. K., LAWRENCE, M. G., SMITHIES, O. & KAKOKI, M. 2014. The role of transforming growth factor β1 in the regulation of blood pressure. *Current Hypertension Reviews,* 10**,** 223-38.

MAZER, N. A., WELBOURN, D., BERNSTEIN, R. K. & RUBINSTEIN, A. 2011. Immunoglobulin Deficiency in Patients with Diabetes Mellitus. *Journal of Allergy and Clinical Immunology,* 127**,** AB12.

MCDERMOTT, M. M., GURALNIK, J. M., CORSI, A., ALBAY, M., MACCHI, C., BANDINELLI, S. & FERRUCCI, L. 2005. Patterns of inflammation associated with peripheral arterial disease: The InCHIANTI study. *American Heart Journal,* 150**,** 276-281.

MCLENNAN, S., YUE, D. & TWIGG, S. 2006. *Molecular Aspects of Wound Healing in Diabetes*, Cambridge Publishing.

MCMILLAN, D. E. 1970. Changes in serum proteins and protein-bound carbohydrates in diabetes mellitus. *Diabetologia,* 6**,** 597-604.

MORBACH, S., FURCHERT, H., GRÖBLINGHOFF, U., HOFFMEIER, H., KERSTEN, K., KLAUKE, G.-T., KLEMP, U., RODEN, T., ICKS, A., HAASTERT, B., RÜMENAPF, G., ABBAS, Z. G., BHARARA, M. & ARMSTRONG, D. G. 2012. Long-Term Prognosis of Diabetic Foot Patients and Their Limbs: Amputation and death over the course of a decade. *Diabetes Care,* 35**,** 2021-2027.

MOULIK, P. K., MTONGA, R. & GILL, G. V. 2003. Amputation and Mortality in New-Onset Diabetic Foot Ulcers Stratified by Etiology. *Diabetes Care,* 26**,** 491-494.

MÜHLFELD, A. S., SPENCER, M. W., HUDKINS, K. L., KIRK, E., LEBOEUF, R. C. & ALPERS, C. E. 2004. Hyperlipidemia aggravates renal disease in B6.ROP Os/+ mice. *Kidney International,* 66**,** 1393-1402.

MUSSA, B. M., SRIVASTAVA, A., AL-HABSHI, A., MOHAMMED, A. K., HALWANI, R. & ABUSNANA, S. 2021. Inflammatory Biomarkers Levels in T2DM Emirati Patients with Diabetic Neuropathy. *Diabetes, Metabolic Syndrome and Obesity,* 14**,** 3389-3397.

NARASIMHULU, C. A. & SINGLA, D. K. 2023. BMP-7 Attenuates Sarcopenia and Adverse Muscle Remodeling in Diabetic Mice via Alleviation of Lipids, Inflammation, HMGB1, and Pyroptosis. *Antioxidants,* 12**,** 331.

NEVES, L. M. G., WILGUS, T. A. & BAYAT, A. 2023. In Vitro, Ex Vivo, and In Vivo Approaches for Investigation of Skin Scarring: Human and Animal Models. *Advances in Wound Care (New Rochelle),* 12**,** 97-116.

NING, J., ZHAO, H., CHEN, B., MI, E. Z., YANG, Z., QING, W., LAM, K. W. J., YI, B., CHEN, Q., GU, J., ICHIM, T., BOGIN, V., LU, K. & MA, D. 2019. Argon Mitigates Impaired Wound Healing Process and Enhances Wound Healing In Vitro and In Vivo. *Theranostics,* 9**,** 477-490.

O'CONNOR-MCCOURT, M. D. & WAKEFIELD, L. M. 1987. Latent transforming growth factor-beta in serum. A specific complex with alpha 2-macroglobulin. *The Journal of Biological Chemistry,* 262**,** 14090-9.

O'ROURKE, S., STEFFEN, C., RAULLI, A. & TULIP, F. 2013. Diabetic major amputation in Far North Queensland 1998–2008: What is the Gap for Indigenous patients? *Australian Journal of Rural Health,* 21**,** 268-273.

OHIGASHI, M., IMAI, N., TOBA, H., KOBARA, M. & NAKATA, T. 2016. Pitavastatin Exhibits Protective Effects on Podocytes Accompanied by BMP-7 Up-Regulation and Rho Suppression. *Pharmacology,* 97**,** 265-276.

OHIGASHI, M., KOBARA, M., TAKAHASHI, T., TOBA, H., WADA, T. & NAKATA, T. 2017. Pitavastatin suppresses hyperglycaemia-induced podocyte injury via bone morphogenetic protein-7 preservation. *Clinical and Experimental Pharmacology and Physiology,* 44**,** 378-385.

OLIVER-KRASINSKI, J. M. & STOFFERS, D. A. 2008. On the origin of the β cell. *Genes & Development,* 22**,** 1998-2021.

OUYANG, C., HUANG, L., YE, X., REN, M. & HAN, Z. 2022. HDAC1 Promotes Myocardial Fibrosis in Diabetic Cardiomyopathy by Inhibiting BMP-7 Transcription Through Histone Deacetylation. *Experimental and Clinical Endocrinology & Diabetes*.

PAGE, M. J., MCKENZIE, J. E., BOSSUYT, P. M., BOUTRON, I., HOFFMANN, T. C., MULROW, C. D., SHAMSEER, L., TETZLAFF, J. M., AKL, E. A., BRENNAN, S. E., CHOU, R., GLANVILLE, J., GRIMSHAW, J. M., HRÓBJARTSSON, A., LALU, M. M., LI, T., LODER, E. W., MAYO-WILSON, E., MCDONALD, S., MCGUINNESS, L. A., STEWART, L. A., THOMAS, J., TRICCO, A. C., WELCH, V. A., WHITING, P. & MOHER, D. 2021. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ,* 372**,** n71.

PARK, K. H., HAN, S. H., HONG, J. P., HAN, S.-K., LEE, D.-H., KIM, B. S., AHN, J. H. & LEE, J. W. 2018. Topical epidermal growth factor spray for the treatment of chronic diabetic foot ulcers: A phase III multicenter, double-blind, randomized, placebo-controlled trial. *Diabetes Research and Clinical Practice,* 142**,** 335-344.

PATSALOS, O., DALTON, B. & HIMMERICH, H. 2020. Effects of IL-6 Signaling Pathway Inhibition on Weight and BMI: A Systematic Review and Meta-Analysis. *International Journal of Molecular Sciences,* 21**,** 6290.

PEAVY, D. E., TAYLOR, J. M. & JEFFERSON, L. S. 1985. Time course of changes in albumin synthesis and mRNA in diabetic and insulin-treated diabetic rats. *American Journal of Physiology-Encrinology and Metabolism,* 248**,** E656-63.

PENG, W., ZHOU, X., XU, T., MAO, Y., ZHANG, X., LIU, H., LIANG, L., LIU, L., LIU, L., XIAO, Y., ZHANG, F., LI, S., SHI, M., ZHOU, Y., TANG, L., WANG, Y. & GUO, B. 2022. BMP-7 ameliorates partial epithelial-mesenchymal transition by restoring SnoN protein level via Smad1/5 pathway in diabetic kidney disease. *Cell Death & Disease,* 13**,** 254.

PERERA, N., RITCHIE, R. H. & TATE, M. 2020. The Role of Bone Morphogenetic Proteins in Diabetic Complications. *ACS Pharmacology & Translational Science,* 3**,** 11-20.

PEREZ-GOMEZ, M. V., PIZARRO-SANCHEZ, S., GRACIA-IGUACEL, C., CANO, S., CANNATA-ORTIZ, P., SANCHEZ-RODRIGUEZ, J., SANZ, A. B., SANCHEZ-NIÑO, M. D. & ORTIZ, A. 2021. Urinary

Growth Differentiation Factor-15 (GDF15) levels as a biomarker of adverse outcomes and biopsy findings in chronic kidney disease. *Journal of Nephrology*.

PHANG, S. J., ARUMUGAM, B., KUPPUSAMY, U. R., FAUZI, M. B. & LOOI, M. L. 2021. A review of diabetic wound models—Novel insights into diabetic foot ulcer. *Journal of Tissue Engineering and Regenerative Medicine,* 15**,** 1051-1068.

POPESCU, M., BOGDAN, C., PINTEA, A., RUGINĂ, D. & IONESCU, C. 2018. Antiangiogenic cytokines as potential new therapeutic targets for resveratrol in diabetic retinopathy. *Drug Design, Development and Therapy,* 12**,** 1985-1996.

POSTLETHWAITE, A. E., RAGHOW, R., STRICKLIN, G., BALLOU, L. & SAMPATH, T. K. 1994. Osteogenic protein-1, a bone morphogenic protein member of the TGF-β superfamily, shares chemotactic but not fibrogenic properties with TGF-β. *Journal of Cellular Physiology,* 161**,** 562-570.

PRADHAN, A. D., MANSON, J. E., RIFAI, N., BURING, J. E. & RIDKER, P. M. 2001. C-Reactive Protein, Interleukin 6, and Risk of Developing Type 2 Diabetes Mellitus. *JAMA,* 286**,** 327-334.

PRIMADHI, R. A. & HERMAN, H. 2021. Diabetic foot: Which one comes first, the ulcer or the contracture? *World journal of orthopedics,* 12**,** 61-68.

PROMPERS, L., HUIJBERTS, M., APELQVIST, J., JUDE, E., PIAGGESI, A., BAKKER, K., EDMONDS, M., HOLSTEIN, P., JIRKOVSKA, A., MAURICIO, D., RAGNARSON TENNVALL, G., REIKE, H., SPRAUL, M., UCCIOLI, L., URBANCIC, V., VAN ACKER, K., VAN BAAL, J., VAN MERODE, F. & SCHAPER, N. 2007. High prevalence of ischaemia, infection and serious comorbidity in patients with diabetic foot disease in Europe. Baseline results from the Eurodiale study. *Diabetologia,* 50**,** 18-25.

PSCHERER, S., FREUDE, T., FORST, T., NUSSLER, A. K., BRAUN, K. F. & EHNERT, S. 2013. Anti-diabetic treatment regulates pro-fibrotic TGF-β serum levels in type 2 diabetics. *Diabetology & Metabolic Syndrome,* 5**,** 48.

QU, D., LIU, J., LAU, C. W. & HUANG, Y. 2014. IL-6 in diabetes and cardiovascular complications. *British Journal of Pharmacology,* 171**,** 3595-603.

RAI, V., MOELLMER, R. & AGRAWAL, D. K. 2022. Clinically relevant experimental rodent models of diabetic foot ulcer. *Molecular and Cellular Biochemistry,* 477**,** 1239-1247.

RAMIREZ, H., PATEL, S. B. & PASTAR, I. 2014. The Role of TGFβ Signaling in Wound Epithelialization. *Advances in wound care,* 3**,** 482-491.

RAMZY, M. M., ABDALLA, A. M., ZENHOM, N. M., OKASHA, A. M., ABDELKAFY, A. E. & SALEH, R. K. 2019. Therapeutic effect of liraglutide on expression of CTGF and BMP-7 in induced diabetic nephropathy. *Journal of Cellular Biochemistry,* 120**,** 17512-17519.

REIBER, G. E. 1996. The Epidemiology of Diabetic Foot Problems. *Diabetic Medicine,* 13**,** S6-S11.

RIBITSCH, I., BAPTISTA, P. M., LANGE-CONSIGLIO, A., MELOTTI, L., PATRUNO, M., JENNER, F., SCHNABL-FEICHTER, E., DUTTON, L. C., CONNOLLY, D. J., VAN STEENBEEK, F. G., DUDHIA, J. & PENNING, L. C. 2020. Large Animal Models in Regenerative Medicine and Tissue Engineering: To Do or Not to Do. *Frontiers in Bioengineering and Biotechnology,* 8.

ROCHER, C. & SINGLA, D. K. 2013. SMAD-PI3K-Akt-mTOR Pathway Mediates BMP-7 Polarization of Monocytes into M2 Macrophages. *PLOS ONE,* 8**,** e84009.

ROCHER, C., SINGLA, R., SINGAL, P. K., PARTHASARATHY, S. & SINGLA, D. K. 2012. Bone morphogenetic protein 7 polarizes THP-1 cells into M2 macrophages. *Canadian Journal of Physiology and Pharmacology,* 90**,** 947-51.

RODRIGUES, M., KOSARIC, N., BONHAM, C. A. & GURTNER, G. C. 2019. Wound Healing: A Cellular Perspective. *Physiological Reviews,* 99**,** 665-706.

ROGERS, L. C., FRYKBERG, R. G., ARMSTRONG, D. G., BOULTON, A. J. M., EDMONDS, M., VAN, G. H., HARTEMANN, A., GAME, F., JEFFCOATE, W., JIRKOVSKA, A., JUDE, E., MORBACH, S., MORRISON, W. B., PINZUR, M., PITOCCO, D., SANDERS, L., WUKICH, D. K. & UCCIOLI, L. 2011. The Charcot Foot in Diabetes. *Diabetes Care,* 34**,** 2123.

SALUJA, S., ANDERSON, S. G., HAMBLETON, I., SHOO, H., LIVINGSTON, M., JUDE, E. B., LUNT, M., DUNN, G. & HEALD, A. H. 2020. Foot ulceration and its association with mortality in diabetes mellitus: a meta-analysis. *Diabetic Medicine,* 37**,** 211-218.

SANAPALLI, B. K. R., YELE, V., SINGH, M. K., THAGGIKUPPE KRISHNAMURTHY, P. & KARRI, V. V. S. R. 2021. Preclinical models of diabetic wound healing: A critical review. *Biomedicine & Pharmacotherapy,* 142**,** 111946.

SCHOLMAN, R. C., GIOVANNONE, B., HIDDINGH, S., MEERDING, J. M., MALVAR FERNANDEZ, B., VAN DIJK, M. E. A., TEMPELMAN, M. J., PRAKKEN, B. J. & DE JAGER, W. 2018. Effect of anticoagulants on 162 circulating immune related proteins in healthy subjects. *Cytokine,* 106**,** 114-124.

SCHULTZ, O., OBERHAUSER, F., SAECH, J., RUBBERT-ROTH, A., HAHN, M., KRONE, W. & LAUDES, M. 2010. Effects of Inhibition of Interleukin-6 Signalling on Insulin Sensitivity and Lipoprotein (A) Levels in Human Subjects with Rheumatoid Diseases. *PLOS ONE,* 5**,** e14328.

SCONOCCHIA, T., HOCHGERNER, M., SCHWARZENBERGER, E., TAM-AMERSDORFER, C., BOREK, I., BENEZEDER, T., BAUER, T., ZYULINA, V., PAINSI, C., PASSEGGER, C., WOLF, P., SIBILIA, M. & STROBL, H. 2021. Bone morphogenetic protein signaling regulates skin inflammation via modulating dendritic cell function. *Journal of Allergy and Clinical Immunology,* 147**,** 1810-1822.e9.

SCONOCCHIA, T. & SCONOCCHIA, G. 2021. Regulation of the Immune System in Health and Disease by Members of the Bone Morphogenetic Protein Family. *Frontiers in Immunology,* 12.

SENDUR, S. N., FIRLATAN, B., BAYKAL, G., LAY, I. & ERBAS, T. 2022. Serum growth differentiation factor-15 levels are associated with the severity of diabetic foot ulcer. *Hormones,* 21**,** 719-728.

SHAKER, O. G. & SADIK, N. A. H. 2013. Transforming growth factor beta 1 and monocyte chemoattractant protein-1 as prognostic markers of diabetic nephropathy. *Human & Experimental Toxicology,* 32**,** 1089-1096.

SHI, Y. & MASSAGUÉ, J. 2003. Mechanisms of TGF-β Signaling from Cell Membrane to the Nucleus. *Cell,* 113**,** 685-700.

SIMPSON, S., KAISLASUO, J., GULLER, S. & PAL, L. 2020. Thermal stability of cytokines: A review. *Cytokine,* 125**,** 154829.

SINGLA, D. K., SINGLA, R. & WANG, J. 2016. BMP-7 Treatment Increases M2 Macrophage Differentiation and Reduces Inflammation and Plaque Formation in Apo E-/- Mice. *PLOS ONE,* 11**,** e0147897.

SKUNDRIC, D. S. & LISAK, R. P. 2003. Role of neuropoietic cytokines in development and progression of diabetic polyneuropathy: from glucose metabolism to neurodegeneration. *Experimental Diabetes Research,* 4**,** 303-12.

SMINA, T. P., RABEKA, M. & VISWANATHAN, V. 2019. Diabetic Foot Ulcer as a Cause of Significant Decline in the Renal Function Among South Indian Population With Type 2 Diabetes: Role of TGF-β 1 and CCN Family Proteins. *International Journal of Lower Extremity Wounds,* 18**,** 354-361.

SORENSEN, D. W. & VAN BERLO, J. H. 2020. The Role of TGF—β Signaling in Cardiomyocyte Proliferation. *Current Heart Failure Reports,* 17**,** 225-233.

SOUZA, J. R., OLIVEIRA, R. T., BLOTTA, M. H. & COELHO, O. R. 2008. Serum levels of interleukin-6 (Il-6), interleukin-18 (Il-18) and C-reactive protein (CRP) in patients with type-2 diabetes and acute coronary syndrome without ST-segment elevation. *Arquivos Brasilerios de Cardiologia,* 90**,** 86-90.

SOVERSHAEV, T. A., UNRUH, D., SVEINBJØRNSSON, B., FALLON, J. T., HANSEN, J. B., BOGDANOV, V. Y. & SOVERSHAEV, M. A. 2016. A novel role of bone morphogenetic protein-7 in the regulation of adhesion and migration of human monocytic cells. *Thrombosis Research,* 147**,** 24-31.

SRIDHARAN, K. & SIVARAMAKRISHNAN, G. 2018. Growth factors for diabetic foot ulcers: mixed treatment comparison analysis of randomized clinical trials. *British Journal of Clinical Pharmacology,* 84**,** 434-444.

STAVNEZER, J. 1995. Regulation of antibody production and class switching by TGF-beta. *The Journal of Immunology,* 155**,** 1647-1651.

STEED, D. L. 1997. The Role of Growth Factors in Wound Healing. *Surgical Clinics of North America,* 77**,** 575-586.

STEFFEN, C. & O'ROURKE, S. 1998. Surgical Management of Diabetic Foot Complications: The Far North Queensland Profile. *Australian and New Zealand Journal of Surgery,* 68**,** 258-260.

STONE, W. L., LEAVITT, L. & VARACALLO, M. 2022. *Physiology, Growth Factor*, StatPearls*,* Treasure Island, viewed 16/11/2023, <https://www.ncbi.nlm.nih.gov/books/NBK442024/>

STOUFFER, G. A., LAMARRE, J., GONIAS, S. L. & OWENS, G. K. 1993. Activated alpha 2-macroglobulin and transforming growth factor-beta 1 induce a synergistic smooth muscle cell proliferative response. *The Journal of Biological Chemistry,* 268**,** 18340-4.

SU, Y., CHEN, Q., JU, Y., LI, W. & LI, W. 2020. Palmitate induces human glomerular mesangial cells fibrosis through CD36-mediated transient receptor potential canonical channel 6/nuclear factor of activated T cell 2 activation. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* 1865**,** 158793.

SULAIMAN, A. H., GHASSAN, Z. I. & OMAR, T. N. 2022. Biochemical Evaluation of Carbonic Anhydrase and Some Antioxidant Markers in Patients with Diabetes Complications. *Archives of Razi Institute,* 77**,** 169-178.

SUN, X., WANG, X., ZHAO, Z., CHEN, J., LI, C. & ZHAO, G. 2020. Paeoniflorin accelerates foot wound healing in diabetic rats though activating the Nrf2 pathway. *Acta Histochemica,* 122**,** 151649.

TALAMINI, M. A., MCCLUSKEY, M. P., BUCHMAN, T. G. & MAIO, A. D. 1998. Expression of α2-macroglobulin by the interaction between hepatocytes and endothelial cells in coculture. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 275**,** R203-R211.

TAN, S. Y., MEI WONG, J. L., SIM, Y. J., WONG, S. S., MOHAMED ELHASSAN, S. A., TAN, S. H., LING LIM, G. P., RONG TAY, N. W., ANNAN, N. C., BHATTAMISRA, S. K. & CANDASAMY, M. 2019a. Type 1 and 2 diabetes mellitus: A review on current treatment approach and gene therapy as potential intervention. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews,* 13**,** 364-372.

TAN, W. S., ARULSELVAN, P., NG, S.-F., MAT TAIB, C. N., SARIAN, M. N. & FAKURAZI, S. 2019b. Improvement of diabetic wound healing by topical application of Vicenin-2 hydrocolloid film on Sprague Dawley rats. *BMC Complementary and Alternative Medicine,* 19**,** 20.

TATE, M., PERERA, N., PRAKOSO, D., WILLIS, A. M., DEO, M., OSEGHALE, O., QIAN, H., DONNER, D. G., KIRIAZIS, H., DE BLASIO, M. J., GREGOREVIC, P. & RITCHIE, R. H. 2021. Bone Morphogenetic Protein 7 Gene Delivery Improves Cardiac Structure and Function in a Murine Model of Diabetic Cardiomyopathy. *Frontiers in Pharmacology,* 12.

THANGARAJAH, H., YAO, D., CHANG, E. I., SHI, Y., JAZAYERI, L., VIAL, I. N., GALIANO, R. D., DU, X. L., GROGAN, R., GALVEZ, M. G., JANUSZYK, M., BROWNLEE, M. & GURTNER, G. C. 2009. The molecular basis for impaired hypoxia-induced VEGF expression in diabetic tissues. *The Proceedings of the National Academy of Sciences,* 106**,** 13505-10.

THEOCHARIDIS, G., THOMAS, B. E., SARKAR, D., MUMME, H. L., PILCHER, W. J. R., DWIVEDI, B., SANDOVAL-SCHAEFER, T., SÎRBULESCU, R. F., KAFANAS, A., MEZGHANI, I., WANG, P., LOBAO, A., VLACHOS, I. S., DASH, B., HSIA, H. C., HORSLEY, V., BHASIN, S. S., VEVES, A. & BHASIN, M. 2022. Single cell transcriptomic landscape of diabetic foot ulcers. *Nature Communications,* 13.

THIRUVOIPATI, T., KIELHORN, C. E. & ARMSTRONG, E. J. 2015. Peripheral artery disease in patients with diabetes: Epidemiology, mechanisms, and outcomes. *World Journal of Diabetes,* 6**,** 961-969.

TSALAMANDRIS, S., ANTONOPOULOS, A. S., OIKONOMOU, E., PAPAMIKROULIS, G.-A., VOGIATZI, G., PAPAIOANNOU, S., DEFTEREOS, S. & TOUSOULIS, D. 2019. The Role of Inflammation in Diabetes: Current Concepts and Future Perspectives. *European Cardiology,* 14**,** 50-59.

TULETA, I. & FRANGOGIANNIS, N. G. 2021. Diabetic fibrosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1867**,** 166044.

TUOMI, T., SANTORO, N., CAPRIO, S., CAI, M., WENG, J. & GROOP, L. 2014. The many faces of diabetes: a disease with increasing heterogeneity. *The Lancet,* 383**,** 1084-1094.

UELAND, T., LEKVA, T., OTTERDAL, K., DAHL, T. B., OLARESCU, N. C., JØRGENSEN, A. P., FOUGNER, K. J., BRIXEN, K., AUKRUST, P. & BOLLERSLEV, J. 2011. Increased serum and bone matrix levels of transforming growth factor β 1 in patients with GH deficiency in response to GH treatment. *European Journal of Endocrinology,* 165**,** 393-400.

USUI, M. L., MANSBRIDGE, J. N., CARTER, W. G., FUJITA, M. & OLERUD, J. E. 2008. Keratinocyte Migration, Proliferation, and Differentiation in Chronic Ulcers From Patients With Diabetes and Normal Wounds. *Journal of Histochemistry & Cytochemistry,* 56**,** 687-696.

VAN DER WORP, H. B., HOWELLS, D. W., SENA, E. S., PORRITT, M. J., REWELL, S., O'COLLINS, V. & MACLEOD, M. R. 2010. Can Animal Models of Disease Reliably Inform Human Studies? *PLOS Medicine,* 7**,** e1000245.

VANDOOREN, J. & ITOH, Y. 2021. Alpha-2-Macroglobulin in Inflammation, Immunity and Infections. *Frontiers in Immunology,* 12.

VANGAVETI, V. N., JHAMB, S., HAYES, O., GOODALL, J., BULBROOK, J., ROBERTSON, K., BIROS, E., SANGLA, K. S. & MALABU, U. H. 2022. Effects of vildagliptin on wound healing and markers of inflammation in patients with type 2 diabetic foot ulcer: a prospective, randomized, double-blind, placebo-controlled, single-center study. *Diabetology & Metabolic Syndrome,* 14**,** 183.

VIROLAINEN, S. J., VONHANDORF, A., VIEL, K. C. M. F., WEIRAUCH, M. T. & KOTTYAN, L. C. 2023. Gene–environment interactions and their impact on human health. *Genes & Immunity,* 24**,** 1-11.

VUKICEVIC, S., BASIC, V., ROGIC, D., BASIC, N., SHIH, M. S., SHEPARD, A., JIN, D., DATTATREYAMURTY, B., JONES, W., DORAI, H., RYAN, S., GRIFFITHS, D., MALIAKAL, J., JELIC, M., PASTORCIC, M., STAVLJENIC, A. & SAMPATH, T. K. 1998. Osteogenic protein-1 (bone morphogenetic protein-7) reduces severity of injury after ischemic acute renal failure in rat. *The Journal of clinical investigation,* 102**,** 202-14.

WANG, L., WANG, H.-L., LIU, T.-T. & LAN, H.-Y. 2021. TGF-Beta as a Master Regulator of Diabetic Nephropathy. *International Journal of Molecular Sciences,* 22**,** 7881.

WANG, S., CHEN, Q., SIMON, T. C., STREBECK, F., CHAUDHARY, L., MORRISSEY, J., LIAPIS, H., KLAHR, S. & HRUSKA, K. A. 2003. Bone morphogenic protein-7 (BMP-7), a novel therapy for diabetic nephropathy¹. *Kidney International,* 63**,** 2037-2049.

WEBB, D. J., WEN, J., KARNS, L. R., KURILLA, M. G. & GONIAS, S. L. 1998. Localization of the binding site for transforming growth factor-beta in human alpha2-macroglobulin to a 20-kDa peptide that also contains the bait region. *Journal of Biological Chemistry,* 273**,** 13339-46.

WEST, M., CHUTER, V., MUNTEANU, S. & HAWKE, F. 2017. Defining the gap: a systematic review of the difference in rates of diabetes-related foot complications in Aboriginal and Torres Strait Islander Australians and non-Indigenous Australians. *Journal of Foot and Ankle Research,* 10**,** 48.

WILDGRUBER, M., WEISS, W., BERGER, H., WOLF, O., ECKSTEIN, H. H. & HEIDER, P. 2007. Association of Circulating Transforming Growth Factor beta, Tumor Necrosis Factor alpha and Basic Fibroblast Growth Factor with Restenosis after Transluminal Angioplasty. *European Journal of Vascular and Endovascular Surgery,* 34**,** 35-43.

WONG, M. G., PERKOVIC, V., WOODWARD, M., CHALMERS, J., LI, Q., HILLIS, G. S., YAGHOBIAN AZARI, D., JUN, M., POULTER, N., HAMET, P., WILLIAMS, B., NEAL, B., MANCIA, G., COOPER, M. & POLLOCK, C. A. 2013. Circulating bone morphogenetic protein-7 and transforming

growth factor-β 1 are better predictors of renal end points in patients with type 2 diabetes mellitus. *Kidney International,* 83**,** 278-84.

WORBY, C. A., VEGA, Q. C., ZHAO, Y., CHAO, H. H. J., SEASHOLTZ, A. F. & DIXON, J. E. 1996. Glial Cell Line-derived Neurotrophic Factor Signals through the RET Receptor and Activates Mitogen-activated Protein Kinase *Journal of Biological Chemistry,* 271**,** 23619-23622.

XIAO, Y., LIANG, D., LI, Z., FENG, Z., YUAN, Z., ZHANG, F., WANG, Y., ZHOU, Y., SHI, M., LIU, L., XIAO, Y. & GUO, B. 2022. BMP-7 Upregulates Id2 Through the MAPK Signaling Pathway to Improve Diabetic Tubulointerstitial Fibrosis and the Intervention of Oxymatrine. *Frontiers in Pharmacology,* 13.

XU, S., WENG, X., WANG, Y., LV, D., ZENG, M., ZHAO, F. & SUN, Y. 2019. Screening and preliminary validation of T lymphocyte immunoregulation-associated long non-coding RNAs in diabetic foot ulcers. *Molecular Medicine Reports,* 19**,** 2368-2376.

YAGIHASHI, S., MIZUKAMI, H. & SUGIMOTO, K. 2011. Mechanism of diabetic neuropathy: Where are we now and where to go? *Journal of Diabetes Investigation,* 2**,** 18-32.

YANG, H., SLOAN, G., YE, Y., WANG, S., DUAN, B., TESFAYE, S. & GAO, L. 2020a. New Perspective in Diabetic Neuropathy: From the Periphery to the Brain, a Call for Early Detection, and Precision Medicine. *Frontiers in Endocrinology,* 10.

YANG, Q., ZHANG, Y., YIN, H. & LU, Y. 2020b. Topical Recombinant Human Epidermal Growth Factor for Diabetic Foot Ulcers: A Meta-Analysis of Randomized Controlled Clinical Trials. *Annals of Vascular Surgery,* 62**,** 442-451.

YANG, S. L., ZHU, L. Y., HAN, R., SUN, L. L. & DOU, J. T. 2017. Effect of Negative Pressure Wound Therapy on Cellular Fibronectin and Transforming Growth Factor-β 1 Expression in Diabetic Foot Wounds. *Foot & Ankle International,* 38**,** 893-900.

YBARRA, J., POU, J. M., ROMEO, J. H., MERCE, J. & JURADO, J. 2010. Transforming growth factor beta 1 as a biomarker of diabetic peripheral neuropathy: cross-sectional study. *Journal of Diabetes and its Complications,* 24**,** 306-312.

YU, X.-J., WANG, Y.-G., LU, R., GUO, X.-Z., QU, Y.-K., WANG, S.-X., XU, H.-R., KANG, H., YOU, H.-B. & XU, Y. 2023. BMP7 ameliorates intervertebral disc degeneration in type 1 diabetic rats by inhibiting pyroptosis of nucleus pulposus cells and NLRP3 inflammasome activity. *Molecular Medicine,* 29**,** 30.

ZENG, J., JIANG, Y., XIANG, S. & CHEN, B. 2011. Serum bone morphogenetic protein 7, insulin resistance, and insulin secretion in non-diabetic individuals. *Diabetes Research and Clinical Practice,* 93**,** e21-e24.

ZHANG, A., FANG, H., CHEN, J., HE, L. & CHEN, Y. 2020a. Role of VEGF-A and LRG1 in Abnormal Angiogenesis Associated With Diabetic Nephropathy. *Frontiers in Physiology,* 11.

ZHANG, F., REN, Y., LIU, P., REN, Y. & WANG, D. 2016. Expression of TGF-β 1 and miRNA-145 in patients with diabetic foot ulcers. *Experimental and Therapeutic Medicine,* 11**,** 2011-2014.

ZHANG, H., ZHANG, X., HU, C. & LU, W. 2012. Exenatide Reduces Urinary Transforming Growth Factor-β ₁ and Type IV Collagen Excretion in Patients with Type 2 Diabetes and Microalbuminuria. *Kidney and Blood Pressure Research,* 35**,** 483-488.

ZHANG, J., CHEN, J. A., GAO, C., SUN, X., WANG, L., HU, Z., LI, G., WANG, J. & WANG, A. 2022a. Maggot treatment promotes healing of diabetic foot ulcer wounds possibly by upregulating Treg levels. *Diabetes Research and Clinical Practice,* 184**,** 109187.

ZHANG, J., GUAN, M., XIE, C., LUO, X., ZHANG, Q. & XUE, Y. 2014. Increased Growth Factors Play a Role in Wound Healing Promoted by Noninvasive Oxygen-Ozone Therapy in Diabetic Patients with Foot Ulcers. *Oxidative Medicine and Cellular Longevity,* 2014**,** 273475.

ZHANG, J., ZHOU, S., XIA, Z., PENG, Z., LUO, W., CHENG, X. & YANG, R. 2022b. Effectiveness of artesunate combined with fractional CO2 laser in a hypertrophic scar model with underlying mechanism. *Burns,* 48**,** 662-671.

ZHANG, P., LU, J., JING, Y., TANG, S., ZHU, D. & BI, Y. 2017. Global epidemiology of diabetic foot ulceration: a systematic review and meta-analysis. *Annals of Medicine,* 49**,** 106-116.

ZHANG, X. L., SELBI, W., DE LA MOTTE, C., HASCALL, V. & PHILLIPS, A. O. 2005. Bone Morphogenic Protein-7 Inhibits Monocyte-Stimulated TGF-β 1 Generation in Renal Proximal Tubular Epithelial Cells. *Journal of the American Society of Nephrology,* 16**,** 79-89.

ZHANG, Y., LAZZARINI, P. A., MCPHAIL, S. M., VAN NETTEN, J. J., ARMSTRONG, D. G. & PACELLA, R. E. 2020b. Global Disability Burdens of Diabetes-Related Lower-Extremity Complications in 1990 and 2016. *Diabetes Care,* 43**,** 964-974.

ZHAO, D.-Y., SU, Y.-N., LI, Y.-H., YU, T.-Q., LI, J. & TU, C.-Q. 2020. Efficacy and safety of recombinant human epidermal growth factor for diabetic foot ulcers: A systematic review and meta-analysis of randomised controlled trials. *International Wound Journal,* 17**,** 1062-1073.

ZIMMET, P., ALBERTI, K. G., MAGLIANO, D. J. & BENNETT, P. H. 2016. Diabetes mellitus statistics on prevalence and mortality: facts and fallacies. *Nature Reviews Endocrinology,* 12**,** 616+.

ZUBAIR, M. & AHMAD, J. 2019. Role of growth factors and cytokines in diabetic foot ulcer healing: A detailed review. *Reviews in Endocrine and Metabolic Disorders,* 20**,** 207-217.

APPENDICES

Appendix 1: Calibration data for TGF-β1 and BMP-7 ELISAs

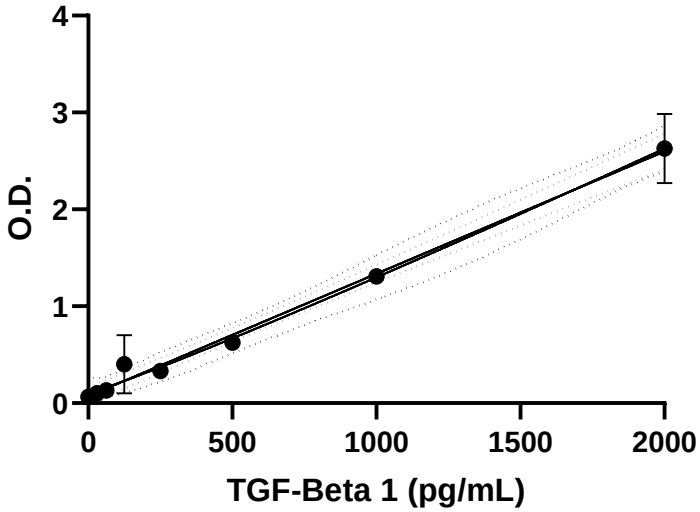

Figure A1: Calibration curve for TGF-β1 ELISA.

Table A1: Calibration data for TGF-β1 ELISA.

Standard (pg/mL)	ABS 1	ABS 2
2000	2.377	2.882
1000	1.276	1.338
500	0.624	0.623
250	0.327	0.335
125	0.191	0.615
63	0.133	0.128
31	0.097	0.107
0	0.067	0.065

Table A2: Raw absorbance values for patient samples used in the TGF-β1 ELISA.

Patient Sample (Patient ID, time point (weeks))	**ABS 1**	**ABS 2**
2,0	0.075	0.107
6,0	0.633	0.695
11,0	0.086	0.867
14,0	0.615	0.674
20,0	0.074	0.07
22,0	0.265	0.061
30,0	0.061	0.061
39,0	0.645	0.059
43,0	0.245	0.381
49,0	0.563	0.667
2,12	0.444	0.455
6,12	0.674	0.774
11,12	1.575	1.486
14,12	0.468	0.433
20,12	0.575	0.414
22,12	0.088	0.201
30,12	0.869	0.841
39,12	0.066	0.074
43,12	0.388	0.078
49,12	0.066	0.067

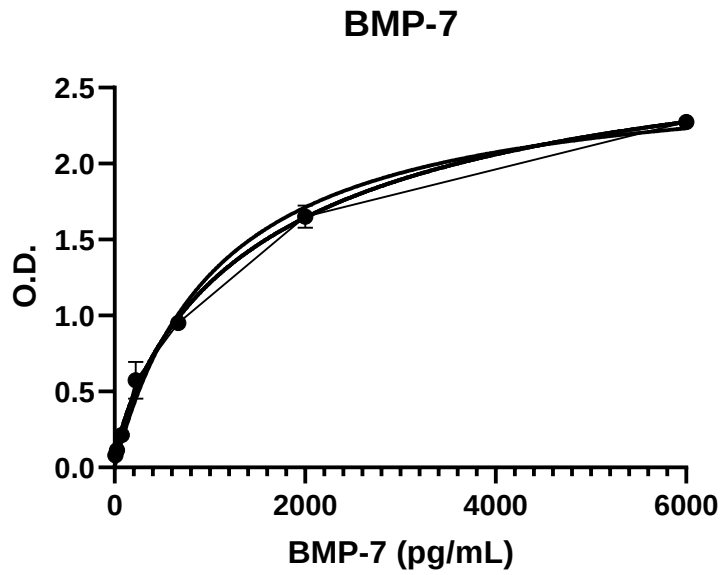

Figure A2: Calibration curve for BMP-7 ELISA.

Table A3: Calibration data for BMP-7 ELISA.

Standard (pg/mL)	**ABS 1**	**ABS 2**
6000	2.271	2.277
2000	1.6	1.704
666.7	0.977	0.926
222.2	0.659	0.488
74.07	0.203	0.224
24.69	0.112	0.117
8.23	0.077	0.082
0	0.069	0.072

Table A4: Raw absorbance values for patient samples used in the BMP-7 ELISA.

Patient Sample (Patient ID, time point (weeks))	**ABS 1**	**ABS 2**
2,0	0.049	0.042
6,0	0.051	0.05
11,0	0.056	0.057
14,0	0.054	0.043
20,0	0.049	0.049
22,0	0.045	0.043
30,0	0.051	0.057
39,0	0.048	0.051
43,0	0.057	0.061
49,0	0.051	0.054
2,12	0.062	0.053
6,12	0.064	0.052
11,12	0.052	0.048
14,12	0.061	0.054
20,12	0.06	0.077
22,12	0.067	0.069
30,12	0.065	0.068
39,12	0.056	0.054
43,12	0.058	0.055
49,12	0.055	0.049

Appendix 2: ANCOVA graphs for relationships between growth factors studied and nominal data categories.

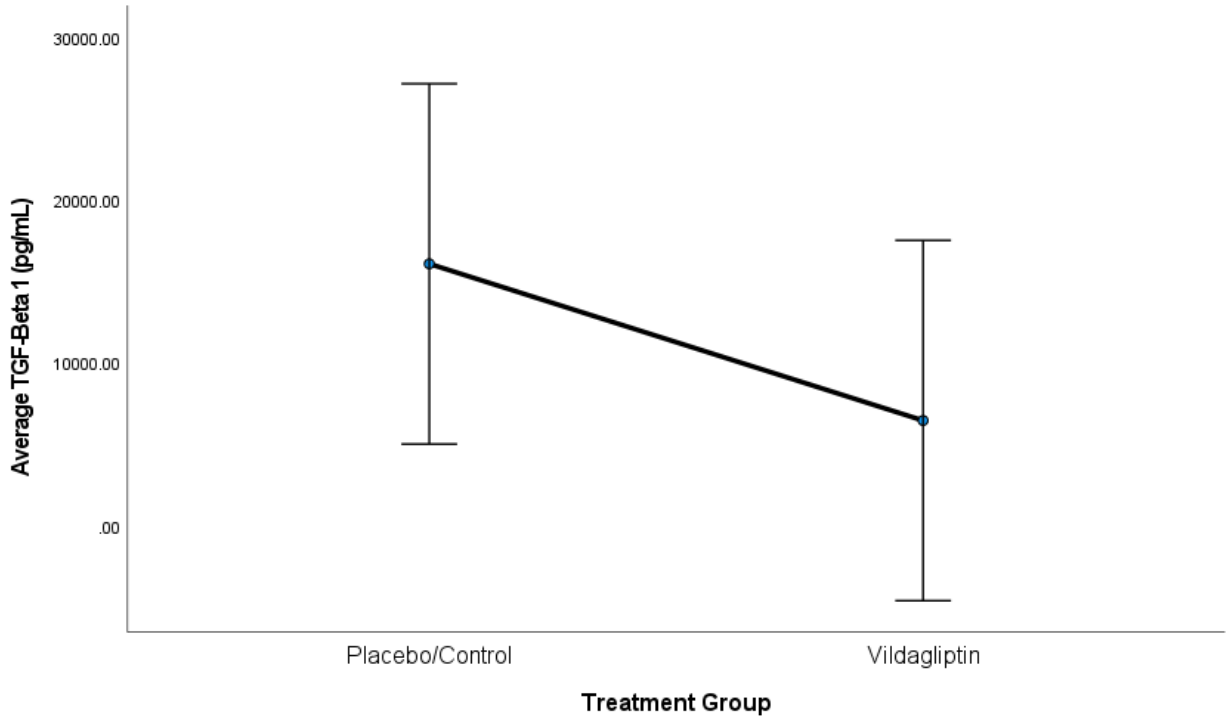

Figure A3: ANCOVA graph showing the effects of treatment group on 12 week TGF-β1 values.

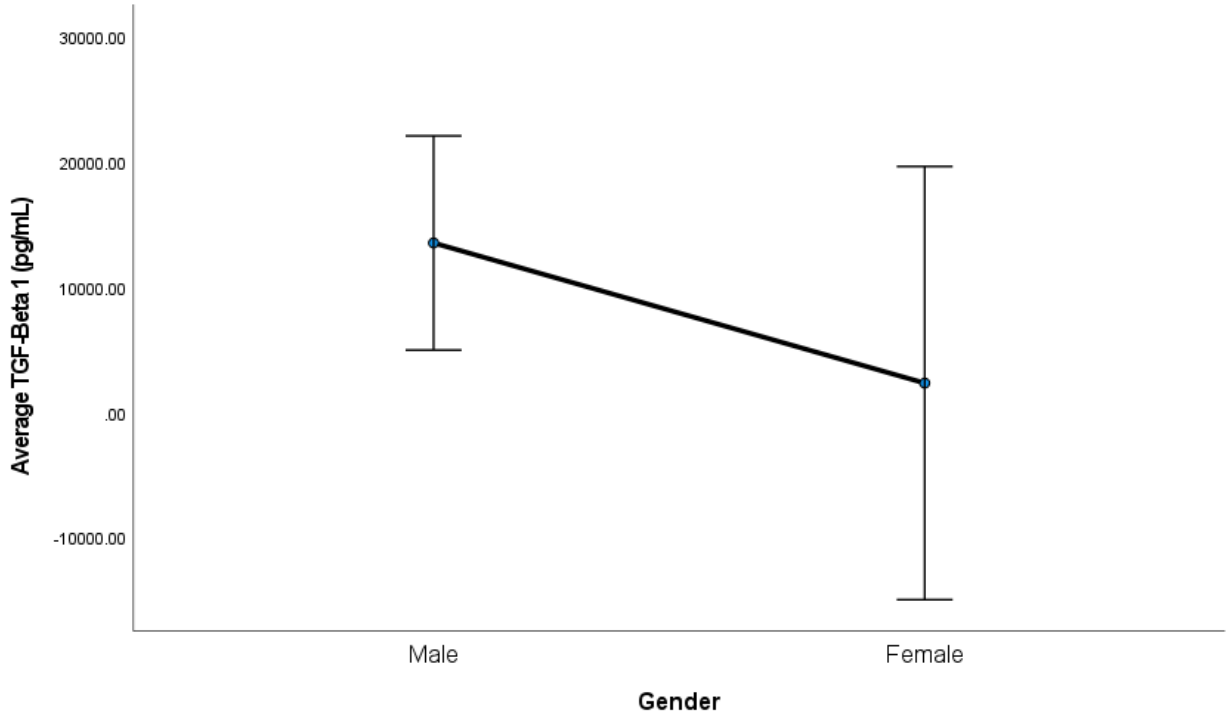

Figure A4: ANCOVA graph showing the effects of gender on 12 week TGF-β1 values.

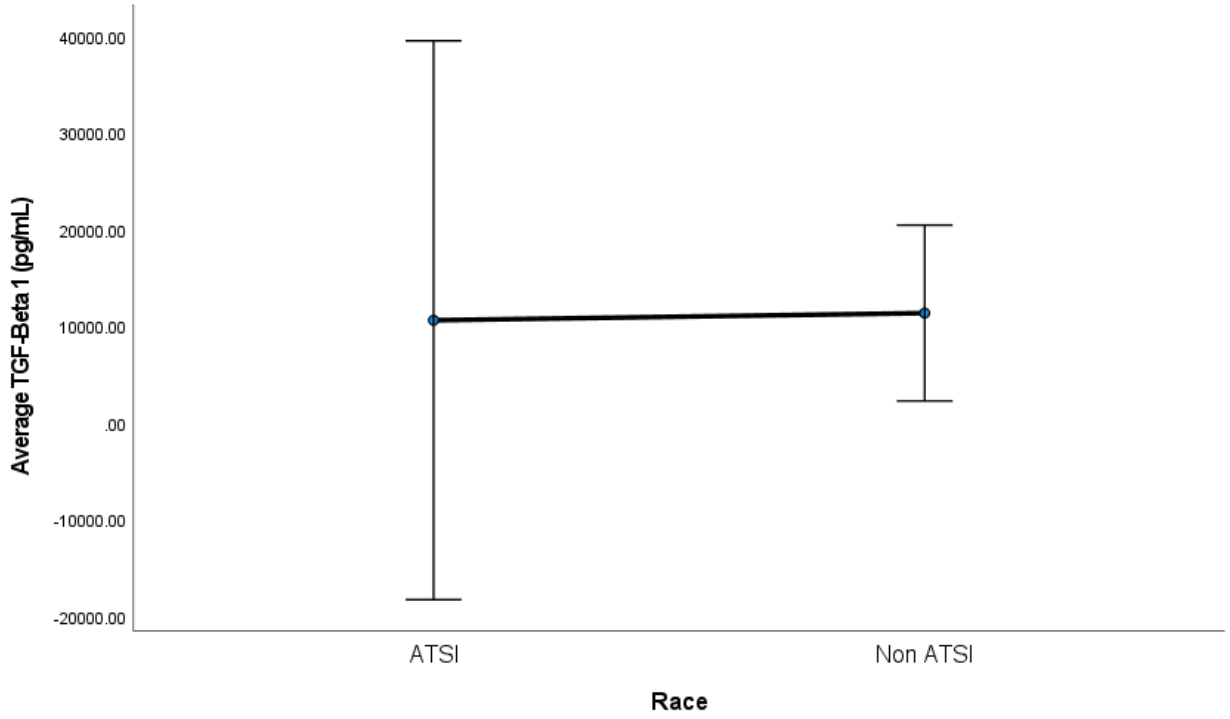

Figure A5: ANCOVA graph showing the effects of race on 12 week TGF-β1 values.

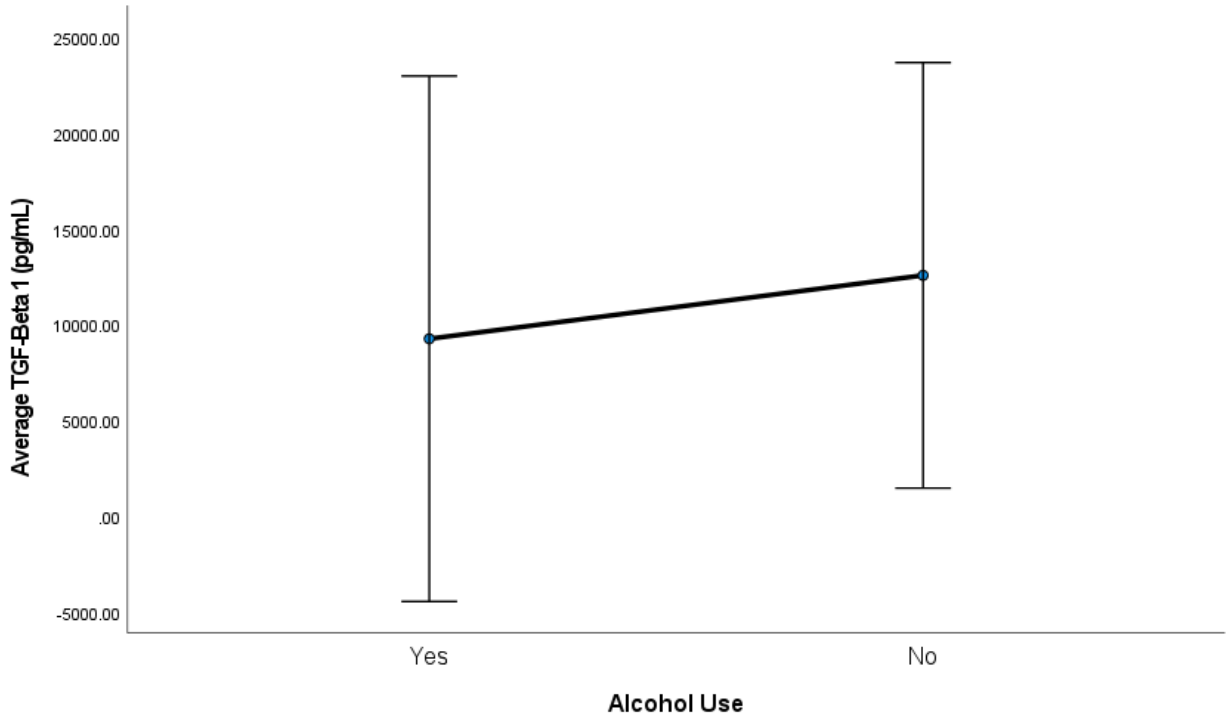

Figure A6: ANCOVA graph showing the effects of alcohol use on 12 week TGF-β1 values.

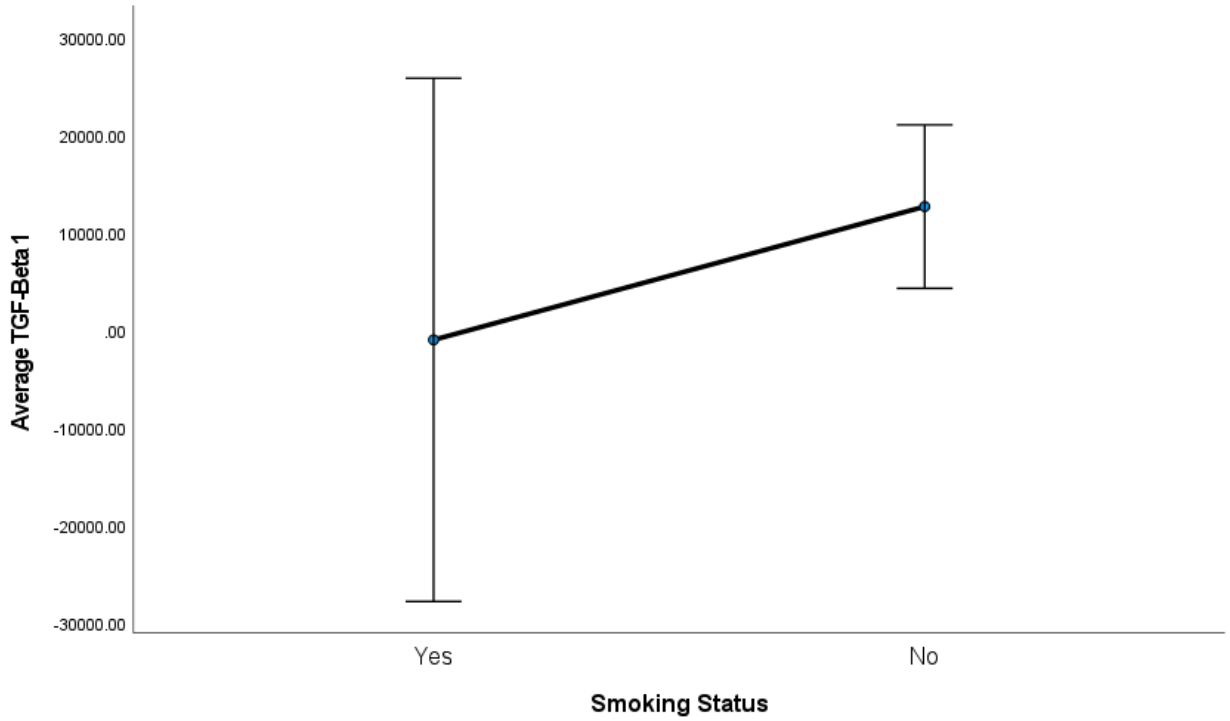

Figure A7: ANCOVA graph showing the effects of smoking status on 12 week TGF-β1 values.

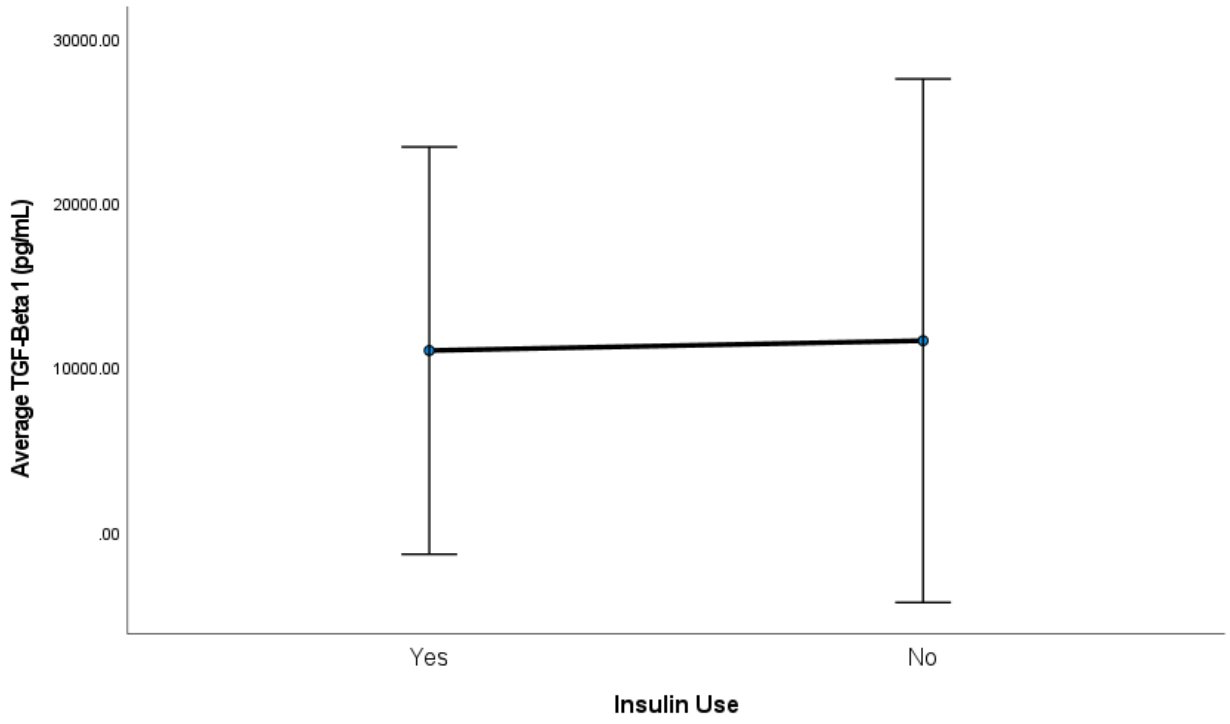

Figure A8: ANCOVA graph showing the effects of insulin use on 12 week TGF-β1 values.

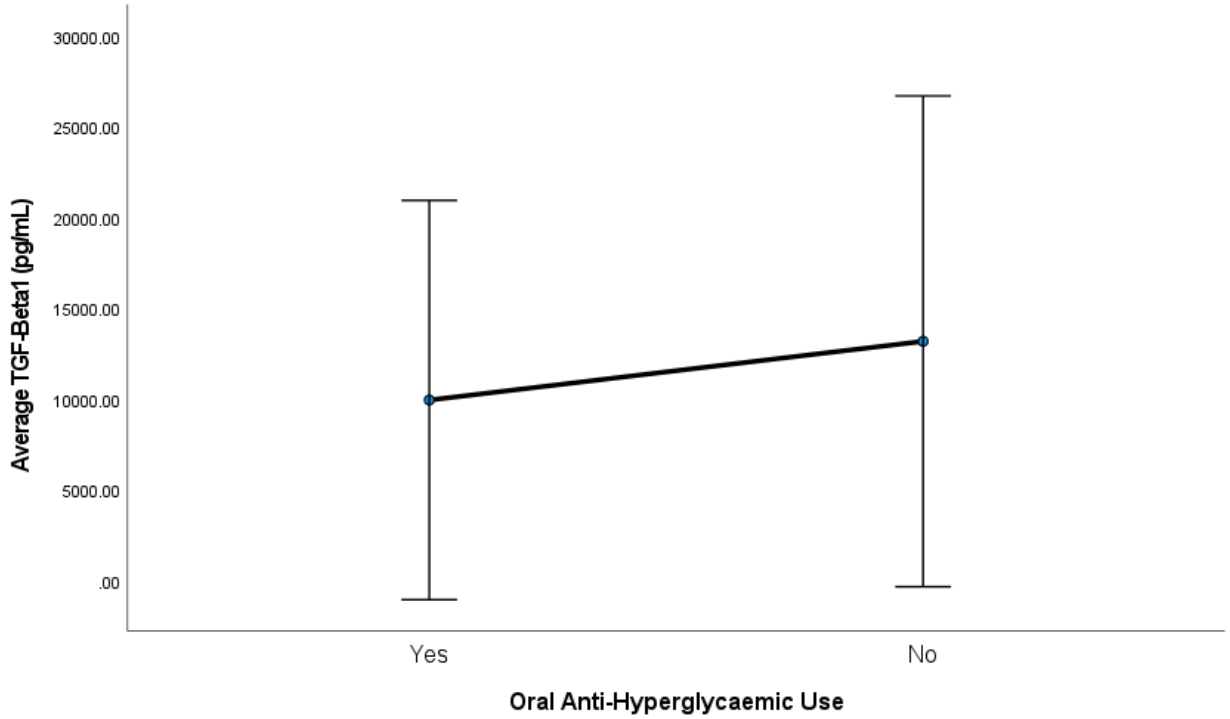

Figure A9: ANCOVA graph showing the effects of oral anti-hyperglycaemic use on 12 week TGF-β1 values.

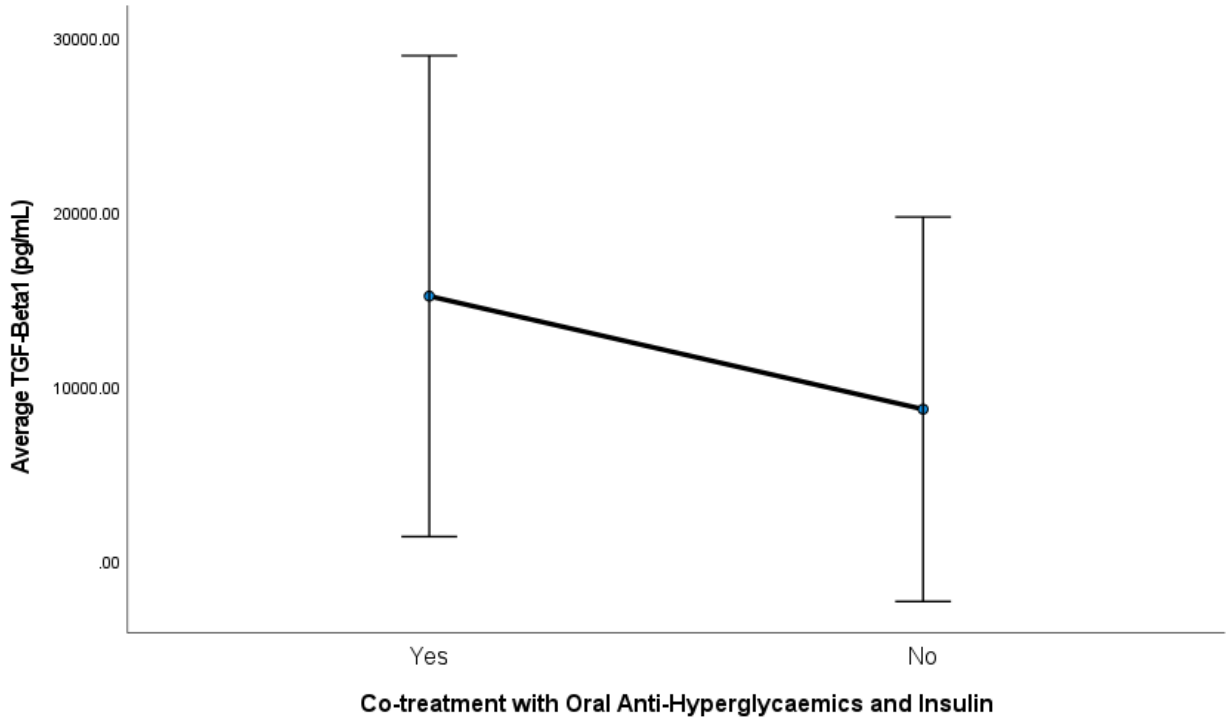

Figure A10: ANCOVA graph showing the effects of insulin and oral anti-hyperglycaemic co-treatment on 12 week TGF-β1 values.

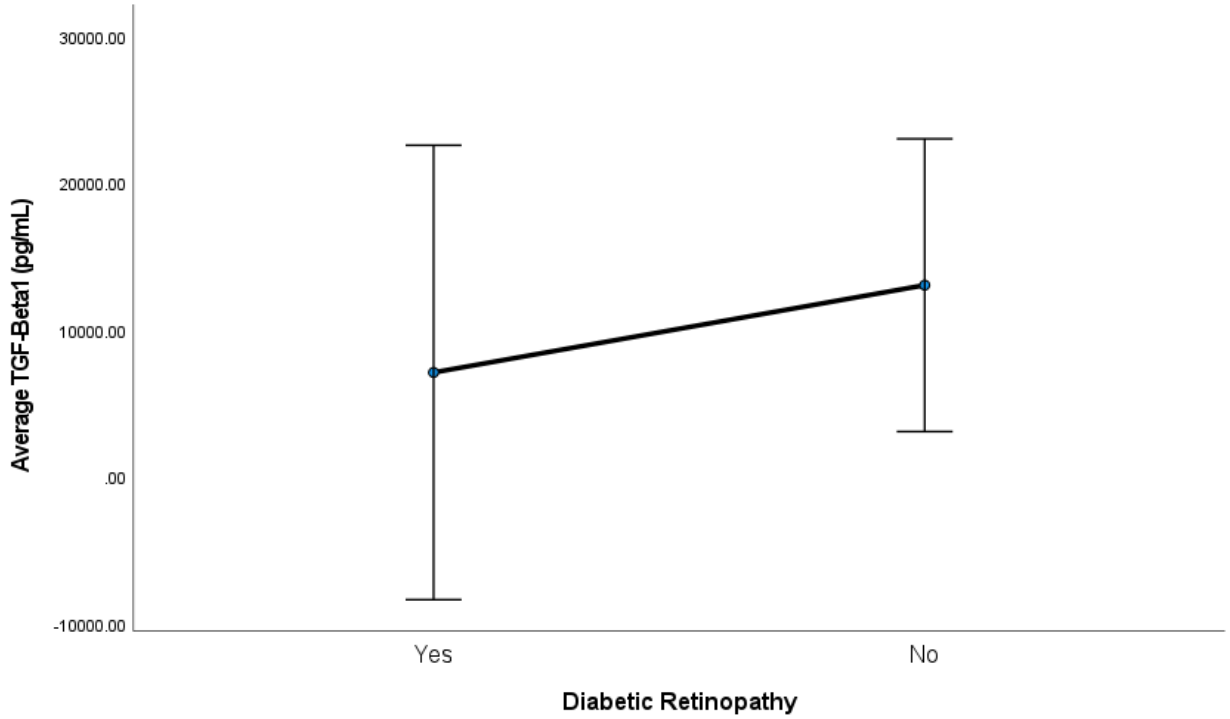

Figure A11: ANCOVA graph showing the effects of Diabetic retinopathy on 12 week TGF-β1 values.

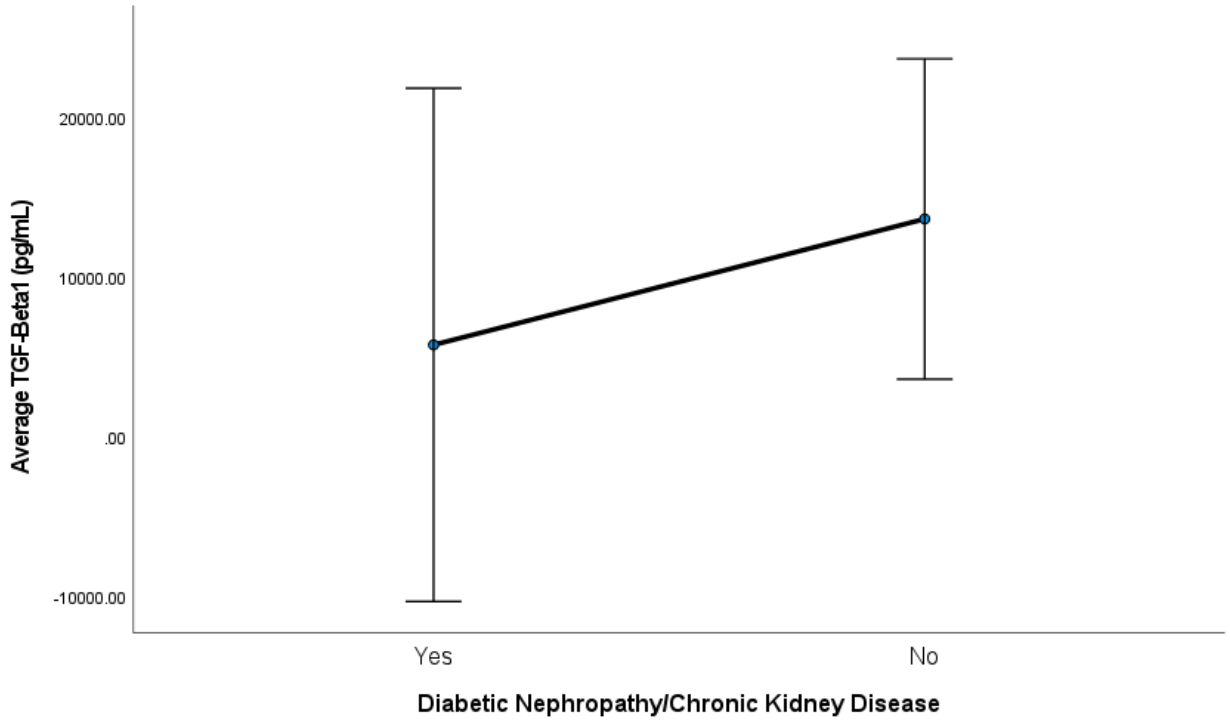

Figure A12: ANCOVA graph showing the effects of diabetic nephropathy/chronic kidney disease on 12 week TGF-β1 values.

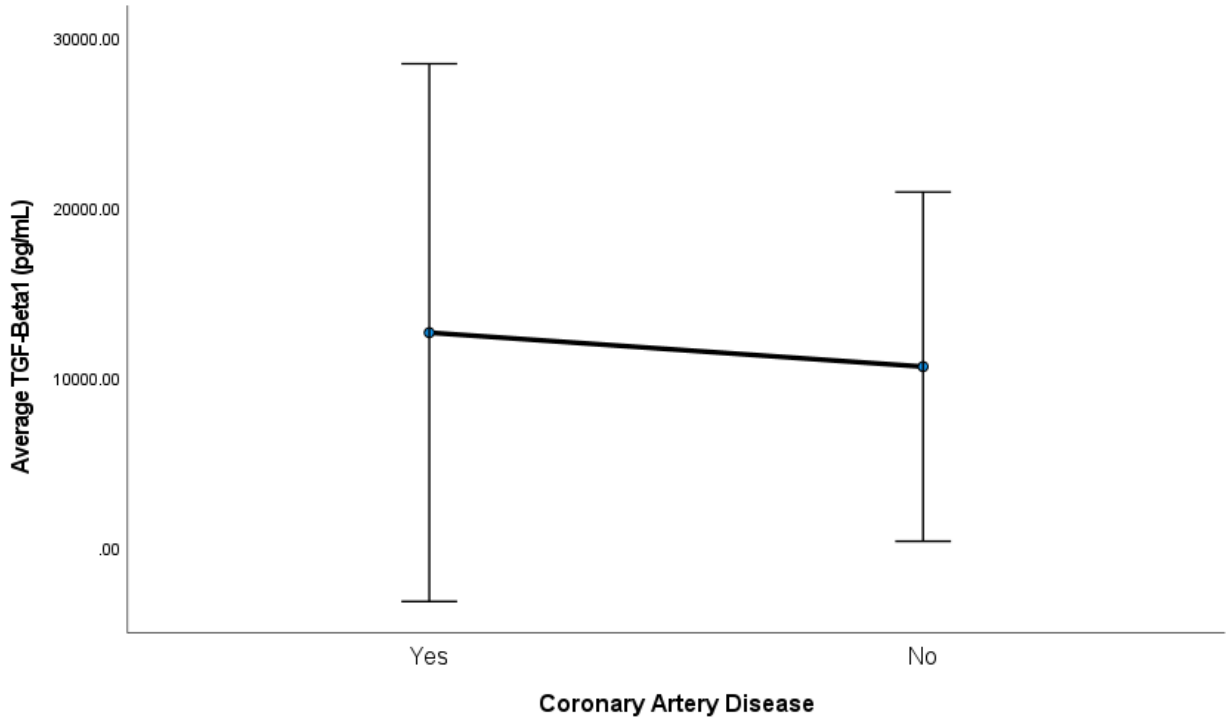

Figure A13: ANCOVA graph showing the effects of Coronary Artery Disease on 12 week TGF-β1 values.

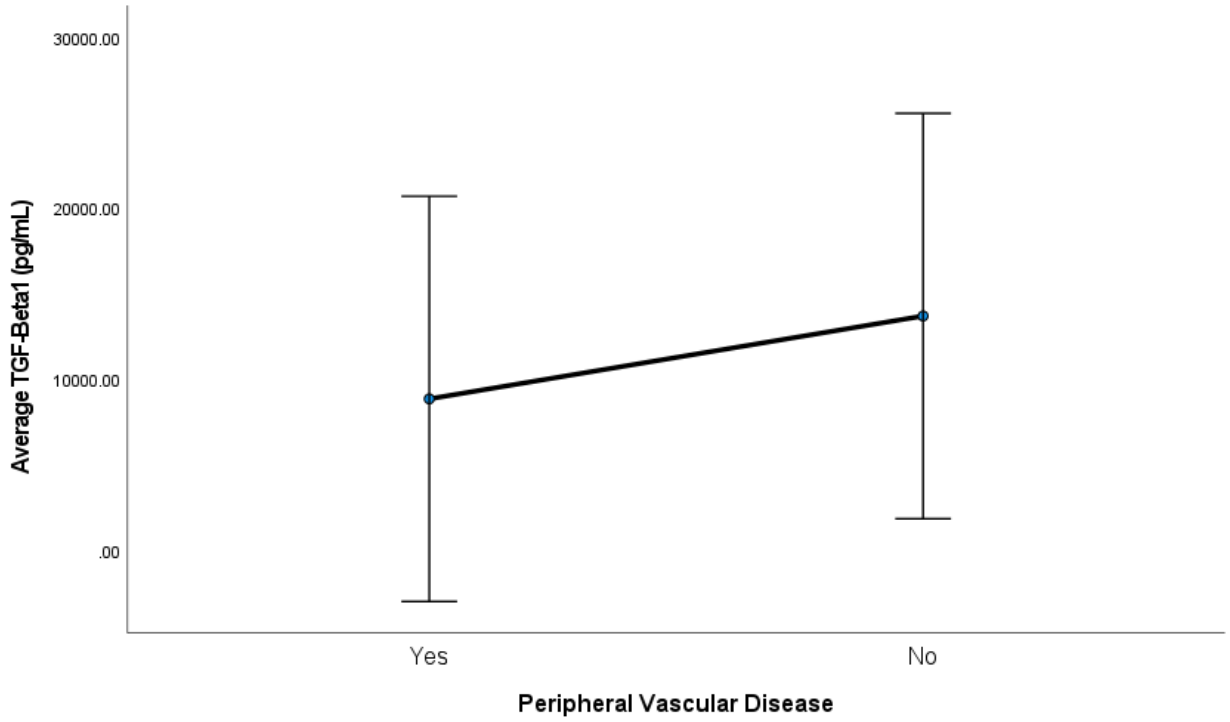

Figure A14: ANCOVA graph showing the effects of Peripheral Vascular Disease on 12 week TGF-β1 values.

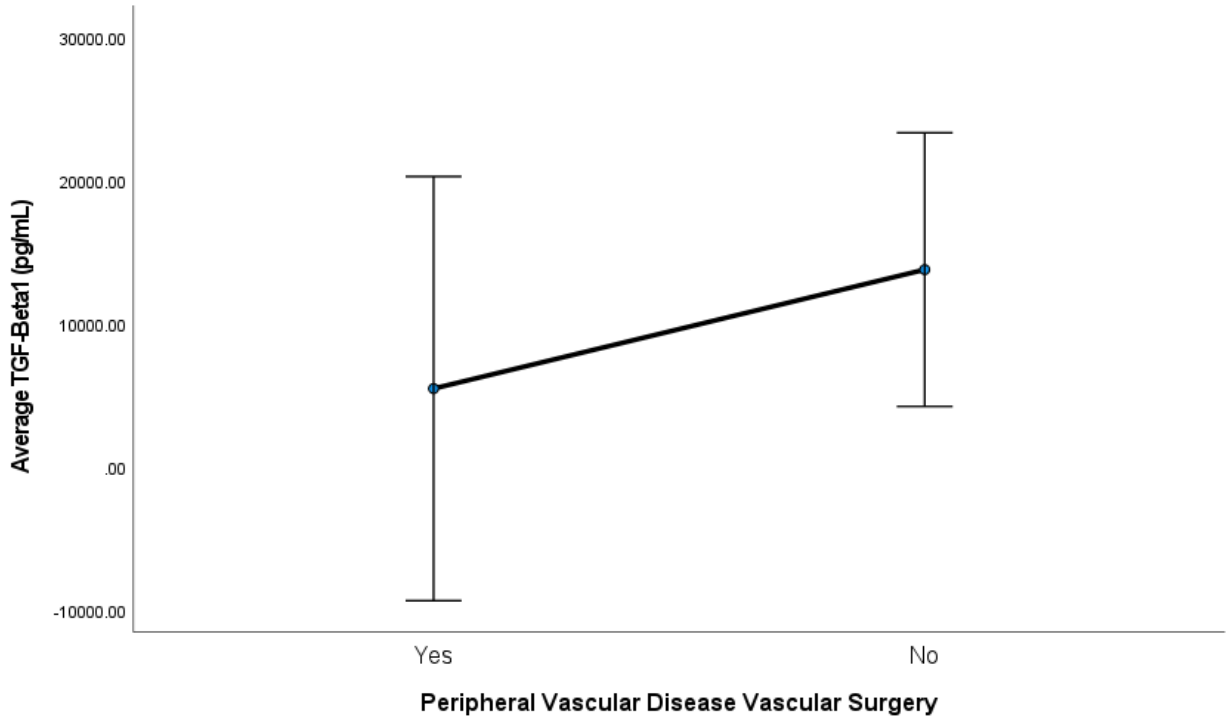

Figure A15: ANCOVA graph showing the effects of Peripheral Vascular Disease Surgery on 12 week TGF-β1 values.

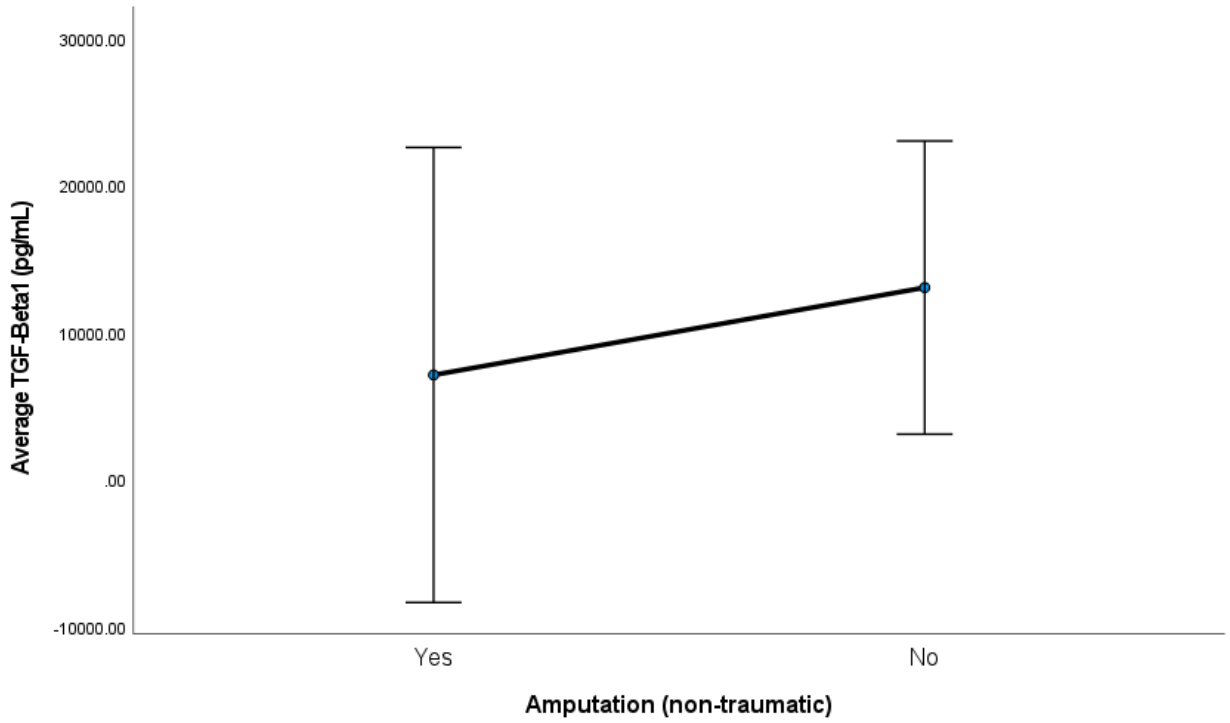

Figure A16: ANCOVA graph showing the effects of non-traumatic amputation on 12 week TGF-β1 values.

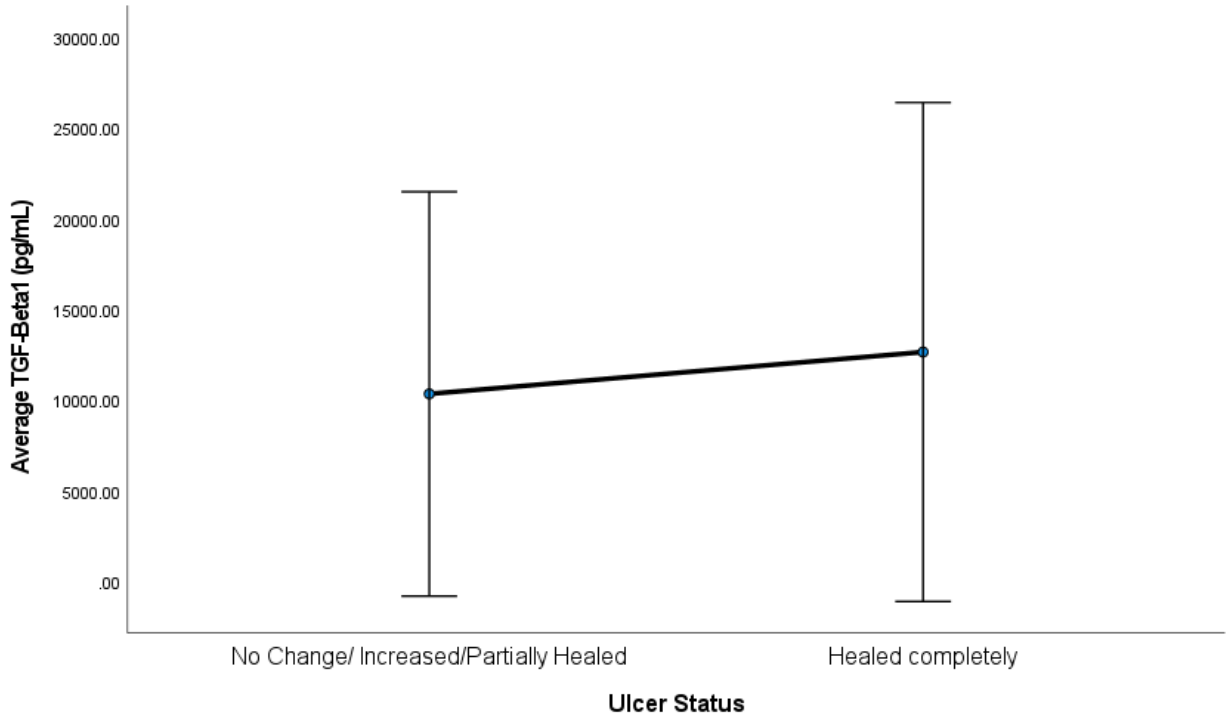

Figure A17: ANCOVA graph showing the effects of ulcer status on 12 week TGF-β1 values.

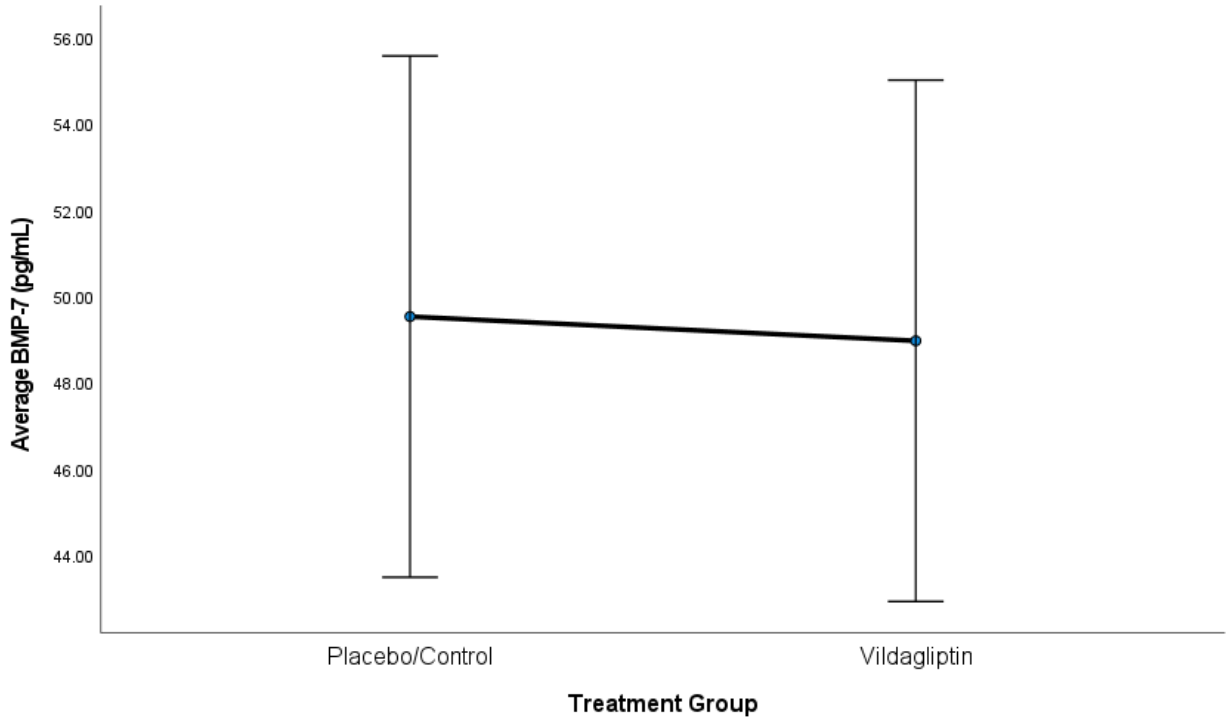

Figure A18: ANCOVA graph showing the effects of treatment group on 12 week BMP-7 values.

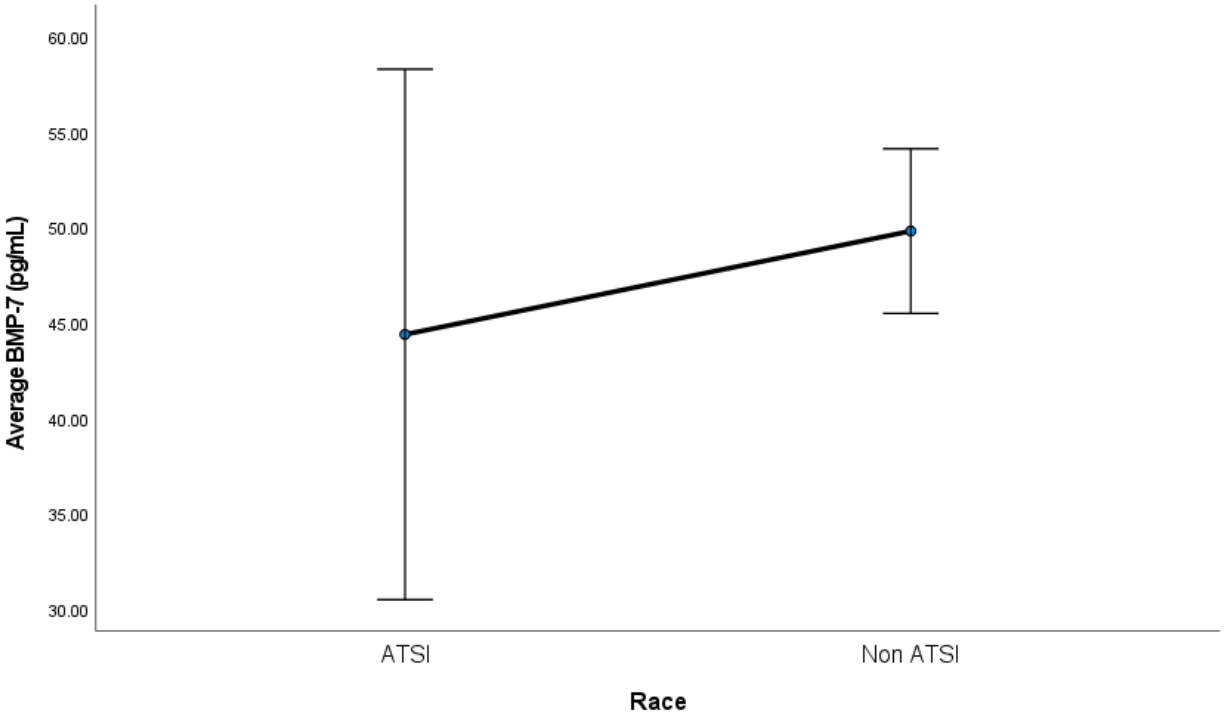

Figure A19: ANCOVA graph showing the effects of race on 12 week BMP-7 values.

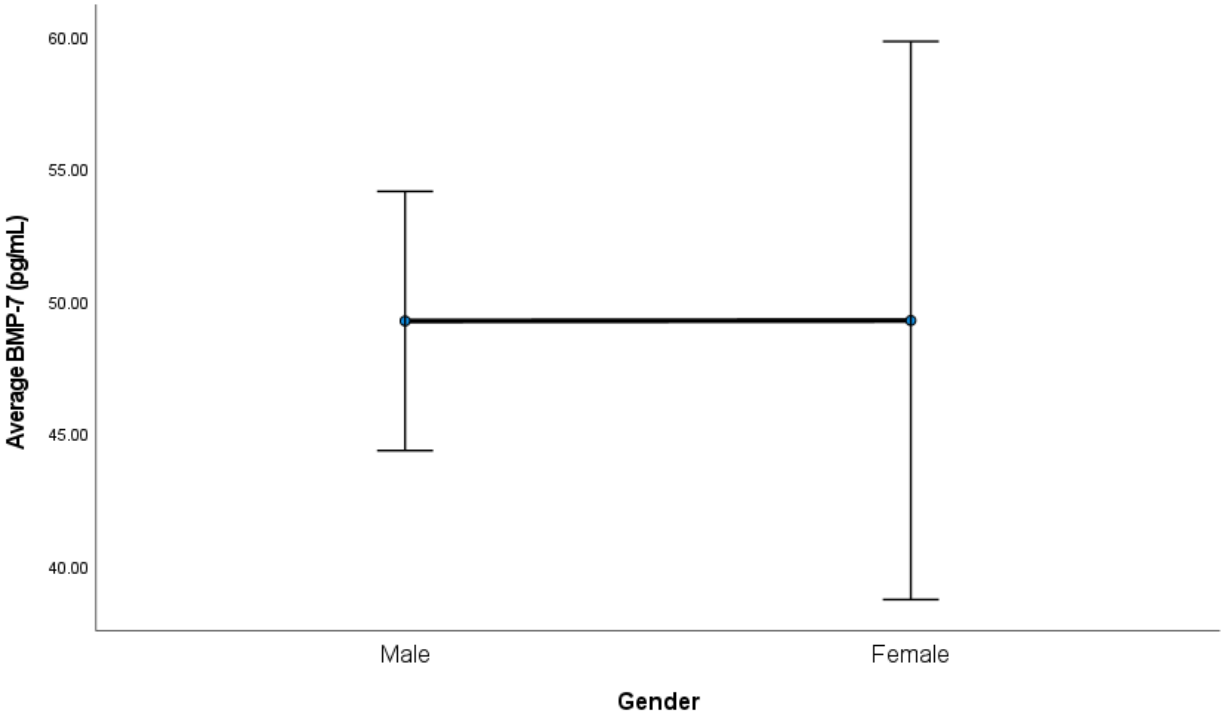

Figure A20: ANCOVA graph showing the effects of gender on 12 week BMP-7 values.

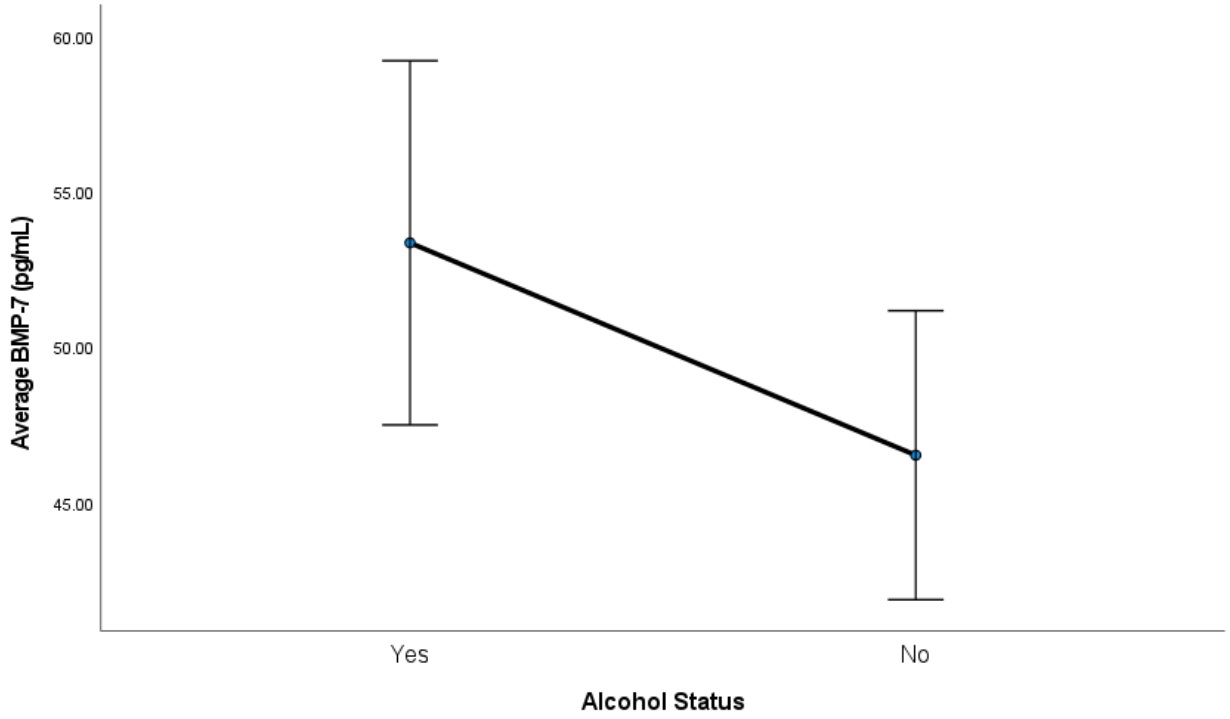

Figure A21: ANCOVA graph showing the effects of alcohol status on 12 week BMP-7 values.

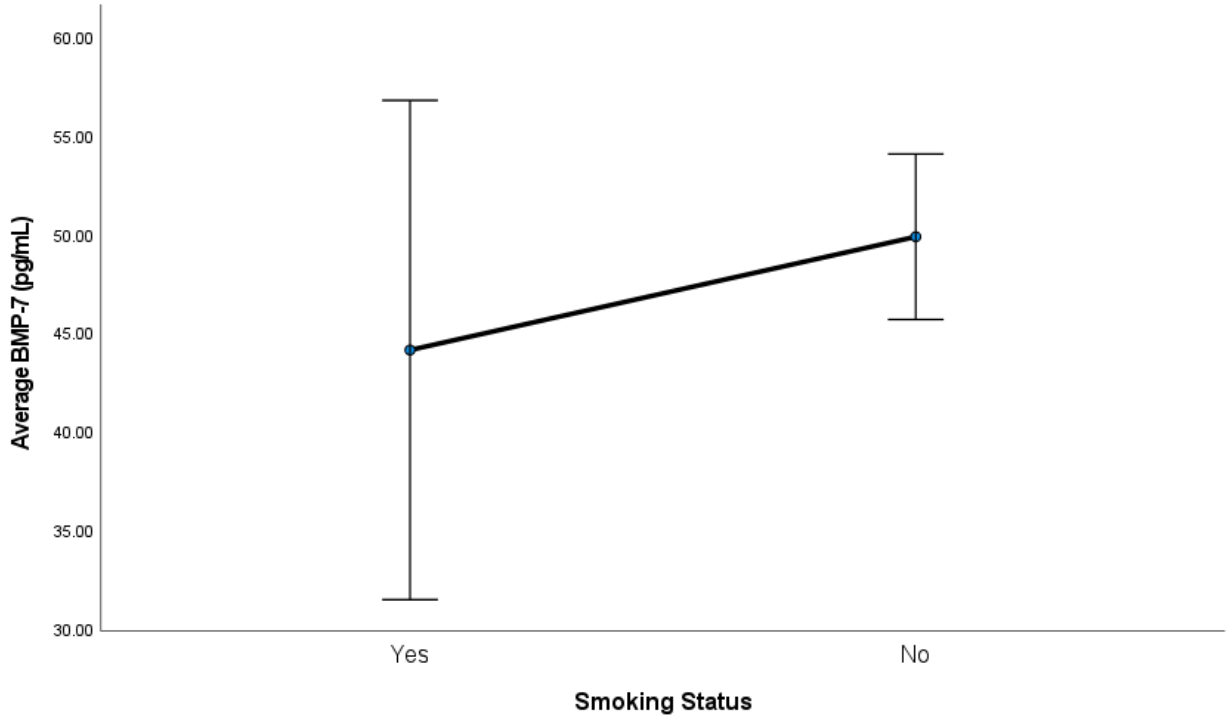

Figure A22: ANCOVA graph showing the effects of smoking status on 12 week BMP-7 values.

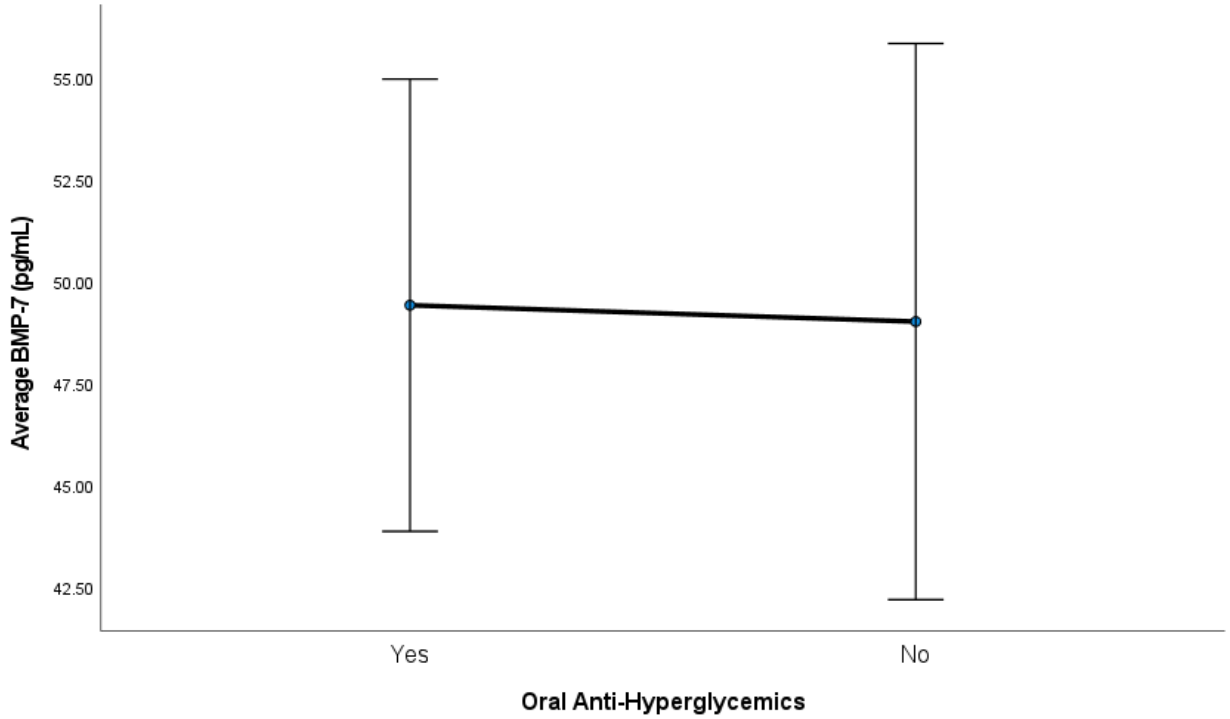

Figure A23: ANCOVA graph showing the effects of Oral anti-hyperglycaemics on 12 week BMP-7 values.

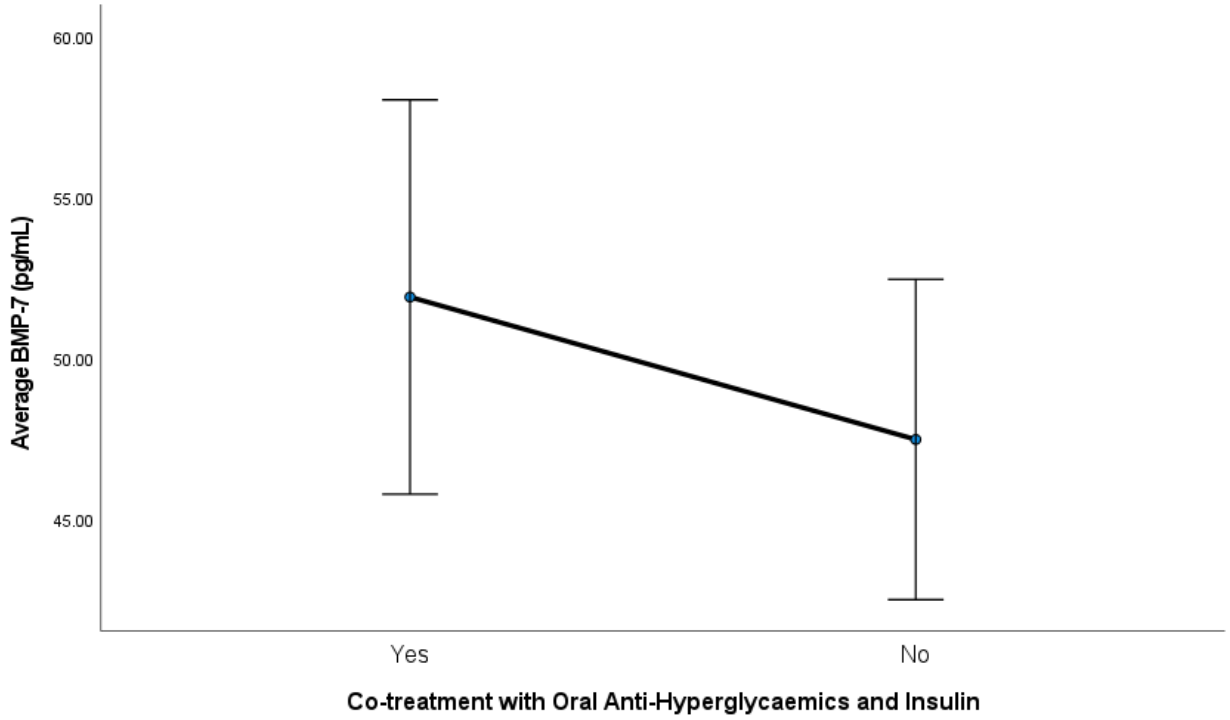

Figure A24: ANCOVA graph showing the effects of co-treatment with oral anti-hyperglycaemics and insulin on 12 week BMP-7 values.

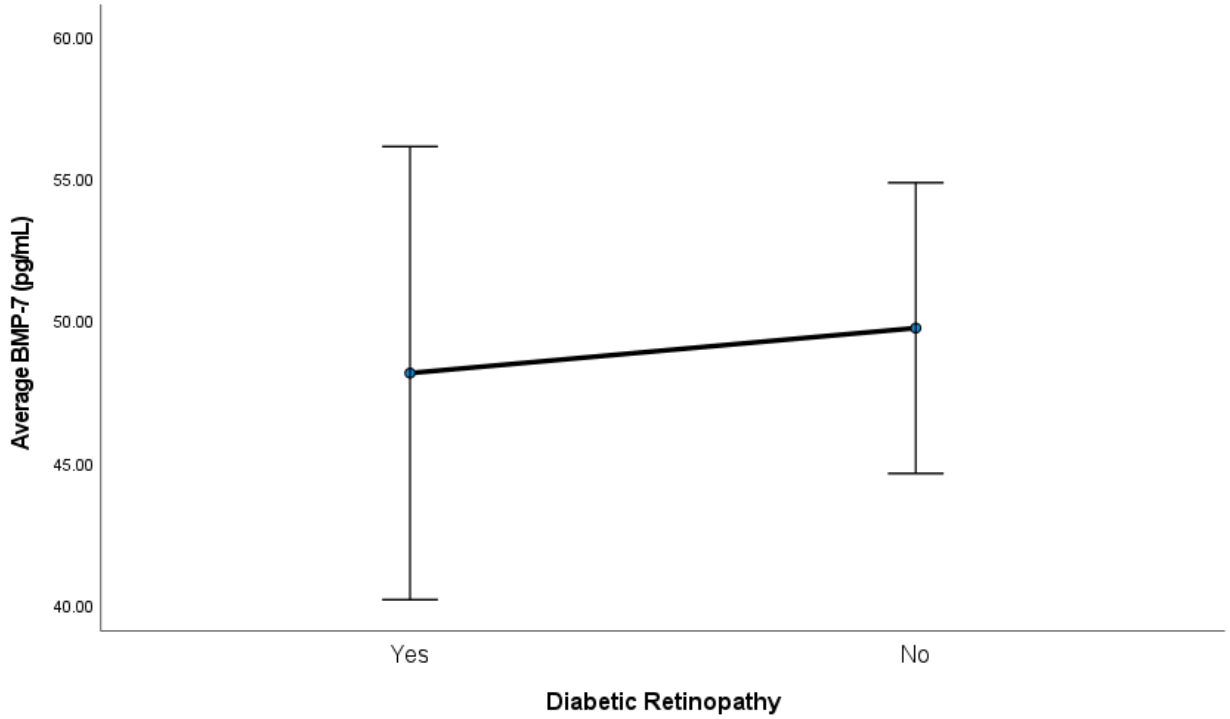

Figure A25: ANCOVA graph showing the effects of Diabetic Retinopathy on 12 week BMP-7 values.

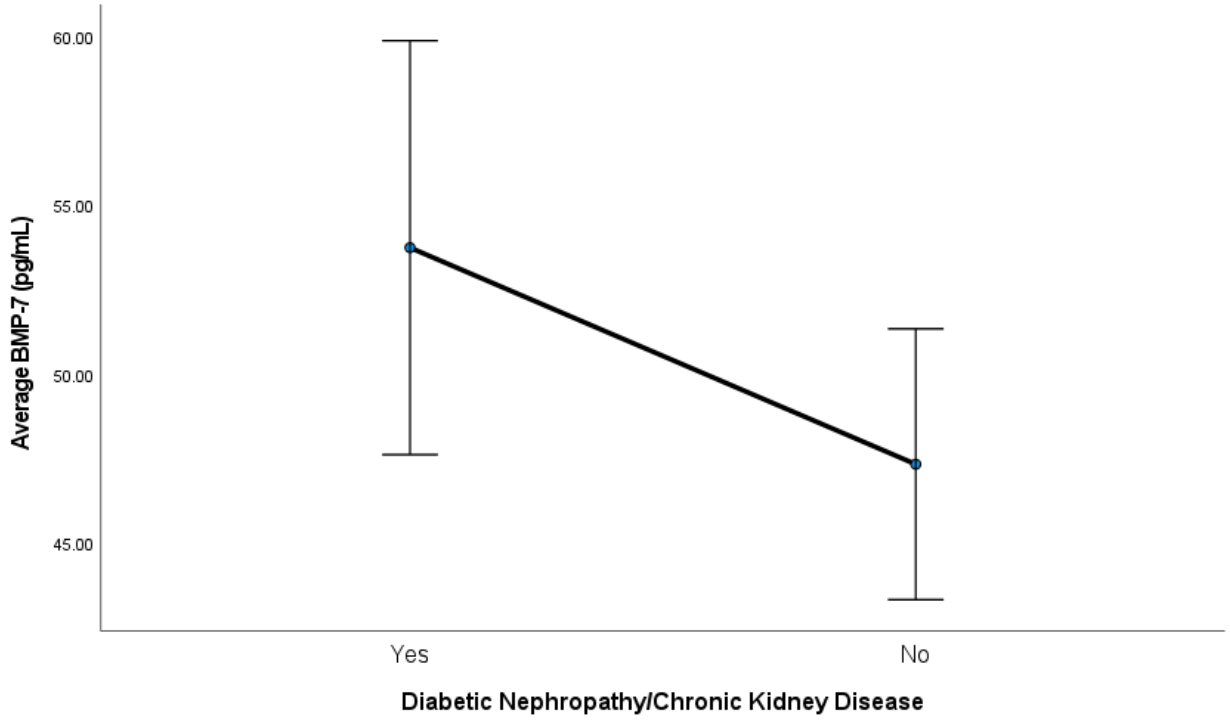

Figure A26: ANCOVA graph showing the effects of Diabetic Nephropathy/Chronic Kidney Disease on 12 week BMP-7 values.

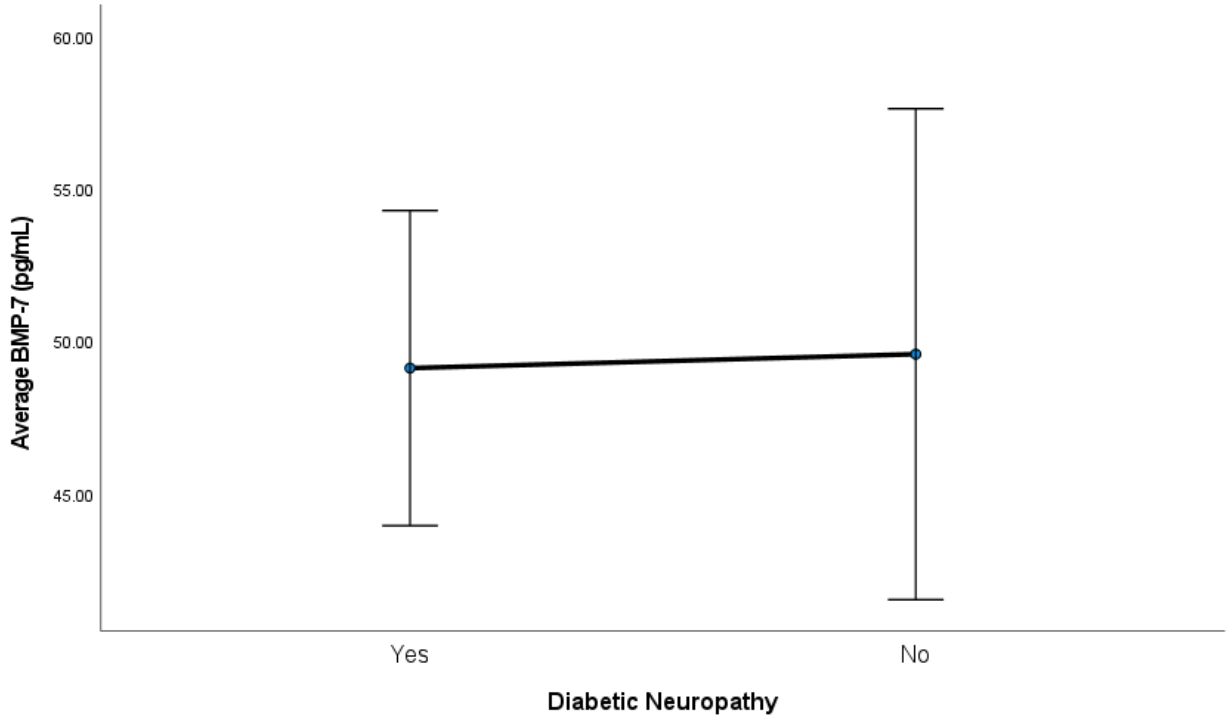

Figure A27: ANCOVA graph showing the effects of Diabetic Neuropathy on 12 week BMP-7 values.

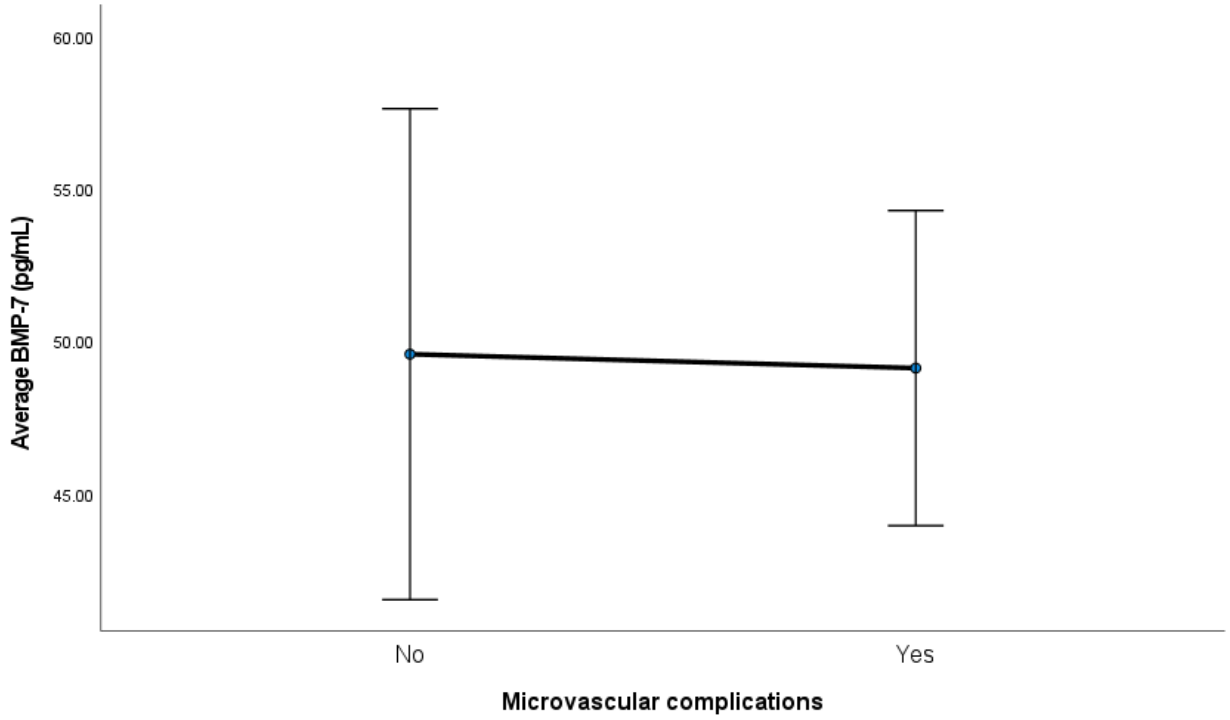

Figure A28: ANCOVA graph showing the effects of microvascular complications on 12 week BMP-7 values.

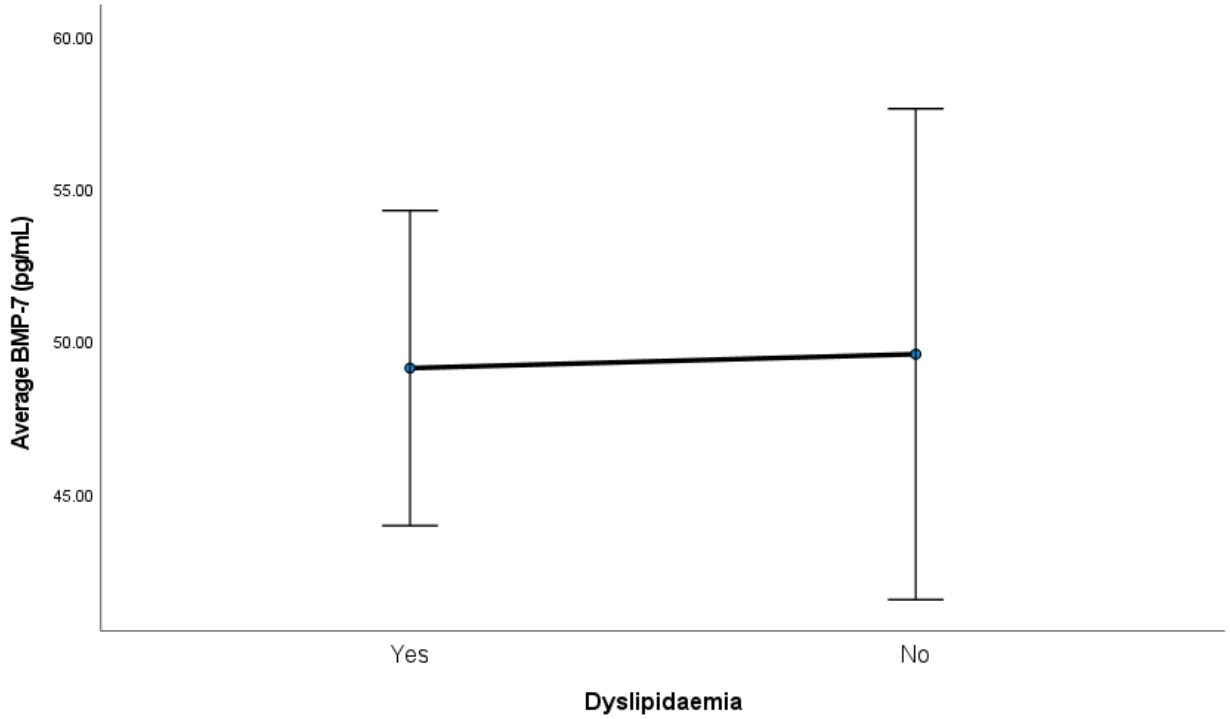

Figure A29: ANCOVA graph showing the effects of dyslipidaemia on 12 week BMP-7 values.

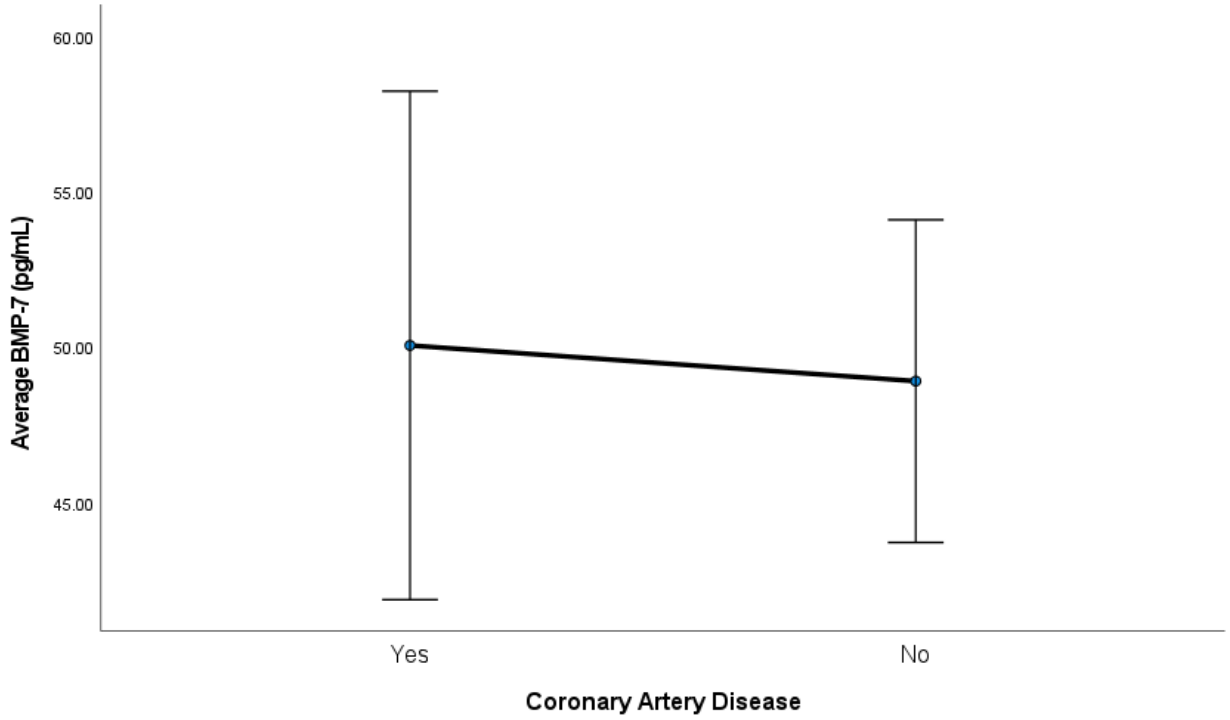

Figure A30: ANCOVA graph showing the effects of coronary artery disease on 12 week BMP-7 values.

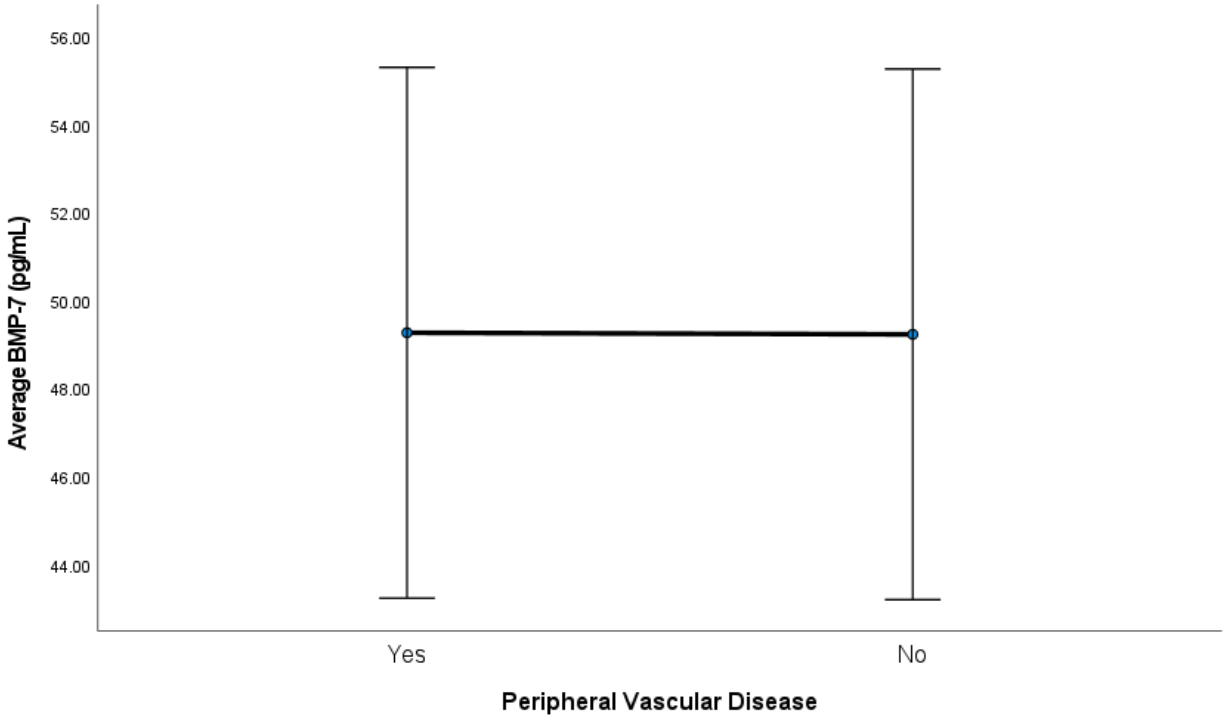

Figure A31: ANCOVA graph showing the effects of peripheral vascular disease on 12 week BMP-7 values.

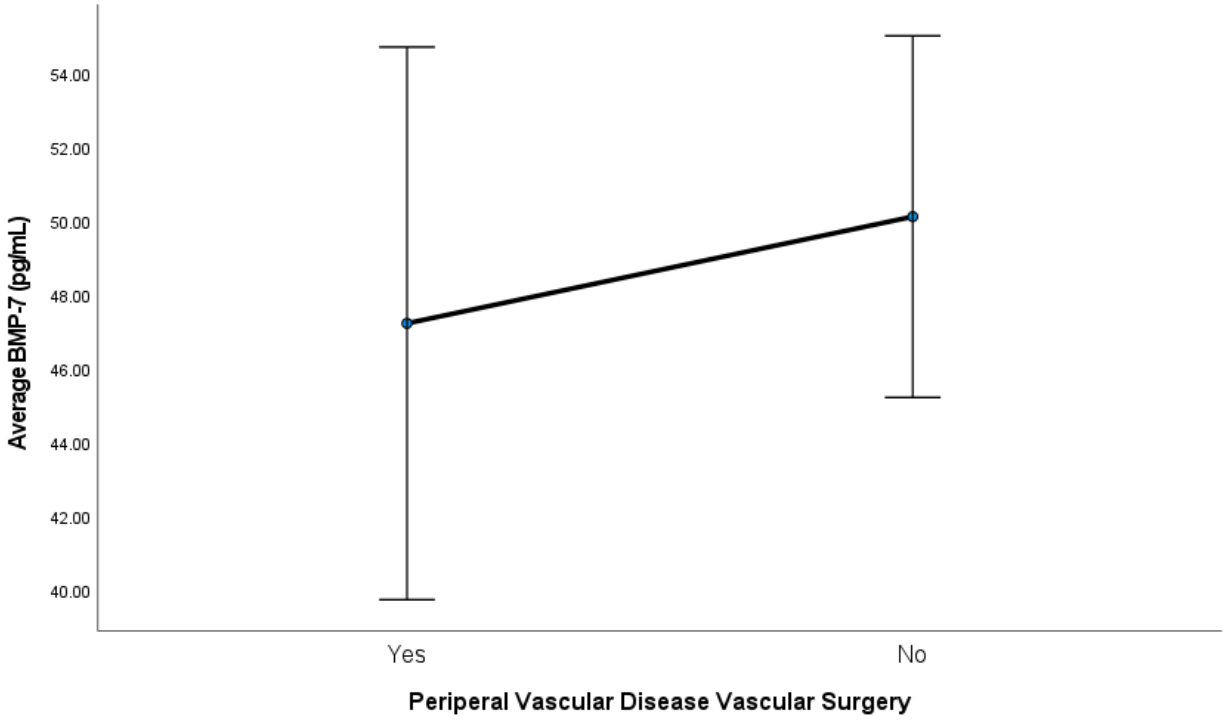

Figure A32: ANCOVA graph showing the effects of Peripheral Vascular Disease Surgery on 12 week BMP-7 values.

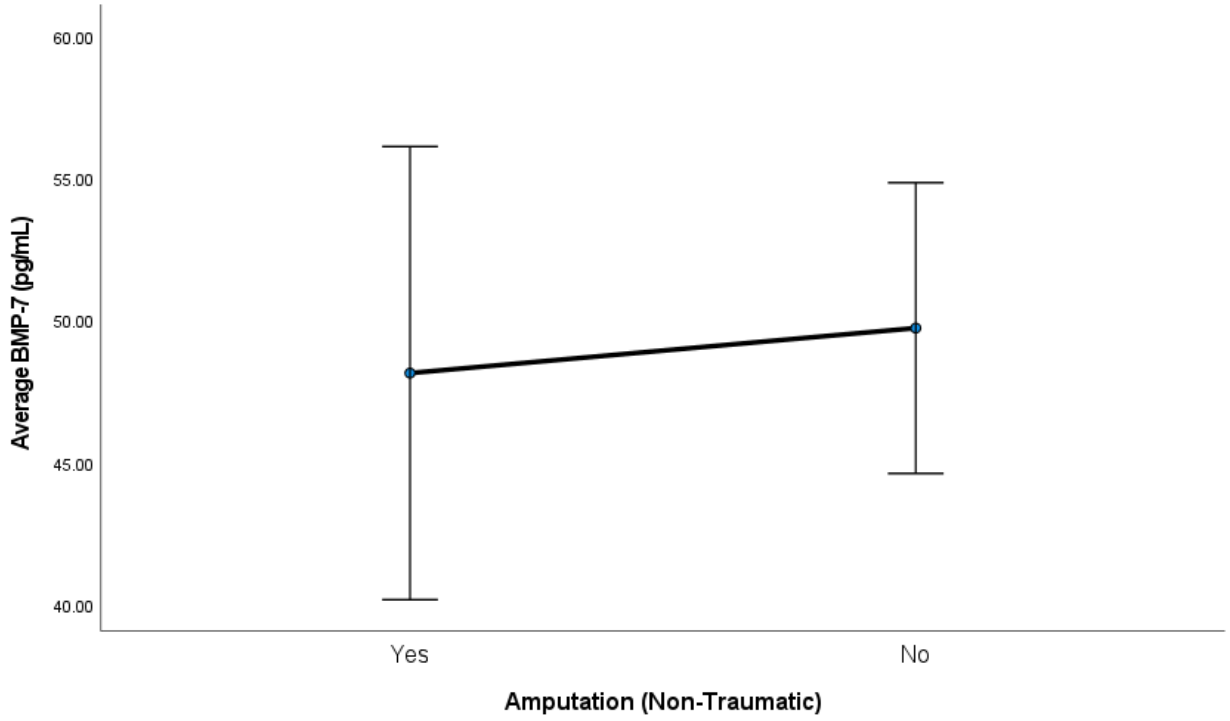

Figure A33: ANCOVA graph showing the effects of Non-Traumatic Amputation on 12 week BMP-7 values.

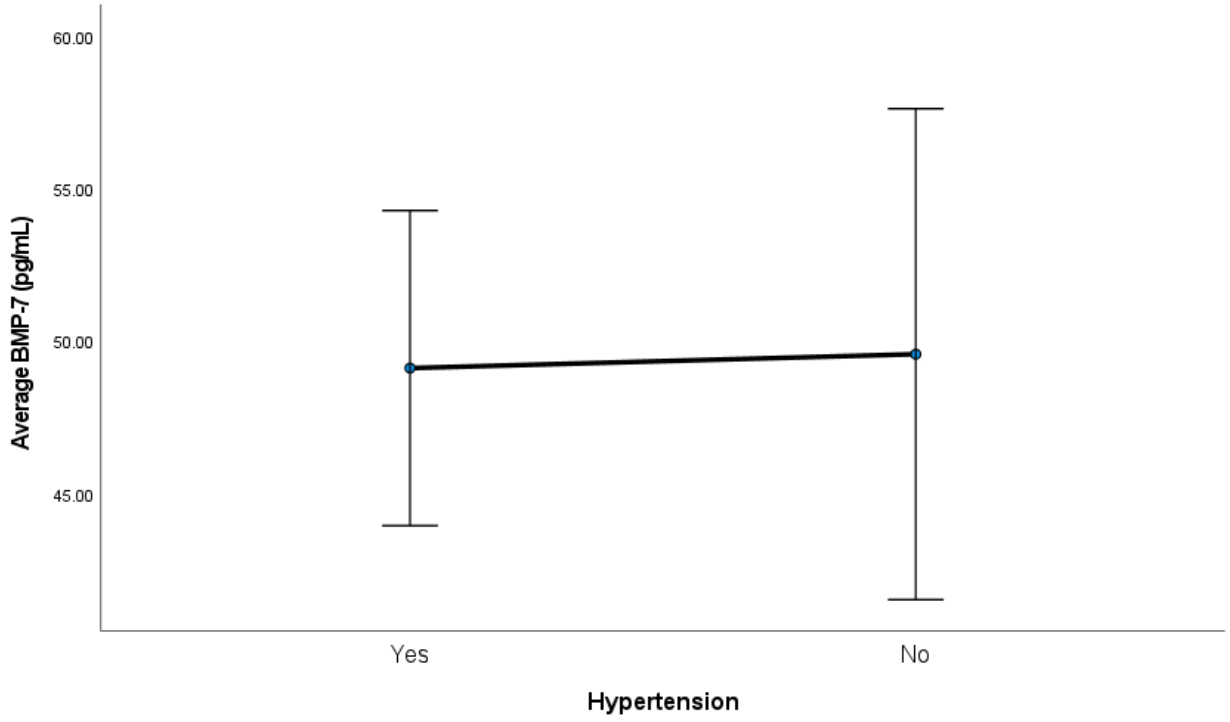

Figure A34: ANCOVA graph showing the effects of hypertension on 12 week BMP-7 values.

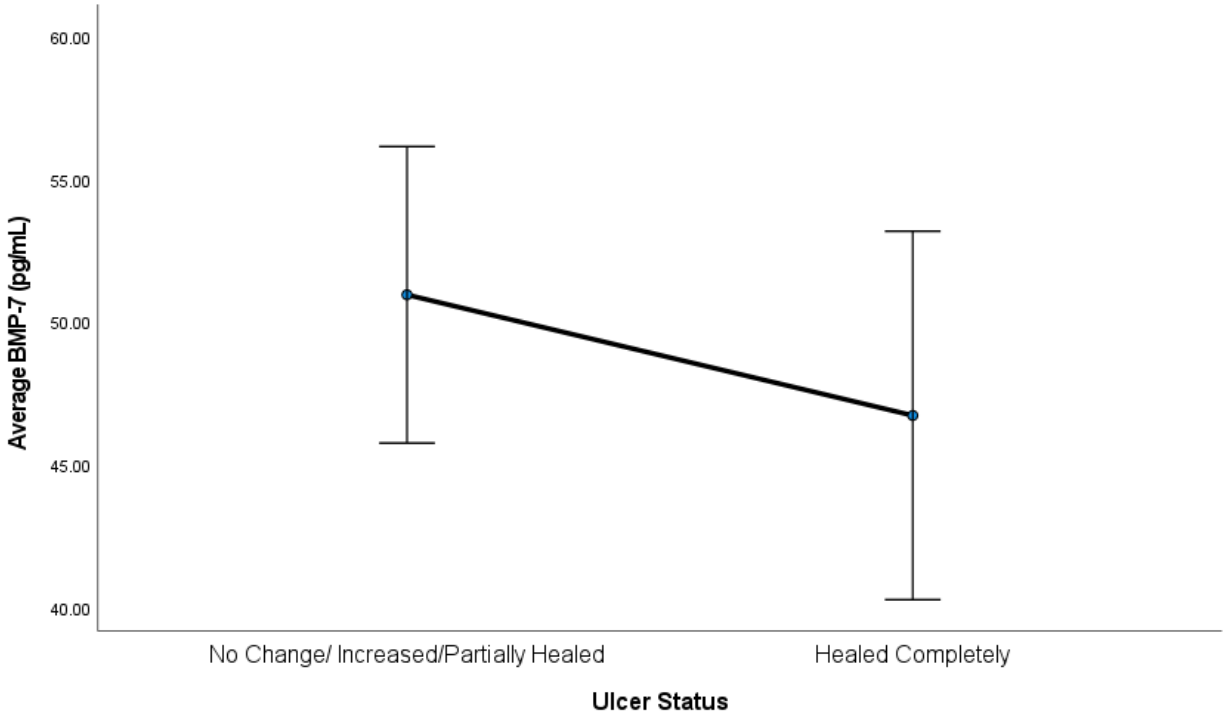

Figure A35: ANCOVA graph showing the effects of ulcer status on 12 week BMP-7 values.

Appendix 3: Correlation tables for relationships between laboratory parameters.

Table A5: Spearman's correlation analysis for intake data collected from the GIED trial. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	age	years of diabetes	T1DM Vs T2DM	Height	Weight	Systolic BP	Diastolic BP	Ulcer SA	Protein (Total)	Albumin	Globulin	Fibrinogen	Urea/ Creatinine
age	1.000	0.818**	.	-0.341	0.085	-0.220	-0.414	-0.277	-0.393	-0.257	0.184	1.000	-0.097
years of diabetes	0.818**	1.000	.	-0.194	0.426	-0.409	-0.669*	-0.117	0.000	-0.173	0.233	1.000	0.030
T1DM Vs T2DM	.	.	.	.	.	.	.	.	.	.	.	.	.
Height	-0.341	-0.194	.	1.000	0.680*	0.492	0.512	0.183	0.464	0.330	-0.313	-1.000	0.050
Weight	0.085	0.426	.	0.680*	1.000	0.052	-0.012	0.354	0.439	0.046	-0.064	-1.000	0.185
Systolic Blood Pressure (BP)	-0.220	-0.409	.	0.492	0.052	1.000	0.644*	0.450	-0.098	0.561	-0.729*	-1.000	-0.097
Diastolic BP	-0.414	-0.669*	.	0.512	-0.012	0.644*	1.000	0.105	0.025	0.077	-0.168	-1.000	0.201
Ulcer SA	-0.277	-0.117	.	0.183	0.354	0.450	0.105	1.000	0.110	0.190	-0.387	-1.000	0.328
Protein (Total)	-0.393	0.000	.	0.464	0.439	-0.098	0.025	0.110	1.000	0.258	0.224	.	-0.110
Albumin	-0.257	-0.173	.	0.330	0.046	0.561	0.077	0.190	0.258	1.000	-0.849**	1.000	0.097
Globulin	0.184	0.233	.	-0.313	-0.064	-0.729*	-0.168	-0.387	0.224	-0.849**	1.000	-1.000	-0.186
Fibrinogen	1.000**	1.000**	.	-1.000**	-1.000**	-1.000**	-1.000**	-1.000**	.	1.000**	-1.000**	1.000	.
Urea/Creatinine	-0.097	0.030	.	0.050	0.185	-0.097	0.201	0.328	-0.110	0.097	-0.186	.	1.000
Estimated Glomerular Filtration Rate (eGFR)	-0.700*	-0.657*	.	-0.162	-0.597	0.117	0.098	0.052	0.000	0.436	-0.274	1.000**	0.223
White Blood cells (WBC)	-0.357	-0.157	.	0.299	0.240	0.265	-0.233	0.381	-0.037	0.719*	-0.651	.	-0.096
Neutrophils	-0.167	0.012	.	0.371	0.359	0.361	-0.356	0.524	-0.259	0.599	-0.699	.	-0.024
Haematocrit	-0.152	-0.032	.	0.530	0.332	-0.090	-0.085	-0.292	0.769*	0.006	0.250	.	-0.759*
Hemoglobin (Hb)	-0.515	-0.491	.	0.693	0.036	0.018	0.327	-0.407	0.505	0.054	0.121	.	-0.524

Table A5: Spearman's correlation analysis for intake data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	age	years of diabetes	T1DM Vs T2DM	Height	Weight	Systolic BP	Diastolic BP	ulcer SA	Protein (Total)	Albumin	Globulin	Fibrinogen	Urea/ Creatinine
Platelets	0.048	0.084	.	0.204	0.228	0.349	0.049	0.714*	-0.556	0.371	-0.542	.	0.707
Cholesterol (Chol)	-0.578	-0.542	.	0.000	-0.359	0.445	0.400	0.116	0.366	0.573	-0.385	1.000**	0.126
Triglyceride	0.433	0.309	.	-0.445	-0.116	0.067	-0.383	0.421	-0.454	-0.315	0.018	-1.000**	-0.315
High Density Lipoprotein	-0.521	-0.559	.	-0.129	-0.534	0.283	0.196	-0.052	0.272	0.637*	-0.370	1.000**	-0.114
Total/ HDL ratio	-0.492	-0.518	.	0.172	-0.179	0.692*	0.619	0.383	0.100	0.289	-0.379	-1.000**	0.175
Low Density Lipoprotein Chol (LDL Chol)	-0.626	-0.652*	.	0.134	-0.377	0.433	0.523	-0.067	0.268	0.598	-0.404	1.000**	0.218
Very LDL Chol (VLDL Chol)	0.422	0.279	.	-0.410	-0.125	0.117	-0.347	0.404	-0.457	-0.291	-0.009	-1.000**	-0.376
Urine Creatinine	0.367	0.286	.	0.259	0.460	0.050	0.255	-0.333	0.037	-0.160	0.034	1.000**	-0.228
Urine Albumin	0.483	0.210	.	0.234	0.218	0.261	0.417	-0.100	-0.556	-0.370	0.067	-1.000**	0.359
Urine Albumin/ Creatinine	0.533	0.261	.	0.075	0.126	0.176	0.315	-0.067	-0.556	-0.311	0.059	-1.000**	0.503
HbA1c	-0.394	-0.288	.	-0.309	-0.260	-0.595	-0.204	-0.180	0.366	-0.255	0.498	1.000**	-0.214
Interleukin (IL)-6	0.262	0.146	.	-0.144	-0.060	-0.096	0.048	0.036	-0.086	-0.862**	0.807*	-1.000**	-0.786*
IL1β	-0.328	-0.277	.	0.128	0.249	0.049	0.425	0.541	-0.073	-0.189	0.055	-1.000**	0.895**
Tumour Necrosis Factor - α (TNF-α)	0.182	0.092	.	0.176	0.237	-0.274	0.265	-0.170	-0.268	-0.720*	0.550	-1.000**	0.351
Adiponectin	-0.170	-0.222	.	0.498	0.298	0.085	0.474	-0.286	0.561	-0.152	0.239	-1.000**	-0.536

Table A5: Spearman's correlation analysis for intake data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	Age	years of diabetes	T1DM Vs T2DM	Height	Weight	Systolic BP	Diastolic BP	ulcer SA	Protein (Total)	Albumin	Globulin	Fibrinogen	Urea/ Creatinine
C-Reactive Protein (CRP)	0.523	0.160	.	-0.116	-0.006	0.091	0.234	0.122	-0.439	-0.262	0.110	-1.000**	0.276
HIF-α	0.048	0.024	.	0.623	0.500	0.671	0.464	0.671	0.116	0.395	-0.611	-1.000**	0.286

Table A5: Spearman's correlation analysis for intake data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	eGFR	WBC	Neutrophils	Haematocrit	Hb	Platelets	Chol	Triglyceride	HDL	Total/ HDL ratio
age	-0.700*	-0.357	-0.167	-0.152	-0.515	0.048	-0.578	0.433	-0.521	-0.492
years of diabetes	-0.657*	-0.157	0.012	-0.032	-0.491	0.084	-0.542	0.309	-0.559	-0.518
T1DM Vs T2DM	.	.	.	.	.	.	.	.	.	.
Height	-0.162	0.299	0.371	0.530	0.693	0.204	0.000	-0.445	-0.129	0.172
Weight	-0.597	0.240	0.359	0.332	0.036	0.228	-0.359	-0.116	-0.534	-0.179
Systolic BP	0.117	0.265	0.361	-0.090	0.018	0.349	0.445	0.067	0.283	0.692*
Diastolic BP	0.098	-0.233	-0.356	-0.085	0.327	0.049	0.400	-0.383	0.196	0.619
ulcer SA	0.052	.0381	0.524	-0.292	-0.407	0.714*	0.116	0.421	-0.052	0.383
Protein (Total)	0.000	-0.037	-0.259	0.769*	0.505	-0.556	0.366	-0.454	0.272	0.100
Albumin	0.436	0.719*	0.599	0.006	0.054	0.371	0.573	-0.315	0.637*	0.289
Globulin	-0.274	-0.651	-0.699	0.250	0.121	-0.542	-0.385	0.018	-0.370	-0.379
Fibrinogen	1.000	.	.	.	.	.	1.000	-1.000	1.000	-1.000
Urea/Creat	0.223	-0.096	-0.024	-0.759*	-0.524	0.707	0.126	-0.315	-0.114	0.175
eGFR	1.000	0.101	-0.025	-0.236	0.108	0.025	0.666*	-0.318	0.757*	0.414
WBC	0.101	1.000	0.905**	0.317	0.275	0.333	0.024	0.012	0.229	-0.419
Neutrophils	-0.025	.0905**	1.000	0.190	0.156	0.524	-0.262	0.275	-0.084	-0.419
Haematocrit	-0.236	0.317	0.190	1.000	0.829*	-0.507	-0.203	-0.287	0.006	-0.500
Hb	0.108	0.275	0.156	0.829*	1.000	-0.347	-0.036	-0.560	0.164	-0.287
Platelets	0.025	0.333	0.524	-0.507	-0.347	1.000	-0.262	0.287	-0.277	-0.140
Cholesterol	0.666*	0.024	-0.262	-0.203	-0.036	-0.262	1.000	-0.371	0.875**	0.757*
Triglyceride	-0.318	0.012	0.275	-0.287	-0.560	0.287	-0.371	1.000	-0.347	-0.078
HDL	0.757*	0.229	-0.084	0.006	0.164	-0.277	0.875**	-0.347	1.000	0.410
Total/ HDL ratio	0.414	-0.419	-0.419	-0.500	-0.287	-0.140	0.757*	-0.078	0.410	1.000

Table A5: Spearman's correlation analysis for intake data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	eGFR	WBC	Neutrophils	Haematocrit	Hb	Platelets	Cholesterol	Triglyceride	HDL	Total/ HDL ratio	LDL Chol
LDL Chol	0.718*	0.024	-0.262	-0.203	0.108	-0.167	0.939**	-0.602	0.856**	0.682*	1.000
VLDL Chol	-0.319	0.024	0.289	-0.244	-0.503	0.253	-0.354	0.997**	-0.326	-0.057	-.0579
Urine Creatinine	-0.694*	-0.095	-0.190	0.203	-0.048	-0.405	-0.050	-0.218	-0.218	-0.026	-0.050
Urine Albumin	-0.529	-0.643	-0.405	-0.469	-0.323	0.238	-0.350	0.059	-0.597	0.279	-0.233
Urine Albumin/ Creatinine ratio	-0.420	-0.667	-0.429	-0.558	-0.443	0.310	-0.333	0.059	-0.563	0.235	-0.217
HbA1c	0.299	0.240	-0.072	0.472	0.494	-0.443	0.085	-0.177	0.345	-0.359	0.067
IL-6	-0.406	-0.714	-0.486	0.395	0.290	-0.657	-0.476	0.647	-0.482	-0.084	-0.524
IL1β	0.071	-0.071	-0.119	-0.469	-0.240	0.619	0.055	-0.176	-0.165	0.269	0.079
TNFα	-0.407	-0.643	-0.452	-0.203	-0.024	0.143	-0.600	-0.103	-0.752*	-0.181	-0.442
Adiponectin	-0.381	0.095	-0.143	0.786*	0.707	-0.667	0.030	-0.347	0.018	-0.050	0.091
CRP	-0.369	-0.286	-0.190	-0.469	-0.395	0.643	-0.418	0.231	-0.373	-0.219	-0.358
HIF-α	-0.122	0.371	0.543	-0.030	-0.143	0.886*	0.000	-0.048	-0.060	0.122	-0.048

Table A5: Spearman's correlation analysis for intake data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	LDL Chol	VLDL Chol	Urine Creatinine	Urine Albumin	Urine Albumin/ Creatinine ratio	HbA1c	IL-6	IL-1β	TNF-α	Adiponectin
age	-0.626	0.422	0.367	0.483	0.533	-0.394	0.262	-0.328	0.182	-0.170
years of diabetes	-0.652*	0.279	0.286	0.210	0.261	-0.288	0.146	-0.277	.092	-0.222
T1DM Vs T2DM	.	.	.	.	.	.	.	.	.	.
Height	0.134	-0.410	0.259	0.234	0.075	-0.309	-0.144	0.128	0.176	0.498
Weight	-0.377	-0.125	0.460	0.218	0.126	-0.260	-0.060	0.249	0.237	0.298
Systolic BP	0.433	0.117	0.050	0.261	0.176	-0.595	-0.096	0.049	-0.274	0.085
Diastolic BP	0.523	-0.347	0.255	0.417	0.315	-0.204	0.048	0.425	0.265	0.474
Ulcer SA	-0.067	0.404	-0.333	-0.100	-0.067	-0.180	0.036	0.541	-0.170	-0.286
Protein (Total)	0.268	-0.457	0.037	-0.556	-0.556	0.366	-0.086	-0.073	-0.268	0.561
Albumin	0.598	-0.291	-0.160	-0.370	-0.311	-0.255	-0.862**	-0.189	-0.720*	-0.152
Globulin	-0.404	-0.009	0.034	0.067	0.059	0.498	0.807*	0.055	0.550	0.239
Fibrinogen	1.000	-1.000	1.000	-1.000	-1.000	1.000	-1.000	-1.000	-1.000	-1.000
Urea/Creat	0.218	-0.376	-0.228	0.359	0.503	-0.214	-0.786*	0.895**	0.351	-0.536
eGFR	0.718*	-0.319	-0.694*	-0.529	-0.420	0.299	-0.406	0.071	-0.407	-0.381
WBC	0.024	0.024	-0.095	-0.643	-0.667	0.240	-0.714	-0.071	-0.643	0.095
Neutrophils	-0.262	0.289	-0.190	-0.405	-0.429	-0.072	-0.486	-0.119	-0.452	-0.143
Haematocrit	-0.203	-0.244	0.203	-0.469	-0.558	0.472	0.395	-0.469	-0.203	0.786*
Hb	0.108	-0.503	-0.048	-0.323	-0.443	0.494	0.290	-0.240	-0.024	0.707
Platelets	-0.167	0.253	-0.405	0.238	0.310	-0.443	-0.657	0.619	0.143	-0.667
Cholesterol	0.939**	-0.354	-0.050	-0.350	-0.333	0.085	-0.476	0.055	-0.600	0.030
Triglyceride	-0.602	0.997**	-0.218	0.059	0.059	-0.177	0.647	-0.176	-0.103	-0.347
HDL	0.856**	-0.326	-0.218	-0.597	-0.563	0.345	-0.482	-0.165	-0.752*	0.018
Total/ HDL ratio	0.682*	-0.057	-0.026	0.279	0.235	-0.359	-0.084	0.269	-0.181	-0.050

Table A5: Spearman's correlation analysis for intake data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	VLDL Chol	Urine Creatinine	Urine Albumin	Urine Albumin/ Creatinine ratio	HbA1c	IL-6	IL-1β	TNF-α	Adiponectin	CRP	HIF-α
LDL Chol	-0.579	-0.050	-0.233	-0.217	0.067	-0.524	0.079	-0.442	0.091	-0.358	-0.048
VLDL Chol	1.000	-0.193	0.067	0.050	-0.196	0.647	-0.220	-0.122	-0.305	0.213	-0.048
Urine Creatinine	-0.193	1.000	0.333	0.183	-0.143	-0.036	-0.117	0.100	0.717*	-0.017	0.000
Urine Albumin	0.067	0.333	1.000	0.967**	-0.790*	0.214	0.200	0.700*	0.000	0.600	0.357
Urine Albumin/ Creatinine ratio	0.050	0.183	0.967**	1.000	-0.798**	0.071	0.267	0.683*	-0.217	0.700*	0.393
HbA1c	-0.196	-0.143	-0.790*	-0.798**	1.000	0.048	0.037	-0.159	0.274	-0.256	-0.675
IL-6	0.647	-0.036	0.214	0.071	0.048	1.000	-0.286	0.452	0.214	0.119	-0.200
IL-1β	-0.220	-0.117	0.200	0.267	0.037	-0.286	1.000	0.442	-0.115	0.406	0.405
TNF-α	-0.122	0.100	0.700*	0.683*	-0.159	0.452	0.442	1.000	0.115	0.467	0.095
Adiponectin	-0.305	0.717*	0.000	-0.217	0.274	0.214	-0.115	0.115	1.000	-0.152	-0.024
CRP	0.213	-0.017	0.600	0.700*	-0.256	0.119	0.406	0.467	-0.152	1.000	0.405
HIF-α	-0.048	0.000	0.357	0.393	-0.675	-0.200	0.405	0.095	-0.024	0.405	1.000

Table A5: Spearman's correlation analysis for intake data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	CRP	HIF-α
age	0.523	0.048
years of diabetes	0.160	0.024
T1DM Vs T2DM	.	.
Height	-0.116	0.623
Weight	-0.006	0.500
Systolic BP	0.091	0.671
Diastolic BP	0.234	0.464
ulcer SA	0.122	0.671
Protein (Total)	-0.439	0.116
Albumin	-0.262	0.395
Globulin	0.110	-0.611
Fibrinogen	-1.000	-1.000
Urea/Creat	0.276	0.286
eGFR	-0.369	-0.122
WBC	-0.286	0.371
Neutrophils	-0.190	0.543
Haematocrit	-0.469	-0.030
Hb	-0.395	-0.143
Platelets	0.643	0.886*
Cholesterol	-0.418	0.000
Triglyceride	0.231	-0.048
HDL	-0.373	-0.060
Total/ HDL ratio	-0.219	0.122

Table A6: Spearman's correlation analysis for 12 week data collected from the GIED trial. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	Ulcer SA	Protein (Total)	Albumin	Globulin	Fibrinogen	Urea/Creat	eGFR	WBC	Neutrophils	Haematocrit	Hb
Ulcer SA	1.000	-0.434	-0.255	-0.223	.	-0.554	0.178	0.270	0.000	-0.143	-0.126
Protein (Total)	-0.434	1.000	0.327	0.325	.	0.163	0.631	0.136	0.378	0.306	0.391
Albumin	-0.255	0.327	1.000	-0.600	.	-0.242	0.221	0.245	0.414	0.018	0.000
Globulin	-0.223	0.325	-0.600	1.000	.	0.181	0.288	-0.396	-0.143	0.286	0.306
Fibrinogen	.	.	.	.	.	.	.	.	.	.	.
Urea/Creat	-0.554	0.163	-0.242	0.181	.	1.000	-0.082	0.164	0.144	-0.541	-0.482
eGFR	0.178	0.631	0.221	0.288	.	-0.082	1.000	0.472	0.802*	0.045	0.135
WBC	0.270	0.136	0.245	-0.396	.	0.164	0.472	1.000	0.793*	0.072	0.136
Neutrophils	0.000	0.378	0.414	-0.143	.	0.144	0.802*	0.793*	1.000	0.179	0.234
Haematocrit	-0.143	0.306	0.018	0.286	.	-0.541	0.045	0.072	0.179	1.000	0.991**
Hb	-0.126	0.391	0.000	0.306	.	-0.482	0.135	0.136	0.234	0.991**	1.000
Platelets	-0.464	0.378	0.487	-0.214	.	0.559	0.535	0.126	0.286	-0.643	-0.613
Cholesterol	0.173	0.018	-0.064	0.500	.	-0.391	0.249	-0.086	0.314	0.429	0.429
Triglyceride	0.064	0.090	0.101	0.200	.	-0.264	0.119	0.174	0.493	0.638	0.638
HDL	0.449	-0.037	0.359	-0.748	.	-0.168	0.123	0.174	-0.029	-0.493	-0.493
Total/ HDL ratio	0.018	-0.144	-0.385	0.718	.	-0.091	0.109	-0.200	0.143	0.314	0.314
LDL Chol	0.173	0.126	-0.064	0.636	.	-0.336	0.517	0.000	0.377	0.406	0.406
VLDL Chol	0.255	-0.225	-0.219	0.293	.	-0.217	0.041	0.030	0.273	0.273	0.273
Urine Creatinine	0.116	-0.429	-0.116	-0.348	.	-0.319	-0.638	0.100	0.000	0.700	0.700

Table A6: Spearman's correlation analysis for 12 week data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	ulcer SA	Protein (Total)	Albumin	Globulin	Fibrinogen	Urea/Creat	eGFR	WBC	Neutrophils	Haematocrit
Urine Albumin	0.145	-0.829*	-0.609	-0.464	.	0.232	-0.880*	-0.100	-0.500	-0.200
Urine Albumin/ Creatinine ratio	-0.319	-0.486	-0.493	-0.290	.	0.812*	-0.698	-0.200	-0.500	-0.900*
HbA1c	-0.034	0.240	-0.313	0.671	.	0.252	0.218	0.144	0.250	0.464
IL-6	0.479	0.541	-0.180	0.306	.	-0.054	0.802*	0.829*	0.657	0.143
IL-1β	0.119	-0.240	-0.759*	0.180	.	0.503	-0.355	-0.198	-0.429	-0.464
TNF-α	-0.532	-0.192	-0.325	0.096	.	0.838**	-0.273	0.162	0.250	-0.536
Adiponectin	-0.300	-0.192	-0.241	0.228	.	-0.323	-0.518	-0.270	-0.143	0.786*
CRP	-0.694*	0.240	0.518	-0.455	.	0.527	-0.136	0.036	0.071	-0.643
HIF-α	-0.759	0.000	0.000	0.316	.	0.800	-0.632	-1.000**	-0.500	-0.500

Table A6: Spearman's correlation analysis for 12 week data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	Platelets	Cholesterol	Triglyceride	HDL	Total/ HDL ratio	LDL Chol	VLDL Chol	Urine Creatinine	Urine Albumin	Urine Albumin/ Creatinine ratio
Ulcer SA	-0.464	0.173	0.064	0.449	0.018	0.173	0.255	0.116	0.145	-0.319
Protein (Total)	0.378	0.018	0.090	-0.037	-0.144	0.126	-0.225	-0.429	-0.829*	-0.486
Albumin	0.487	-0.064	0.101	0.359	-0.385	-0.064	-0.219	-0.116	-0.609	-0.493
Globulin	-0.214	0.500	0.200	-0.748	0.718	0.636	0.293	-0.348	-0.464	-0.290
Fibrinogen	.	.	.	.	.	.	.	.	.	.
Urea/Creat	0.559	-0.391	-0.264	-0.168	-0.091	-0.336	-0.217	-0.319	0.232	0.812*
eGFR	0.535	0.249	0.119	0.123	0.109	0.517	0.041	-0.638	-0.880*	-0.698
WBC	0.126	-0.086	0.174	0.174	-0.200	0.000	0.030	0.100	-0.100	-0.200
Neutrophils	0.286	0.314	0.493	-0.029	0.143	0.377	0.273	0.000	-0.500	-0.500
Haematocrit	-0.643	0.429	0.638	-0.493	0.314	0.406	0.273	0.700	-0.200	-0.900*
Hb	-0.613	0.429	0.638	-0.493	0.314	0.406	0.273	0.700	-0.200	-0.900*
Platelets	1.000	-0.314	-0.406	0.203	-0.314	-0.290	-0.455	-0.900*	-0.600	0.300
Cholesterol	-0.314	1.000	0.882**	-0.337	0.882**	0.918**	0.916**	0.551	-0.116	-0.667
Triglyceride	-0.406	0.882**	1.000	-0.337	0.773*	0.745	0.925**	0.754	0.174	-0.319
HDL	0.203	-0.337	-0.337	1.000	-0.673	-0.449	-0.291	-0.058	0.058	0.116
Total/ HDL ratio	-0.314	0.882**	0.773*	-0.673	1.000	0.882**	0.859*	0.406	0.029	-0.377
LDL Chol	-0.290	0.918**	0.745	-0.449	0.882**	1.000	0.774*	0.116	-0.464	-0.841*
VLDL Chol	-0.455	0.916**	0.925**	-0.291	0.859*	0.774*	1.000	0.833*	0.339	-0.309
Urine Creatinine	-0.900*	0.551	0.754	-0.058	0.406	0.116	0.833*	1.000	0.714	0.143

Table A6: Spearman's correlation analysis for 12 week data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	Hb	Platelets	Cholesterol	Triglyceride	HDL	Total/ HDL ratio	LDL Chol	VLDL Chol	Urine Creatinine	Urine Albumin
Urine Albumin	-0.200	-0.600	-0.116	0.174	0.058	0.029	-0.464	0.339	0.714	1.000
Urine Albumin/ Creatinine ratio	-0.900*	0.300	-0.667	-0.319	0.116	-0.377	-0.841*	-0.309	0.143	0.714
HbA1c	0.450	-0.107	0.306	0.342	-0.927**	0.613	0.523	0.243	-0.143	-0.257
IL-6	0.290	0.086	0.203	0.261	0.030	0.145	0.551	0.177	-0.400	-0.600
IL-1β	-.0414	-0.107	-0.360	-0.342	0.259	-0.198	-0.523	-0.075	0.143	0.771
TNF-α	-0.505	0.321	-0.036	0.144	-0.185	0.216	-0.144	0.262	0.200	0.657
Adiponectin	0.757*	-0.893**	0.595	0.613	-0.408	0.559	0.324	0.617	0.771	0.429
CRP	-0.613	0.929**	-0.523	-0.342	0.408	-0.613	-0.631	-0.487	-0.029	0.029
HIF-α	-0.500	1.000**	-0.316	-0.200	-0.105	-0.200	-0.400	-0.316	0.200	0.400

Table A6: Spearman's correlation analysis for 12 week data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test

Categories	HbA1c	IL-6	IL-1β	TNF-α	Adiponectin	CRP	HIF-α
ulcer SA	-0.034	0.479	0.119	-0.532	-0.300	-0.694*	-0.759
Protein (Total)	0.240	0.541	-0.240	-0.192	-0.192	0.240	0.000
Albumin	-0.313	-0.180	-0.759*	-0.325	-0.241	0.518	0.000
Globulin	0.671	0.306	0.180	0.096	0.228	-0.455	0.316
Fibrinogen	.	.	.	.	.	.	.
Urea/Creat	0.252	-0.054	0.503	0.838**	-0.323	0.527	0.800
eGFR	0.218	0.802*	-0.355	-0.273	-0.518	-0.136	-0.632
WBC	0.144	0.829*	-0.198	0.162	-0.270	0.036	-1.000**
Neutrophils	0.250	0.657	-0.429	0.250	-0.143	0.071	-0.500
Haematocrit	0.464	0.143	-0.464	-0.536	0.786*	-0.643	-0.500
Hb	0.450	0.290	-0.414	-0.505	0.757*	-0.613	-0.500
Platelets	-0.107	0.086	-0.107	0.321	-0.893**	0.929**	1.000**
Cholesterol	0.306	0.203	-0.360	-0.036	0.595	-0.523	-0.316
Triglyceride	0.342	0.261	-0.342	0.144	0.613	-0.342	-0.200
HDL	-0.927**	0.030	0.259	-0.185	-0.408	0.408	-0.105
Total/ HDL ratio	0.613	0.145	-0.198	0.216	0.559	-0.613	-0.200
LDL Chol	0.523	0.551	-0.523	-0.144	0.324	-0.631	-0.400
VLDL Chol	0.243	0.177	-0.075	0.262	0.617	-0.487	-0.316
Urine Creatinine	-0.143	-0.400	0.143	0.200	0.771	-0.029	0.200

Table A6: Spearman's correlation analysis for 12 week data collected from the GIED trial continued. *: $p < 0.05$, **: $p < 0.01$ 2 tailed test.

Categories	Urine Albumin/ Creatinine ratio	HbA1c	IL-6	IL-1β	TNF-α	Adiponectin	CRP	HIF-α
Urine Albumin	0.714	-0.257	-0.600	0.771	0.657	0.429	0.029	0.400
Urine Albumin/ Creatinine ratio	1.000	-0.200	-0.700	0.886*	0.886*	-0.086	0.486	0.800
HbA1c	-0.200	1.000	0.385	-0.195	-0.201	0.036	-0.559	-0.058
IL-6	-0.700	0.385	1.000	-0.067	-0.217	-0.550	-0.483	-0.900*
IL-1β	0.886*	-0.195	-0.067	1.000	0.358	0.127	0.030	0.257
TNF-α	0.886*	-0.201	-0.217	0.358	1.000	0.248	0.539	0.600
Adiponectin	-0.086	0.036	-0.550	0.127	0.248	1.000	-0.006	0.600
CRP	0.486	-0.559	-0.483	0.030	0.539	-0.006	1.000	1.000**
HIF-α	0.800	-0.058	-0.900*	0.257	0.600	0.600	1.000**	1.000

Appendix 4: Power analysis output from G*Power.

F tests – ANOVA: Repeated measures, between factors

Analysis:	A priori: Compute required sample size		
Input:	Effect size f	=	0.25
	α err prob	=	0.05
	Power (1−β err prob)	=	0.95
	Number of groups	=	2
	Number of measurements	=	4
	Corr among rep measures	=	0.5
Output:	Noncentrality parameter λ	=	13.2000000
	Critical F	=	3.9139890
	Numerator df	=	1.0000000
	Denominator df	=	130
	Total sample size	=	132
	Actual power	=	0.9501436