UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

School Of Health Professions and
Rehabilitation Sciences

INTRINSIC FACTORS IMPLICATED IN THE SEQUENCE OF DIABETIC ULCERATION:
The potential role of core2 β1,6-N-acetylglucoseaminyltransferase (core2GlcNAcT-I) [core 2 transferase]

by

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ABSTRACT

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INTRINSIC FACTORS IMPLICATED IN THE SEQUENCE OF DIABETIC ULCERATION: The potential role of core2 β1,6-N-acetylglucoseaminyltransferase (core2GlcNAcT-I) [core 2 transferase]

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Peripheral neuropathy is an insidious complication of diabetes mellitus with notable secondary events, such as vascular dysfunction and plantar ulceration. Traditional doctrine maintains that diabetic ulceration is a direct consequence of concurrent neuropathy and pressure. Whilst these two variables were accepted as contributory, it was supposed that preceding events actually have a metabolic cause. In accordance with study into parallel diabetic complications, it was hypothesised that neuropathic ulceration is promoted by capillary occlusion, the resultant hypoxia leading to expeditious cell death. The golgi enzyme, core 2 transferase, was implicated in this event, given its mediation of intercellular signalling and leukocyte / endothelial adhesion. Hence, an upregulation of this particular facilitator would increase leukocyte / endothelial binding and thereby, effect microcirculatory stasis and post-occlusion ischaemia.

Type II diabetic study groups, with and without neuropathy (n=20), were canvassed and set against aged matched non-diabetic controls (n=5). All participants were subjected to anthropometric testing prior to venous blood sampling for the key marker, core 2 transferase. Additional blood chemistry and clinical testing (VPT and

10g monofilament) was further undertaken to demonstrate possible correlations with core 2 transferase upregulation.

The outcome of this study identified that core 2 transferase was significantly elevated in both diabetic study groups, in comparison to control participants ($p<0.001$). This trend was further continued, when comparing diabetic neuropathic individuals to both remaining groups ($p<0.001$). Subsequent linear regression modelling identified three principal correlations with core 2 transferase over-expression: VPT, 10g monofilament and creatinine levels. Using each of the above correlations as independent co-variates, adjusted models identified a very robust *Rsq* of 0.911 (91% predictability) for VPT and creatinine, as clinical markers for core 2 transferase specificity.

Consequently, these findings positively implicate core 2 transferase activity within a diabetic population and moreover, offer validated clinical tools to facilitate its early detection.

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1.0 INTRODUCTION

Diabetic individuals with peripheral neuropathy (diagnosis criterion of a vibratory perception threshold [VPT] > 25 V) show a significantly increased propensity towards plantar ulceration (Ctercteko et al., 1981, Cavanagh et al., 1993, Albert and Chen 1996, Raginarson et al., 2001). This association has been historically founded upon the theory that compromised protective sensation induces higher temporal and peak plantar pressures during gait, leading to increased force translation onto key weight bearing sites (Armstrong 1999, Lord and Hosein, 2000). To date, however, no definitive model has been successfully generated to predict a pressure / time integral or peak plantar force synonymous with ulceration in the neuropathic foot. Notably, patients with Rheumatoid Arthritis suffer similar point loading of prominent structures, yet ulceration remains the anomaly (Masson et al., 1989). Hence, pressure alone cannot be regarded as singularly attributable, but rather, a component of an alternative mechanism.

Although the stresses induced by increased plantar pressure remain entirely valid, their role may actually be that of the "trigger" to plantar ulceration. Indeed, it is supposed that certain metabolic factors contrive to create an underlying organic flaw, which is exacerbated by subsequent external loading. This goes some way to explaining the inherent uniqueness of ulceration and most particularly, the lack of symmetry between patients (Lavery et al., 2003). Hence, an appreciation of tangential diabetic complications provides invaluable insight.

Recent advances in the research of retinopathy and microangiopathies have implicated the role of leukocytes in diabetic complications (Kantar et al., 1991, Pecsvarady et al., 1994, Lutty et al., 1997, Miyamoto et al., 1997, Miyamoto et al., 1998). Moreover, there is accumulating evidence, in both human and animal models, which shows that diabetes leads to an increase in leukocyte / endothelial adhesion activity (Morigi et al., 1998, Chibber et al., 2000). One particular key factor within this sequence appears to be the activation of the protein kinase C (PKC) pathway via elevated glucose concentrations at both acute and chronic stages (Porte and Schwartz, 1996, Hempel et al., 1997, Ceolotto et al., 1999).

PKC exists in various isoforms and their level of specificity and redundancy within cellular communication still continues to pose questions (Newton, 1995, Idris et al., 2001, Cotter et al., 2002). Notably, the stimulation of PKCβ seems to increase the expression of the adhesion proteins, P- and E-selectin (Tesfaye et al., 1996). However, hyperglycaemia is not the only mediator of PKC stimulation, as Vascular Endothelial Growth Factor (VEGF) and Tumour Necrosis Factor Alpha (TNFα) are also known to initiate this pathway (Kunkel and Ley, 1996, Xia et al., 1996).

Seminal work by Tesfaye et al., (1994) was the first to study the vasa nervosum in living diabetic human patients. Data recorded from this study revealed new acellular vessel formation and tortuous capillaries with associated arterio-venous shunting. Seemingly, these findings compliment the theory of capillary occlusion triggered by the accumulation of neutrophils (Cameron et al., 2001). The observed tortuous vessels within diabetic individuals tend to support this

notion, as the section immediately prior to the occlusion site is subjected to "back-pressure", leading to distension (Tesfaye et al., 1996). Accordingly, it would follow that the area post occlusion is potentially exposed to a reduction in oxygen tension, creating a hypoxic environment (Yasuda and Dyck, 1987, Ibrahim et al., 1999).

This factor would, in turn, act as a catalyst for the favoured production of vascular endothelial growth factor (VEGF), thereby accounting for the recognised new acellular vessel formation, as seen on the vasa nervosum in diabetic neuropathic patients (Obrosova et al., 2001). Moreover, the recorded arterio-venous shunting would help promote the "steal effect" theory (Tesfaye et al., 1996). Yet, such diversion of nutrient supply from sections of the capillary further expedites an environment ripe for the on-going production of VEGF (Shih and Claffey, 1998). This relative perpetuation is illustrated by Aiello et al., (1997) and Koya et al., (1998), who recorded a correlation between the presence of VEGF and PKC, thereby establishing the potential for mutuality.

Nevertheless, the increased expression of P- and E-selectins on the vascular endothelial cells merely represents half of the equation for leukocyte / endothelial cell adhesion (Cominacini et al., 1995, Olson et al., 1997, Dreβler et al., 1998). The element which controls the leukocyte's adhesion signalling continues to remain elusive, but is supposed as core 2 transferase, a golgi enzyme responsible for the biosynthesis and elongation of o-linked oligosaccharides (Datti and Dennis, 1993). These are protein structures present on the surface of the leukocyte, which determine inter-cellular communication /

adhesion (Li et al., 1996). A rat diabetic model study showed a proportionate relationship between elevated levels of PKC and core 2 transferase, ultimately leading to ischaemia and capillary bed destruction at chronic levels (Schroder et al., 1991).

Hence, the relationship between PKC expression and core 2 transferase may be regarded as that of a "hook and eye", in terms of endothelial / leukocyte cell adhesion. However, it is the cytotoxic nature of the leukocyte, which is believed to be most destructive in diabetes mellitus (Chibber et al., 2000). Indeed, the leukocyte's attachment to the endothelial cell promotes what may be termed, an "Unholy Trinity" of reactions: direct cellular attack via superoxide, combination with other free-radicals to produce more potent reactive oxygen species and lastly, inactivation of nitric oxide to reduce vasodilation (Schroder et al., 1991, Halliwell, 1995, Honing et al., 1998, Bardell and Macleod, 2001, Zou et al., 2002, Maritim et al., 2003). Given the further postulation by Chibber et al., (2003) that core 2 transferase up regulation is positively linked to diabetic retinopathy, possible associations with diabetic neuropathy therefore require exploration. In view of the detrimental effects of leukocyte adhesion and the increased potential for stasis within the vasculature, it is supposed that the cellular structure of the neuropathic foot is sufficiently undermined, rendering it incapable of resisting regular assault from daily life.

1.1 Research Questions

The aim of this research piece was to investigate whether diabetic neuropathic individuals display higher concentrations of the specific marker (core 2

transferase), against two alternative study groups (Study I). Study II expands upon the sample population and in addition, attempts to validate certain clinical markers, capable of assisting in the early identification of "at risk" patients. Therefore, the outline research questions are as follows:

1.1.1 Research Questions for the Analysis of Core 2 Transferase

a. Do diabetic neuropathic individuals with manifest ulceration display higher absolute levels of the enzyme, core 2 transferase, compared to diabetic individuals without complications and a control group?
b. Do diabetic neuropathic individuals without ulceration display higher absolute levels of the enzyme, core 2 transferase, compared to diabetic individuals without complications and a control group?
c. Do diabetic individuals without complications display higher absolute levels of the enzyme, core 2 transferase, compared to non-diabetic individuals (control group)?

1.1.2 Research Questions for the Identification of Correlations between Standard Clinical Markers and Elevated Levels of Core 2 Transferase

a. Is there a correlation between the rate of detection of a 10g monofilament and the presence of elevated core 2 transferase in diabetic neuropathic individuals (with and without ulceration) and diabetic individuals without complications, against the background of an age matched control group?
b. Is there a correlation between Vibration Perception Threshold and the presence of elevated core 2 transferase in diabetic neuropathic individuals

(with and without ulceration) and diabetic individuals without complications, against the background of an age matched control group?

c. Is there a correlation between standard blood chemistry markers and the presence of elevated core 2 transferase in diabetic neuropathic individuals (with and without ulceration) and diabetic individuals without complications, against the background of an age matched control group?

d. Is there a correlation between biometric outcomes and the presence of elevated core 2 transferase in diabetic neuropathic individuals (with and without ulceration) and diabetic individuals without complications, against the background of an age matched control group?

1.2 Diabetes Mellitus - Epidemiology and Economic Implication

Diabetes mellitus is one of the single most chronic illnesses with a prevalence approaching 8% in many developed countries (Amos et al., 1997). In Europe alone, approximately 27 million people suffer from diabetes, the majority presenting Type II diabetes [80-90%] (Ragnarson Tennvall et al., 2001). However, the enormity of this situation is not truly reflected by these figures, as increased life expectancy, lifestyle changes, as well as improved screening methods and frequency, is projected to severely distort the status quo (Clarke et al., 2003, Raikou and McGuire, 2003). Indeed, a 50% rise in the overall number of European cases over the next 15 years has been forecast (Bagust et al., 2001). However, due to the insidious onset of Type II diabetes, around half of all cases may remain undiagnosed for up to 7 years, thus marring the true demographics of this condition (Zimmet, 2003)

Within the UK population, the economic implications of diabetes have been estimated by the Kings' Fund to be in the region of £2 billion a year (Booth, 2001). The necessary cost commitment to the management of diabetes and its related complications is consequently set to demand 10% of the total NHS spending by 2011 (Boulton, 1996.)

1.3 Diabetic Foot Ulceration

The manifestation of foot ulceration (mixed aetiology) in the UK resident diabetic population lies between 4% and 10%, versus 2 - 3% in the United States (Reiber et al., 1999, Stacpoole et al., 1999). Whilst there are certain prevailing socio-economic factors, which may assist in perverting results, there is no apparent correlation between incidence and geography to explain this three-fold difference (Caddick et al., 1994, Chaturvedi et al., 1996, Mason et al., 1999). It may, however, be stated with relative security that a combination of factors are ultimately responsible for effecting this particular result. To attempt to identify the singular factors would extent the scope of a given study to immense proportions and moreover, prove exhaustive both in financial and human terms. Therefore, a more pragmatic approach would be to define those influences, which may be termed as directly attributable or "causal".

One traditional area of investigation is based around a reduction in peak and temporal plantar pressures, with specific reference to callus debridement (Boulton et al., 1987, Fernando et al., 1991, Veves et al., 1995, Cavanagh et al., 1996). However, given that high plantar pressures are evident in other non-diabetic groups (Mason et al., 1989), all with seemingly negligible incidences of

ulceration (Woodburn and Helliwell, 1996, Pitei et al., 1999, Lavery et al., 2003), it is proposed that alternative pathological processes are responsible (Mayfield et al., 1998). This is not to say that plantar pressure is inconsequential. Rather, it is likely that there are other mechanisms working in disfigured harmony to create the net result.

1.4 Plantar Pressure

There remains an undeniable association between elevated foot pressures in diabetic individuals and plantar ulceration (Ctercteko et al., 1981, Duckworth et al., 1985, Veves et al., 1992, Armstrong et al., 1998). However, there is no absolute correlation, as not all individuals with high plantar pressures necessarily ulcerate (Masson et al., 1989). Thus said, this would advocate the existence of some variable in cases of this nature. One principal factor in long-term complications of diabetes is peripheral neuropathy (Boulton et al., 1983, Cavanagh et al., 1993, Albert and Chen, 1996, Katoulis et al., 1996).

The plantar pressure / neuropathy parallel has been further investigated by Boulton et al. (1987), whose findings suggest that elevated plantar pressures are observed prior to the manifestation of clinically recognised neuropathies. If this were the case absolutely, it would infer that pressure is the principle factor in the cascade to neuropathy. Similarly, Ctercteko's et al. (1981) supposition that neuropathic ulceration correlates with the site of maximum plantar pressure must also follow. However, if one considers that neuropathic individuals suffer from compromised cellular health, resultant ulceration would logically occur at the point of maximum force, i.e. tissue breakdown.

Fryberg et al. (1998) found that after the site of a plantar ulcer had healed, elevated pressures were still present, thereby implying a channel for re-ulceration. However, the continued presence of elevated plantar pressure raises an awkward question. Given that elevated pressure is still present, why are there periods without ulceration? If plantar pressure remains constant, there must be another variable.

1.4.1 **Plantar Pressure Attenuation**

Plantar pressure attenuation can be undertaken in certain foot deformities by the use of orthoses. Although work conducted by Pitei et al. (1996) and McPoil and Cornwall (1998) has examined the efficacy of various types of orthotic using centre of pressure patterns, there is no direct evidence to show that plantar pressure attenuation using orthoses actually reduces the frequency of ulceration and/or re-ulceration. The latter point is particularly poignant, as re-ulceration tends to occur in the very group with bespoke footwear equipped with orthoses. Hence, Tooke's (1995) supposition that interrupted blood flow to soft tissue may be an integral cause of ulceration, implies that an orthotic design should attempt to reduce both temporal and peak pressures simultaneously.

1.4.2 **Temporal and Peak Loading**

Alteration of temporal loading is clearly present in the cavoid / intrinsic minus foot, with increases in peak and temporal pressure correlating with the metatarsal heads, the most prevalent ulcer sites in the diabetic neuropathic foot

(Lord and Hosein, 1994). Moreover, increased forefoot loading is an accepted aspect of neuropathic gait (Cavanagh et al., 1996, Katoulis et al., 1997). Yet, despite these observations, the direct role of pressure in the expedition of neuropathic ulceration remains tenuous at best. Were it otherwise, the UK diabetic population would arguably suffer a far higher incidence of ulceration than the accepted 4%-10% (Reiber et al., 1999, Stacpoole-Shea et al., 1999). A comparison with the Charcot Foot complication provides a far better example, as the remodelling and inflammatory process during its "active" phase potentially creates a proapoptic environment, so facilitating the role of plantar pressure in degrading cell integrity (Jude and Boulton, 2001, Rajbhandari et al., 2002).

A compromised tissue environment would also work in unison with Landsman's et al., (1995) proposal, that primary atrophy of the anterior muscle group places the posterior group in a dominant position, thus altering the foot function, sequencing and application of load. The following physical law best illustrates the implication of increased velocity on forefoot strike:

Figure 1.4.2.i **Calculation of kinetic energy**

$$\textit{Kinetic Energy } (E_k) = \frac{\textit{Mass x Velocity}^2}{2}$$

Extracted from Adler U., Bauer H., Cypra A., Dinkler F., Bosch Automotive Handbook, 3rd Edition, Stuttgart, 1993, pp 45

Consequently, the foot is subject to increased energy translation by the very action of walking, expediting tissue damage. Once again, however, it should be recognised that muscle atrophy will occur in elderly individuals in both the general and diabetic populations. Nevertheless, it is only a relatively small subsection of the diabetic neuropathic population, which will ultimately present ulceration (Mason et al., 1999).

1.5 Diabetic Peripheral Neuropathy

Diabetic peripheral polyneuropathy (DN) is a frequent complication of diabetes mellitus (Litzelman et al., 1993, Abbott et al., 1998, Fryberg et al., 1998, Booth and Young, 2000, Rosoklija et al., 2000). The prevalence has been shown to range slightly between studies, but a figure of approximately 60% of all Type II diabetic individuals go on to develop this condition within the first 10 years of diagnosis (Dyck et al., 1993, Young, et al., 1993, Tesfaye et al., 1996, Tentolouris et al., 2001, Vinik et al., 2000). DN is defined pathologically as a loss of axonal fibres with accompanying demyelination [0.5-0.7 m/s per year has been recorded] (Wang et al., 1999). The progression of this complication follows a course of distal to proximal, i.e. the toes frequently are first to exhibit neurological disturbances (Boulton et al., 1994). These observations also substantiate studies, which have demonstrated a diminished functionality to the axonal retrograde transport mechanism, thus inhibiting adequate nutrient supply (Thomas and Lascelles, 1995, Dyck et al., 1986).

Diabetic neuropathy has been proposed as a key synergistic factor for the development of ulceration in the diabetic foot (Boulton et al., 1986, Boulton et

al., 1987 and Cavanagh et al., 1993). However, whilst Edmonds et al., (1996) and Reiber et al., (1999) have demonstrated the value of peripheral neuropathy as a predictive indicator in determining the increased risk of ulceration, not all diabetic individuals with DN actually ulcerate (Masson, 1989). A recent study has maintained that alteration to plantar pressures, coupled with peripheral neuropathy and foot deformity work in unison to create an increased risk of foot ulceration (Cavanagh et al., 1996). But, as demonstrated by Masson (1989), rheumatoid arthritic patients do not show the same predilection for ulceration as diabetic patients, despite similar manifest deformity and increased load translation. Hence, the case for plantar pressure becomes further weakened, as it is difficult to see how DN alone could subvert physical law:

Figure 1.5.1 **Pascal's law**

$$Pressure = \frac{Force}{Area}$$

Extracted from Adler U., Bauer H., Cypra A., Dinkler F., Bosch Automotive Handbook, 3rd Edition, Stuttgart, 1993, pp 14

Undoubtedly, diabetic peripheral neuropathy will diminish or potentially remove the protective withdrawal response to noxious stimuli and affect proprioception (Boulton et al., 1994). Naturally, this will leave the foot subject to any given traumatic insult, i.e. stepping onto sharp objects or exposure to extremes of temperature. This being the case, such incidents will tend to effect an immediate and apparent wound, not an ulcer. Hence, it appears to be the

actual process of metabolic wound repair distinctive to diabetic neuropathic or neuroischaemic individuals, which gives rise to ulceration:

> ***Ulceration: a circumscribed, crater-like lesion of the skin or mucous membrane resulting from necrosis that accompanies some inflammatory, infectious or malignant processes. An ulcer may be shallow, involving only the epidermis or deep penetrating to the bone.*** (Anderson et al., 1990).

This proposal has particular significance, as many non-pathological events, even a blister, can form into an ulcer in the neuropathic or neuroischaemic foot (Koivukangas et al., 1999). Consequently, it would seem to suggest that the underlying metabolic changes associated with diabetes mellitus actually facilitate ulceration and that neuropathy, rather than being a cause, is more likely to be a symptom of the same pathological process. This supposition reflects the inherent non-linearity of diabetic foot ulceration and whilst there is no attempt to dismiss DN as a valuable predictive tool per se, some doubt must be cast as to its true culpability in the prelude to ulceration.

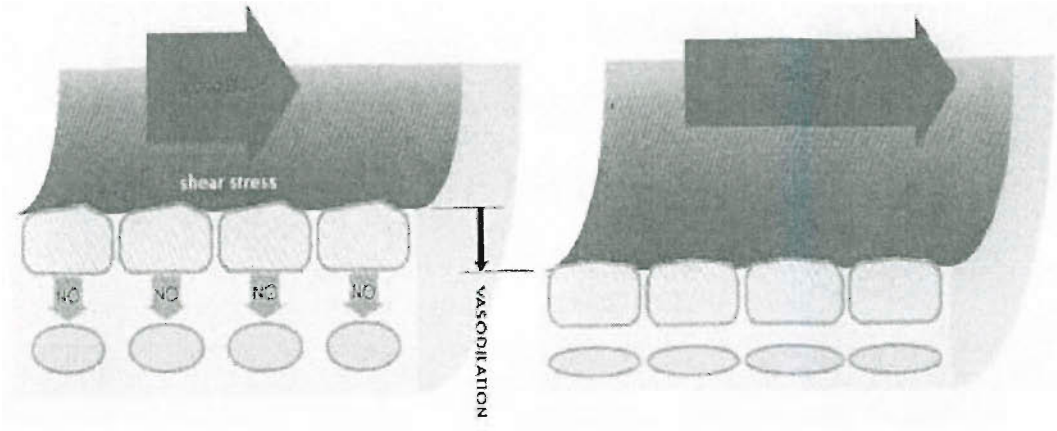
1.6 Microvascular Hypothesis of Neuropathy

The microvascular hypothesis is founded upon the principles that irreversible damage to the vasa nervosum leads to a compromised or complete loss of nutrient supply, thereby causing cellular and tissue death (Young et al., 1992, Tooke, 1999). Histopathic studies have shown a thickening of the endoneurial and perineurial vessel walls, hyperplasia of capillary endothelial cells and increased occlusion of vessels in neuropathic patients (Stevens et al., 1995, Jude

et al., 1998, Kennedy and Zochodne, 2002). Tooke (1999) and Nabuurs-Franssen et al., (2002) further demonstrated evidence for microvascular alterations by observing an initial blood flow increase and a subsequent elevation in capillary pressure in early diabetes. These findings potentially corroborate those of Jude et al., (1998), which showed that haemodynamic stresses bring about endothelial injury, which in turn, causes membrane thickening and impaired gaseous diffusion.

A resultant factor of the haemodynamic alteration can be a potential increase in the release of nitric oxide (NO), as the heightened shear stresses invoke the normal vasodilatory action, i.e. the release of nitric oxide and vasodilation via the stimulation of endothelial nitric oxide synthase [eNOS] (Calles-Escandon and Cipolla, 2001, Weston, 2001). This would provide the classic feature of "over perfusion", a characteristic of the neuropathic foot (Figure 1.6.1). However, it should be noted that protracted deviation beyond the normal levels of NO may actually lead to the generation of a proapoptic environment (Byrne et al., 2001).

Figure 1.6.1 **Schematic of over perfusion**



Reproduced from Weston. C, (2001) NO hiding place: nitric oxide in cardiovascular disease. The British Journal of Cardiology 8 (10): 544-547.

In the last stages of microangiopathy, there is a functional loss of maximal hyperaemia together with autoregulatory impairment (Tooke, 1999). The clinical implications of reduced hyperaemia will render the affected cells and tissues subject to a reduced immune response, as the number of phagocytes and antibody complexes are proportionately decreased (Nash et al., 2001). The immune response is further depressed, as diapedesis through the capillary membrane is also impaired due to thickening (Pecsvarady et al., 1994). Consequently, this may go some way to providing a clinical springboard, from which to explore the apparent increased susceptibility to infection shown by many diabetic individuals.

In a study conducted by Tesfaye et al. (1996), the vasa nervosum in established neuropathy was investigated and observed to display acellular new vessel formation, increased capillary permeability, arterio-venous shunting, tortuous epineurial vessels and the attenuation of arterial supply. It has been proposed that the activation of the arterio-venous shunting mechanism and occlusion of

the arterial supply leads to a "steal" effect, so rendering the endoneurium ischaemic (Tesfaye et al., 1994). This is consistent with additional studies, which have displayed the multi-focal nature of nerve degeneration (Britland et al., 1990, Malik et al., 1992, Giannini and Dyck, 1995). Animal models of diabetes also confirm the association between reduced neural tissue blood flow and impaired nerve conduction velocity (Cameron et al., 1999). More recently, however, Cameron and Cotter (2001) have demonstrated that specific free radical scavengers were able to significantly restore both blood flow and nerve conduction velocity.

1.7 Oxidative Stress

Since the discovery of certain reactive oxygen species (ROS) or more vernacularly, "free radicals", in the late Eighties, their implication in the pathogenesis of diabetic complications has been investigated (West, 2000). Seemingly, oxidative stress results as the consequence of an imbalance between ROS (e.g. superoxide anion, hydroxyl radicals, peroxynitrite and hydrogen peroxide) and antioxidants, such as superoxide dismutase, catalase, glutathione, as well as vitamins C and E (Kourie, 1998, Sytze van Dam, 2002). Indeed, increased oxidative stress and/or the attenuated clearance of ROS are now accepted mechanisms for the initiation and progression of long-term diabetic complications (Van Dam et al., 1995, Greene et al., 1999, Ohmori et al., 2000, Cameron et al., 2001, Maritim, et al., 2003). The following table demonstrates the results of oxidative stress:

Table 1.7.1 **Pathological alterations as a result of oxidative stress**

ALTERATIONS AND EFFECTS OF OXIDATIVE STRESS
Glucose autoxidation - producing excess amounts of free radicals
Depletion of free radical scavengers and antioxidants
Excessive production of superoxide by mitochondrial cells
Increased free radical production by cytochrome p450, xanthin oxidase and PKC-dependent activation of NADH/NADPH oxidase
AGE receptor triggered cellular oxidant stress
Depletion of high-density lipoprotein (HDL). Glycation of HDL, thus reducing its anti-oxidative role
Excessive glycation of albumin - important antioxidant
Hyperglycaemia - protein glycation, which can be a source of free radicals. Also, use of the polyol pathway and generation of superoxide anion

It has been shown that the oxidant / anti-oxidant balance within the diabetic individual is greatly altered, with hyperglycaemia particularly favouring free radical generation, as a consequence of accumulated Advance Glycated End-stage [AGE] products (Boel et al., 1995, Ceriello, 1999 and Singh et al., 2001). Moreover, a coincident reduction in nitric oxide (NO) bioavailability enhances platelet aggregation and leukocyte adhesion, leading to further peripheral vascular complications (Halliwell, 1991, Keaney and Loscalzo, 1999, Stefanec, 2000).

Therefore, given that free radical damage can occur within tissue subjected to ischaemia and subsequent reperfusion (Hokama et al., 2000), the microcirculation within the diabetic foot is extremely vulnerable. Indeed, during normal gait, the occlusion of vessels and the resultant pseudo-hypoxia

may be instrumental in the expedition of tissue necrosis and subsequent ulceration (Williamson et al., 1993). By definition, this mechanism is clearly not restricted to the extremities, but rather the entire vascular tree, to include the cerebral supply. Support for this theory has been demonstrated by Salom et al., (2000), who showed that the administration of nitric oxide donors reduced the size of cerebral infarcts, thus displaying the protective role of NO generally.

1.8 Reactive Oxygen Species – "Free Radicals"

The term "free radical" is given to atoms capable of independent existence, which possess one or more unpaired electrons within its associated orbitals (Halliwell, 1991). The result of this configuration is that the unpaired electron itself becomes an unstable and reactive entity (Halliwell, 1991). The foundation for this instability is the singular electron's ability to rotate in one of two orthogonal planes (Slater, 1984). However, the mere criterion for existence as a radical species will not determine the extent of its reactive nature, as free radicals cover a gamut of reactionary potentials (Giugliano et al., 1996, Baynes and Thorpe, 1999). Given the intrinsic stability of paired electrons, the interactions observed to date between radical and non-radical entities tend to follow three distinct pathways (Slater, 1984).

The reducing radical is one type of interaction and is characterised by the donation of an electron by the radical to the non-radical molecule (Halliwell, 1991). Secondly, the free radical may sequester an electron from the non-radical molecule, thus performing an oxidising function (Thomson and Manuel, 1996). Finally, a radical may actually join onto a non-radical, although in a

mutual context. However, it should be noted that irrespective of the selected pathway, a common end point is ultimately reached - the conversion of a non-radical to a radical species (Aruoma et al., 1989). The potential domino effect of this relationship can lead to numerous chain reactions causing the generation of a variety of radical species, which may work either symbiotically or pathologically within the body (Majima et al., 1998).

The potential significance of free radical tissue damage was first expounded by Fidovich in 1979, when he suggested the role of reactive oxygen species generation during transitory ischaemia (Soncul, 1999). Subsequent studies have observed that two of the reactive oxygen species potentially involved in this process are superoxide ($O_{2}^{\cdot-}$) and nitric oxide [NO] (Miles et al., 1996, Hishikawa and Luscher, 1998, Veves et al., 1998). The generation of these species occurs during the early period of reperfusion following ischaemia and can be stimulated in epithelial cells and mast cells during degeneration (Wang and Zweier, 1996). Consequently, an important link between reperfusion, inflammation and tissue injury is exhibited. However, the combination of these two reactive species in normal states represents the classic "brake and accelerator" relationship, so frequently observed in vivo (Pieper, 1998).

1.9 Advanced End Stage Glycation (AGE) Products

The interaction between the mechanisms for raised oxidative stress levels emanating from hyperglycaemia is paramount within the context of the long-term diabetic complications (Boel, et al., 1995, Friedman, 1999, Singh et al., 2001, Sugimoto and Yagihashi, 2002). In particular, the formation of

irreversible AGE products has been shown by Wautier et al., (2001) to activate nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidase and ROS after only 16 hours. Similarly, initiation of the NADPH oxidase system may actually potentiate the formation of further ROS (Berg et al., 1998a). Hence, irregular glycaemic control offers immense scope for alteration to the redox state and the mediation of further cellular damage, both directly and indirectly.

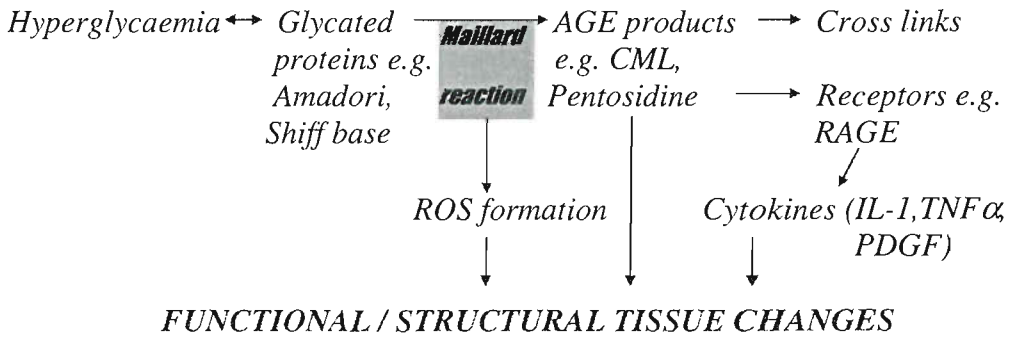
Protracted hyperglycaemia leads to excessive levels of glucose in circulation, which allows an "over flow" to migrate both readily and insulin independently, into the intracellular sites of vascular endothelial cells and peripheral nerves (Aaseth and Stoa-Birketvedt, 2000). Conversely, movement into other cells, such as muscle cells, is insulin-dependent and consequently restricted (Aaseth and Stoa-Birketvedt, 2000). The potential reduction of an adequate nutrient supply to muscle tissue in poorly controlled diabetes is also consistent with classic diabetic muscle atrophy to the anterior compartment of the leg and intrinsic muscles of the foot, leading to increased foot slap and toe deformities (Boulton et al., 1994, Landsman et al., 1995).

The former of these two musculoskeletal alterations is important, as increased velocity during forefoot contact will ultimately result in elevated foot pressures, a factor which has been positively correlated as a contributing factor in diabetic foot ulceration (Landsman et al., 1995 and Ward et al., 1998). Furthermore, increased forefoot loading exacerbated by lesser toe deformities, will serve to point-load areas of the foot, causing the potential for further elevated pressures. The importance of this, in relation to diabetic foot ulceration is potentially great, given that AGE formation alters the tensile and elastic properties of the skin,

leaving it open to an increased insult from unchecked trauma (Melling et al., 2000).

The excessive influx of glucose in both the circulation and cells leads to glycation of haemoglobin (HbA1c) and structures including proteins, enzymes, transport and structural entities (Ceriello, 1999). There are two key steps involved leading to the formation of AGE products. Firstly, glucose interacts with proteins causing the formation of Amadori products and the Shiff base. Secondly, the Maillard reaction occurs, forming AGE products, such as N-(carboxymethyl)lysine [CML] and pentosidine (Ahmed et al., 1986). Concurrently, ROS are also generated, which, in turn, promote further AGE products (Baynes and Thorpe, 1999, Takeda et al., 2001) [Figure 1.9.1].

Figure 1.9.1 **Process of non-enzymatic glycation**



Adapted from Dickinson, P. J., Carrington, A. L., Frost, G. S. and Boulton, A. J. M. (2002) Neurovascular disease, antioxidants and glycation in diabetes. Diabetes/Metabolism Research and Reviews 18: 260-272

Clearly, the potential for a positive feedback mechanism is in place, as ROS also accelerate the formation of AGE products (Dickinson et al., 2002). Historically, AGE products have been implicated in the formation of vascular

injury in diabetes (Radoff et al., 1990). This process appears to be mediated by macrophages displaying an increased uptake of glycated low-density lipoproteins (LDL's) by AGE specific receptors [RAGEs] (Hoff et al., 1992, Stitt and Friedman, 1997). Ultimately, this leads to "foam cell" formation in the arterial subintimal space and the generation of further ROS due to RAGE interaction (Dickinson et al., 2002). Additionally, as high-density lipoproteins (HDL's) undergo glycation, substantial modification occurs, resulting in a reduction in their cholesterol ester transfer protein [CETP] availability (Lemkadem et al., 1999). As a consequence, their cardiovascular protective factor is negatively affected. This supports findings by Litzelman et al., (1997) that low HDL levels are predictive of diabetic foot lesions.

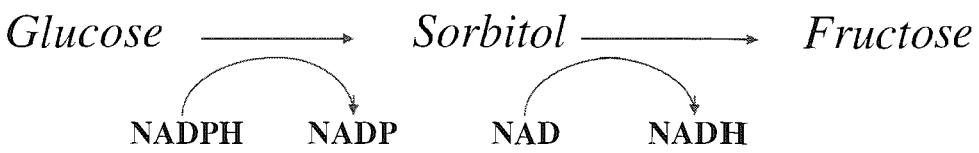
Another important aspect of AGE products in reducing blood flow, is the effect that they have on increasing the expression of the vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cells (Schmidt et al., 1995, Sengoelge et al., 1995). Accordingly, the potential for interruption to the nutrient supply is exacerbated (Williams et al., 1980, Timperly et al., 1985, Fasching et al., 1996, Jude et al., 1998, Cameron et al., 2001). The import of this becomes apparent, when considering the delicate structure of the microcirculation, particularly the vasa nervosum, which would easily become occluded by accumulating cells (McMillan et al., 1978, Wautier et al., 1981, Dyck, 1985, Malik et al., 1989, Ford et al., 1992). This is vital, as AGE products quench the anti-proliferative effects of nitric oxide on smooth muscle cells, whilst also invoking a reduction in its anti-aggregate effect (Honing et al., 1998, Bardell and MacLeod, 2001, Maejima et al., 2001). As a consequence, reduced oxygen tension will prevail (Cameron et al., 2001).

However, whilst AGE products have been clearly linked to an increase in oxidative stress and the pathogenesis of atherosclerosis, compounds that block the formation of AGE products, such as aminoguanidine, have failed to produce conclusive clinical results in the abatement of diabetic complications (Forbes et al., 2001, King, 2001, Van Dam, 2002). Conversely, certain studies involving the investigation of AGE levels in both animal and cultured tissue models have found that vitamin E and C, aminoguanidine and desferrioxamine have successfully prevented AGE formation (Cameron et al., 1993, Cameron et al., 1994, Nickander et al., 1994, Nagamastsu et al., 1995, Van Dam et al., 1999). Given this degree of disparity, it would appear that another part of the metabolic puzzle remains, as yet, unidentified.

1.10 **Raised Polyol Pathway Activity**

Excessive amounts of glucose also lead to the activation of the polyol pathway (Kador, 1988, Greene, et al., 1993, Yabe-Nishimura, 1998, Kamiya et al., 2003). Given that elevated levels of glucose are synonymous with diabetes, the polyol pathway has been suggested by many authors as playing an important role in the pathogenesis of diabetic neuropathy (Greene et al., 1984, Cameron, et al., 1994, De Mattia et al., 1994, Hotta, 1995, Ciuchi et al., 1996, Cameron et al., 2001). The process itself leads to the transformation of glucose to fructose, however, this is dependent upon the available levels of the coenzyme, nicotinamide-adenine-dinucleotide phosphate [NADPH] (Aaseth and Stoa-Birketvedt, 2000) {Figure 1.10.1}

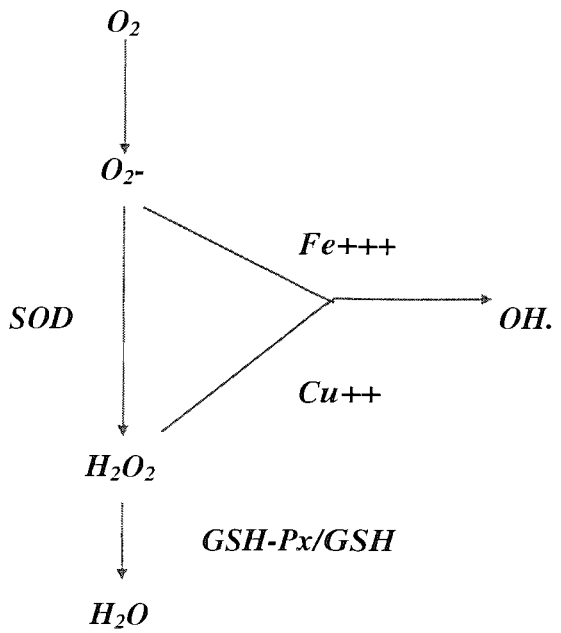
Figure 1.10.1 **The polyol pathway**



Adapted from Aaseth, J., Stoa- Birketvedt, G. (2000) Glutathione in overweight patients with poorly controlled type 2 diabetes. The Journal of Trace Elements in Experimental Medicine 13: 105-111

As aldose reductase will consume the co-enzyme NADPH, a further reaction to fructose is catalysed by sorbitol dehydrogenase, leading to the production of nicotinamide-adenine-dinucleotide hydrate (NADH), but not NADPH (Aaseth and Stoa-Birketvedt). Depletion of the essential co-enzyme NADPH will ultimately have a profound effect on the redox state of the cell, as NADPH is vital for the regeneration of oxidised glutathione (GSH), which acts as an antioxidant in concert with superoxide dismutase for the successful removal of superoxide (Cheng and Gonzalez, 1986, Williams, 1993, Gonzalez et al., 1986, Santini et al., 1997). Furthermore, NADPH acts to negate the potential generation of the highly toxic hydroxyl radical (Kashiwagi et al., 1996).

Figure 1.10.2 **Superoxide formation and interaction**

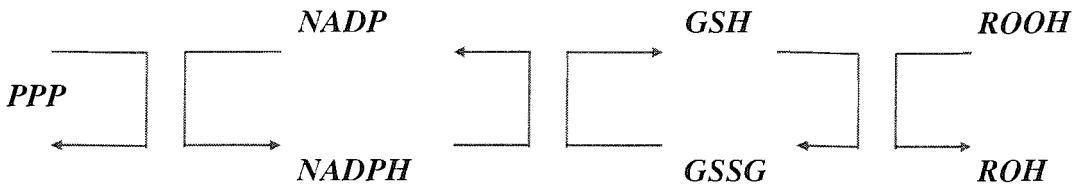


Explanatory Notes:

Superoxide (O_2-) is formed from oxygen by an uptake of an unpaired electron and converted to H_2O_2 by the action of SOD (superoxide dismutase). H_2O_2 is converted to H_2O by GSH-px (glutathione peroxidase), using GSH as a cofactor. High levels of O_2- and H_2O_2 in the presence of free iron or copper ions give rise to formation of the highly toxic OH. radical (Cohen, 1985).

The only cellular mechanism for the generation of NADPH is via the pentose-phosphate shunt (PPP) [Figure 1.10.3], although this is inhibited by ≈ 50% of its normal rate, when glucose levels are increased from ≈5 - ≈30 mmol/l (Jocelyn, 1972, Cameron, and Cotter, 1997).

Figure 1.10.3 **Pentose-phosphate shunt**



Explanatory Notes:

Conversion of toxic peroxides (ROOH) to non-toxic alcohols (ROS) by glutathione peroxidase. The reduction involves oxidation of GSH to GSSG, which has to be regenerated by the action of glutathione reductase, an enzyme that depends upon the presence of reduced NADPH, which can only be produced from NADP by the pentose phosphate shunt (PPP)

Adapted from Aaseth, J., Stoa-Birketvedt, G. (2000) Glutathione in overweight patients with poorly controlled type 2 diabetes. The Journal of Trace Elements in Experimental Medicine 13: 105-111

Alteration to either the PPP and or GSH level potentially leaves the endothelial cells open to damage, due to a reduction in the antioxidant protection levels. Furthermore, imbalance in this antioxidant / oxidant relationship could lead to a shift to proapoptic conditions (Kern et al., 2000, Droge, 2001, Obrosova, et al., 2001, Segura et al., 2002). Consequently, this notion supports previous observations of reduced GSH levels against an activation of superoxide in poorly controlled diabetic patients, when compared to controls (Maxwell et al., 1997, Berg et al., 1998b, Ruiz et al., 1999). Similarly, exogenous supplements of antioxidants (lipoic acid) have restored nerve blood flow and stopped / reversed nerve conduction velocity abatement (Chow, 1991, Cotter et al., 1995, Keegan et al., 1995, Baynes, 1991, Laight, et al., 2000, Cameron et al., 2001).

However, whilst initial trials of aldose reductase inhibitors mooted promising results in the treatment of diabetic peripheral neuropathy in animal models, later studies have failed to yield satisfactory advancement in combating this particular diabetic complication (Jiang et al., 1991, Cameron et al., 1994, Hotta, 1995, Cameron et al., 1997, Greene et al., 1999, Obrosova et al., 1999).

Therefore, this suggests that it is the oxidative stress / ROS element to the polyol pathway, which is most pertinent. Nevertheless, even if this theory represents only part of the pathogenesis of peripheral diabetic neuropathy, the question still remains as to why it is initiated distally and progresses proximally. Consequently, it appears that identification of another unknown component(s) must still be made to form the link between hyperglycaemia and oxidative stress.

1.11 Nitric Oxide Imbalance

During the 1980's, research principally focused upon cells' ability to synthesize oxides of nitrogen and in 1985, it was discovered by Stuehur and Marletta (1985) that macrophages could produce notable amounts of nitrite and nitrate. Subsequently, Marletta et al., (1988) identified nitric oxide as the primary substance, which undergoes oxidation to nitrate and nitrite accordingly. Simultaneously, Furchgott and Zawadzki (1980) and Furchgott et al., (1984) discovered endothelium-derived relaxing factor (EDRF). Further studies on EDRF-mediated activity revealed positive associations between vasodilation and increased cyclic GMP (guanine monophosphate) activation and GMP kinase activity in smooth muscle cells, coupled with the ability to directly stimulate purified soluble guanylyl cyclase (Fiscus et al., 1983, Rapoport and Murad, 1983, Ignarro et al., 1984, Forstermann et al., 1986, Ignarro et al., 1986). Finally, in 1987, it was established that nitric oxide could account for the biological properties of EDRF and that L-arginine was the essential substrate for EDRF / NO synthesis in endothelial cells (Palmer et al., 1988, Schmidt et al., 1988).

To date, three isozymes of nitric oxide synthase (NOS) have been both identified and implicated in the synthesis of NO (Honing et al., 1998, Jude et al., 1999, Ding et al., 2000, Li and Forstermann, 2000, Shimpo et al., 2000). Table 1.11.1 highlights the currently known isoforms:

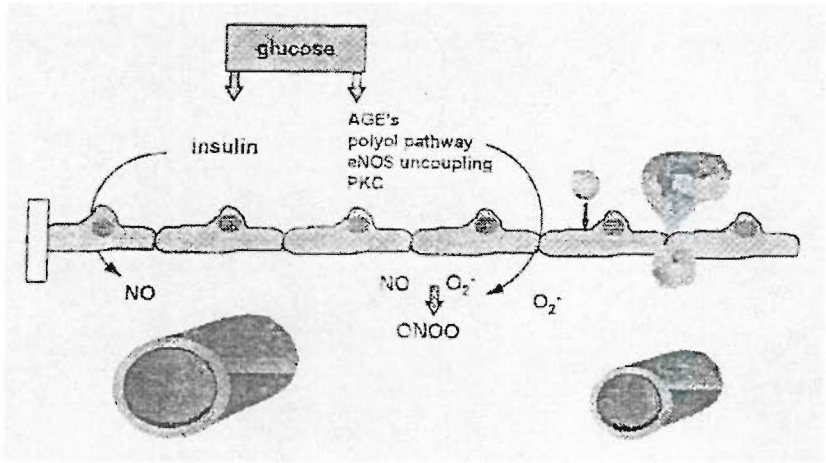
Table 1.11.1 **Classification of NOS isoforms**

NOS ISOFORM	CELL TYPE
NOS I (or nNOS)	Mainly found in central and peripheral neuronal cells
NOS II (or iNOS)	Inducible isoform found in many cells types. First found in murine macrophages
NOS III (or eNOS)	Primarily found in endothelial cells

Note: all isoforms require L-arginine as the substrate and molecular oxygen as the essential co-substrate

Co-factors required by all three isoforms include nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6R)-5,6,7,8-tetrahydrobiopterin [BH_4] (Knowles and Moncada, 1994, Li and Forstermann, 2000). Consequently, the previously observed alterations to NADPH availability through hyperglycaemia, the polyol pathway and GSH depletion, may potentially reduce the bioavailability of NO. Additionally, the shift in redox state may unduly influence the availability of BH_4 (Schmidt et al., 1992, Consention and Katusic, 1995). This can lead to the "uncoupling" of NOS via a specific receptor-Gi protein and initiate the increased superoxide production over that of NO (Klatt et al., 1996, Miller et al., 1997, Wever et al., 1997) [Figure 1.11.2]

Figure 1.11.2 **Pathophysiology of the vascular endothelium**



Taken from Honing et al., Nitric oxide availability in Diabetes Mellitus, Diabetes/Metabolism Reviews 14; 241-249

In chronic hyperglycaemia, numerous biochemical pathways are initiated. These include the formation of AGE products, the polyol pathway and activation of protein kinase C, all ultimately culminating in the generation of ROS, most particularly, the superoxide anion (Halliwell and Gutteridge, 1990, Chang, et al., 1993, Young, 1995, Low et al., 1997, Baynes and Thorpe, 1999, Cameron and Cotter, 1999, Chistyakov et al., 2001). This has the ability to rapidly interact with NO to form peroxynitrite (ONOO-), potentially causing impaired vasodilation, further generation of ROS and cellular injury (Ohara et al., 1993, Beckman and Keoppenol, 1996, Wang and Zweier, 1996, Warnholtz et al., 1999).

This issue is further compounded, as in vitro exposure of endothelial cells to glucose has been shown to elevate NOS expression, as well as increase its specific activity (Cosentino et al., 1997). Whilst it has been intimated that NO

is actually up regulated in diabetes, its apparent reduced bioavailability in diabetic patients is almost certainly a result of its breakdown / quenching / interaction of with other ROS (Ceriello et al., 1996, Donnini et al., 1996, Giugliano et al., 1996). The theory of oxidative stress damage in long-term diabetes is further supported by findings, which indicate a reduced radical-trapping antioxidant parameter (TRAP) and increased lipid peroxidation levels in diabetes (Frei, 1994, Donnini et al., 1996, Pieper et al., 1997). A possible explanation for this increase / bioavailable reduction paradox could be lie within the investigation of iNOS (the inducible type of NOS).

This particular isoform has been demonstrated in the cardiomyocytes in diabetic streptozotocin-induced rats and platelets in patients with Type I and Type II diabetes (Smith et al., 1997 and Tannous et al., 1999). Additionally, in vascular smooth muscle cells (VSMC), iNOS is induced by various cytokines, i.e. interleukin-1 [IL1], tumour necrosis factor [TNFα], nuclear factor-κB and protein kinase C (De Vera et al., 1996, Paul et al., 1997). The particular importance of this isoform activation may be better appreciated, when noting that iNOS synthesises between 10- and 50-fold more NO than the constitutive NOS types (Moncada and Higgs, 1995). Furthermore, the half-life of NO synthesised from iNOS is increased and has a prolonged release, when compared to that of eNOS or nNOS (Bardell and MacLeod, 2001).

The scope for detrimental effects increases, as the free radical NO. can interact with oxygen derived radicals (molecular oxygen is the essential co-substrate in the synthesis of NO) to produce peroxynitrite, which is thought to mediate the cytotoxic effects of NO (Beckman, 1990, Snyder and Bredt, 1992). Further

evidence of this was shown by Tannous et al., (1999), who found an increased production of peroxynitrite in the platelets of diabetic individuals.

Abnormal NO production may also contribute to endothelial dysfunction as a result of an imbalance in other endothelial synthesised factors. This was demonstrated by Takeda et al., (1991), who showed that endothelin-1 (ET-1), a powerful vasoconstrictor, is altered in diabetes. An interaction between NO and ET-1 was forwarded by Kiff et al., (1991), when they discovered that the inhibition of NO increased ET-1 release. Additionally, the Warner et al., (1989) study supported this regulatory relationship by finding that ET-1 diminished NO saturation levels. Therefore, vasodilatory tone could well be lost due to NO quenching or NO / O_{2^-} interaction, as ET-1 will tend to predominate, leading to a pro-vasoconstrictive state (Bardell and MacLeod, 2001).

In combination, this would potentially lead to increased shear stress on the vessel endothelium, prompting the further release of NO, which would again be quenched and/or aid the production of further free radicals, creating a classic "*Teufelskreis*". However, it is important to remember that this event is not an immediate pathological or non-pathological switch, as "slack water" between the two states will exist. This could be attributed to certain conditions, when antioxidant levels and/or as yet unidentified factors are sufficient to combat such alterations. This may account for the variation in the development of long-term complications of diabetes and perhaps more readily, why ulceration occurs in some diabetic neuropathic individuals, but not in others.

In its natural role, NO has the effect of modulating the release of superoxide, so maintaining equilibrium (Table 1.11.3).

Table 1.11.3 **Effects of reduced NO-bioavailability**

EFFECTS OF REDUCED NO-BIOAVAILABILITY
Vasoconstriction
Increased smooth muscle cell proliferation (thus lumen encroachment and increased distance for diffusion)
Increased expression of adhesion molecules
Increased leukocyte and platelet leukocyte-vessel wall adhesion (leading to increased local release of cytokines [IL1 and TNFα] and the stimulation of growth factors, i.e. vascular endothelial growth factor [VEGF])
Increased aggregation of platelets (leading to the potential stimulation of VEGF)
Increased lipoprotein oxidation (thus increased oxidative stress and forming the pathogenesis process for atherosclerosis)

Adapted from Honing et al., (1998) Nitric oxide availability in Diabetes Mellitus, Diabetes/Metabolism Reviews 14; 241-249

Moreover, NO further promotes endothelial relaxation, blood pressure regulation, vascular tone, immunological function, inhibition of the expression of cellular adhesion molecules (CAM's), namely VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular adhesion molecule-1), so preventing neutrophil adhesion, infiltration and conditioning for an anti-inflammatory state (Jude, et al., 1998, Okayama et al., 1997, Li and Forstermann, 2000). The inhibitory effect of NO on platelet adhesion also

prevents smooth muscle cell proliferation and the cellular matrix (Duby, et al., 1995).

The anti-aggregate properties of NO may prove an interesting investigation, as it has been suggested that the production of NO is not diminished in diabetes (Smits et al., 1993, Avogaro et al., 1997, Kelm et al., 1997). Indeed, the stimulatory factors for NO synthesis are actually increased: elevated shear stress on the vascular endothelium via ET-1, activation of iNOS synthesis by means of PKC activation and cytokine activation (IL1, TNFα and Nuclear Factor-κB) [Takagi, et al., 1996, Chen, et al., 2000, Park, et al., 2000, Idris, et al., 2001]. Consequently, localised overproduction, as a result of increased leukocyte / endothelial adhesion and the subsequent release of stimulatory cytokines, could actually impede cell migration, so reducing the immune response in diabetes (Sperandio et al., 2001). Additionally, increased cellular adhesion augments the direct release of cytokines, such as IL1, TNFα and superoxide, thereby acting as a catalyst for further free radical generation and cellular damage (Bagge et al., 1980, Fantone and Ward, 1982, Glowacka et al., 2002).

Oxidative stress and the generation of cytotoxic free radicals have been held up as the key outcomes of NO production imbalance (Tesfamariam and Cohen, 1992, Ceriello et al., 1993, Cohen, 1993, Dai et al., 1993, Tesfamarian, 1994, Giugliano et al., 1995, Angulo et al., 1996, Ting et al., 1996, Cosentino et al., 1997, Mayhan and Patel, 1998, Cohen, 1999). Furthermore, recent studies have now focused on ROS generated by NO / O_2^- interaction as primary mediators, both directly and indirectly for long-term complications associated with diabetes

[neuropathy, retinopathy, nephropathy and atherosclerosis] (Ceriello, 2000, Fitzgerald and Brands, 2000, Ohmori, et al., 2000, Maejima et al., 2001). A recent study has further demonstrated that NF-κB is stimulated by intracellular oxidative stress (Mohamed et al., 1999), facilitating the up regulation of specific target genes, which enhance the production of NO. Hence, the likelihood of a positive feedback mechanism may not be necessarily that remote.

1.12 Nitric Oxide and VEGF Interaction

NF-κB has also been identified as an oxidative stress-responsive transcription factor for vascular endothelial growth factor [VEGF] (Shih and Claffey, 1998). Therefore, alteration of NO outside the normal homeostatic boundaries would lead to a tilt in the vasodilatory / vasoconstrictive balance towards a pro-aggregation state (Henk et al., 2000). Consequently, there would be a dip in oxygen tension (Tesfaye et al., 1994). Indeed, Newrick et al., (1986) demonstrated a reduction in sural nerve oxygen tension within diabetic individuals. The apparent shift towards hypoxia would further stimulate the release of VEGF, as cytokines from the increased adhesion of leukocytes (TNFα and platelet derived growth factor [PDGF]), would continue to favour its production (Kimura et al., 2000, Clauss et al., 2001).

VEGF is one of the most potent factors in the promotion of angiogenesis (Leung et al., 1989, Plouet et al., 1989, Risau, 1997, Ferrara, 2000). It induces the degeneration of the extracellular matrix of existing vessels via proteinases and causes the migration and proliferation of capillary endothelial cells (Altavilla et al., 2001). In addition to its neovascularisation properties, VEGF has also been

shown to enhance vascular permeability in the retina and kidney (Urata et al., 2002).

The pathogenic properties of VEGF have already been established in diabetic complications, such as retinopathy and nephropathy (Urata et al., 2002). Furthermore, the observed structural changes to the vasa nervosum in diabetic peripheral neuropathy mirror the activity of VEGF, i.e. increased vascular permeability and increased acellular angiogenesis (Tesfaye et al., 1994). This is particularly important, if the theory supporting alteration to the vasa nervosum is followed through. Both leukocytes and platelets transport VEGF and would release the same as oxygen tension is reduced as a consequence of occlusion (Mayer, 1990, Kummer et al., 1992, Pinedo et al., 1998). Tesfaye et al., (1994) further demonstrated that the epineurial vessels of diabetic neuropathic patients displayed notable changes, namely an increased presence of arterio-venous shunting. This would further correlate with an attenuation of the nutrient vessel and redirection to the vein.

Hypoxia stimulated VEGF via increased gene transcription activity of the specific factor Hypoxia Inducible Factor (HIF-1), displays an increased half-life of 2-3 fold, compounding the potential pathological effects in retinopathy (Gasmi et al., 2002). Consequently, given the co-existence between neuropathy and retinopathy, it appears entirely feasible that VEGF is linked to the pathogenesis of diabetic peripheral neuropathy and accordingly, the increased risk of ulceration. However, whilst VEGF and ROS play an important role in diabetic complications, the link between the initiation of cellular adhesion and VEGF is still not fully understood. Despite investigation into NO inhibitors, as

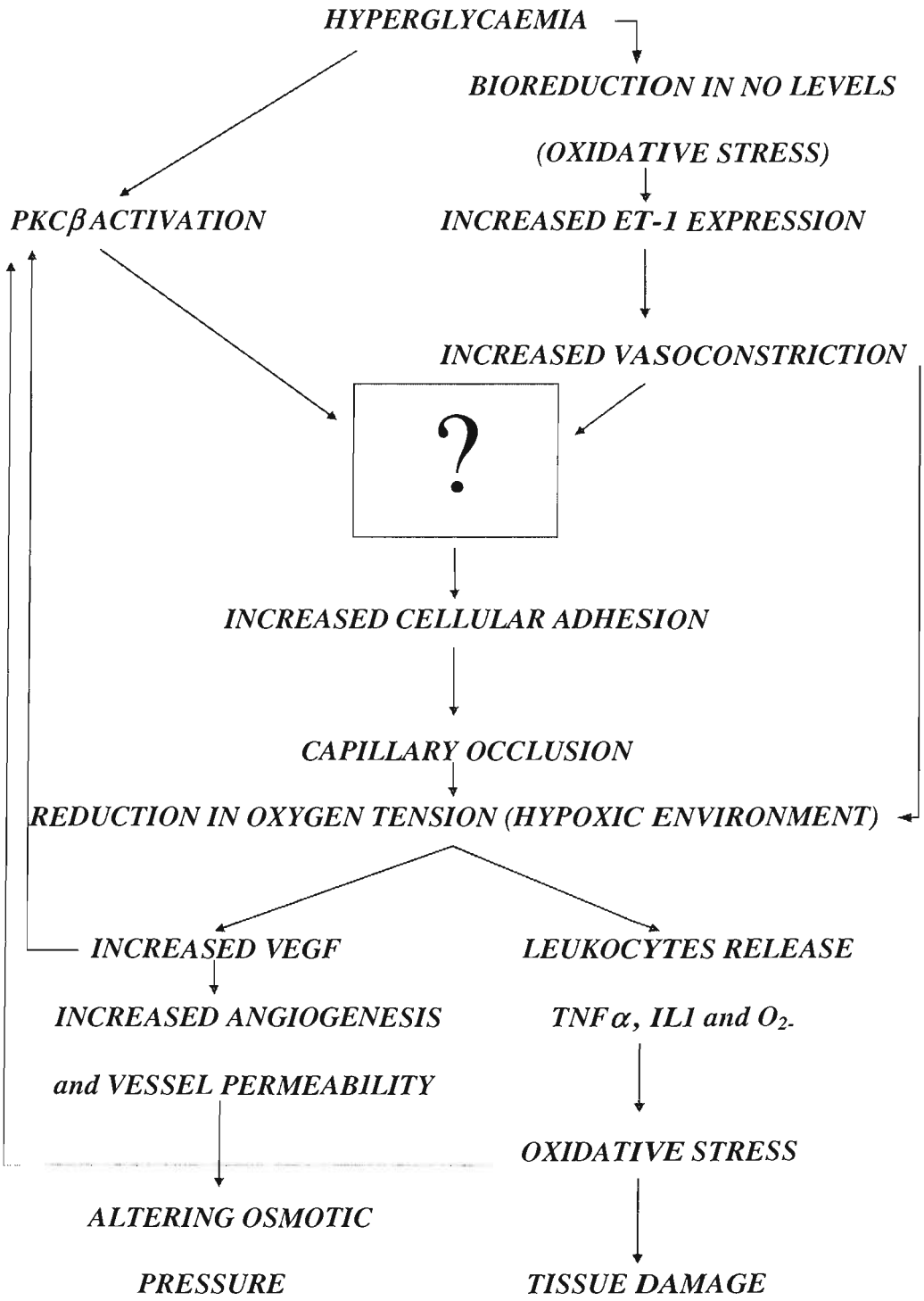
well as compounds to both prevent the formation of AGE products and inhibit aldose reductase, diabetic complications continue unchecked (Odetti et al., 1996, Jude et al., 1999, Cameron et al., 2001, Cameron and Cotter, 2002, Dickinson et al., 2002, Kamiya et al., 2003).

Further studies have also demonstrated that ROS induce the rapid expression of VEGF in many cells (Bunn and Poyton, 1996, Kuroki et al., 1996, Shu-Ching et al., 1998). Most particularly, the presence of VEGF stimulates endothelial cells to become prothrombotic and exhibit an increased level of tissue factor on their membranes, leading to the activation of the coagulation cascade (Verheul et al., 2002). This may explain why aspirin has provided some success in curbing oxidative stress in diabetes (Maritim et al., 2003), particularly as VEGF stimulates endothelial cells to promote platelet adhesion. The ability to halt this event is particularly vital, considering that a tissue factor increase will lead to the expression of VEGF and likewise, VEGF will augment tissue factor concentrations (Verheul et al., 2002).

The multiple metabolic interaction of VEGF can further potentiate an environment ripe for diabetic complications, as VEGF initiates the translocation of protein kinase C, specifically the α and β isoforms (Grugerl et al., 1995, Kieser et al., 1994, Aiello et al., 1997, Koya and King, 1998, Wellner et al., 1999). This is achieved by VEGF binding to VEGF-2 (KDR - kinase inset domain containing receptor), a specific receptor found predominantly on the endothelium (Kieser et al., 1994). The follow-on event is thought to be the activation of phospholipase Cγ, in turn, causing the conversion of DAG (diacylglycerol) to initiate the translocation of PKC (Xia et al., 1996). This is

most important, as PKC has been implicated in the generation of ROS and increased leukocyte / endothelial adhesion, although the direct mediating factor linking the two events has not yet been fully identified (Idris, et al., 2001, Chibber et al., 2000). The further negative effects associated with PKC activation include increased endothelin-1 (ET-1) expression, effecting vasoconstriction and a potential reduction in blood flow (Bursell et al., 1995, Takagi et al., 1996, Park et al., 2000). Figure 1.12.1 outlines the potential factors, which may play a key role in diabetic complications and expeditious foot ulceration:

Figure 1.12.1 **Potential cascade to ulceration**



The interplay between VEGF and PKCβ may also hold further insight, as it has been demonstrated that specific PKCβ inhibitors can significantly reduce the mitogenic effects of VEGF, thus a mutual stimulation or unknown co-element may exist (Wolf et al., 1991, Aiello et al., 1997, Williams, 1997, West et al., 2001).

Whilst VEGF and NO mutuality exists, given the predilection of an oxidant / antioxidant imbalance in individuals with diabetes, it is likely that any increase in NO will result in ROS formation due to the rapid interaction of NO and O_{2}^{-} (Szabo, 2003). Hence, VEGF may represent a double-edged sword in diabetic complications, as its level within keratinocytes may help to explain why neuropathic ulcers heal quite readily in the diabetic foot (Werner et al., 1992, Altavilla et al., 2001). However, this is small consolation, given its involvement in the potential pathogenesis of peripheral diabetic neuropathy, namely the cause of the wound.

1.13 Superoxide (O_{2}^{-})

Aerobic cells possess antioxidant systems, which combat the production of ROS, in particular, the superoxide anion (O_{2}^{-}) during cellular respiration and substrate oxidation (Martin, 1984, Hinshaw et al., 1992, Berman and Martin, 1993, Meharg et al., 1993, Doan et al., 1994, Okayama et al., 1997). The presence of ROS at low levels is vital for biochemical survival, as they often represent an integral part of intracellular signalling and immunological defence mechanisms (Kelm et al., 1997, Fukai et al., 2002, Klink et al., 2003). However, high levels or the inadequate removal of O_{2}^{-} result in oxidative stress

and the implicated pathogenesis of neuropathy, atherosclerosis, hypertension, retinopathy, heart failure and hypercholesterolaemia (Fukai et al., 2002). Indeed, hyperglycaemia / diabetes, smoking, hypertension and hypercholesterolaemia have all been positively associated with the generation of O_{2}- (Tesfaye et al., 1996, Baynes and Thorpe, 1999, Kojda and Harrison, 1999).

The mode of superoxide in promoting tissue damage has been proposed via two routes. The first is an alteration in the disposal of superoxide, through a reduction of the superoxide dismutase [SOD] enzyme (Beckman et al., 1994, Kocak and Karasu, 2002). All human tissues contain SOD and to date, three types of SOD have been identified: Cu/ZnSOD (SOD1), MnSOD (SOD2) and ecSOD (SOD3) [Beyer et al., 1991]. The differentiation of the isoforms has been based upon the apparent location and structures, which house the various isoforms (Chistyakov et al., 2001):

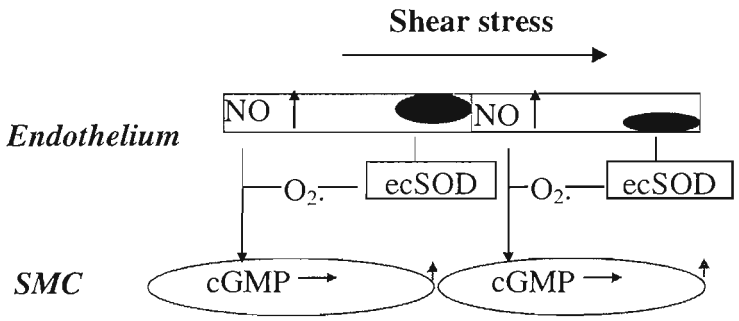
Table 1.13.1 **SOD isoforms and locations**

SOD ISOFORMS	LOCATION
Cu/ZnSOD (SOD1)	Cytosol
MnSOD (SOD2)	Mitochondria
ecSOD (SOD3)	Extracellular space

Investigations by Stralin et al., (1995) and Oury, Day and Crapo, (1996) have proposed that ecSOD may adopt the pivotal role in regulating oxidative stress within the vascular wall. The local concentration of this isoform would also

support this tenet, as its activity has been recorded at ≈ 10-fold higher than in other tissues (Stralin et al., 1995). In a normal state, it is made in the vascular smooth muscle cells and not directly via the endothelium (Marklund, 1990). However, once the isoform has been produced, it binds to heparin sulphates on the endothelial cell surface, which can be ingested by the endothelial cells themselves (Ohta et al., 1994). Figure 1.13.2 indicates the homeostatic balance between NO and ecSOD in the vessel wall:

Figure 1.13.2 **NO and ecSOD interaction**



Explanatory Notes:

As shear stress induces NO from eNOS via the endothelium, it acts to stimulate ecSOD from the smooth muscle cells. The secreted ecSOD adheres to the extracellular matrix and endothelial surface. ecSOD can protect NO degradation by superoxide produced extracellular. Adapted from Fukai et al., 2002, Extracellular superoxide dismutase and cardiovascular disease, Cardiovascular Research 55;239-249.

Secondly, and perhaps more importantly, is the interaction between nitric oxide and superoxide to produce cytotoxic products capable of localised and potentially widespread tissue destruction (Stefanec, 2000).

1.13.1 **Superoxide Dismutase Non-availability Theory**

The first proposed theory was founded upon the discovery of superoxide dismutase, the enzyme responsible for the removal of superoxide (Maritim et al., 2003). The removal notionally occurs by means of a conversion of two superoxide molecules into oxygen and subsequently, hydrogen peroxide (Halliwell, 1990). However, it was observed that in hyperglycaemic conditions, the bioavailable levels of this enzyme appear to be reduced (Kaul et al., 1995, Kaul et al., 1996, Aragno et al., 1999, West, 2000). This is particularly important, as both SOD and GSH-Px / GSH are involved in the successful detoxification of O_{2^-} (Aaseth and Stoa-Birkedtvedt, 2000). As previously noted, GSH has been shown to be reduced in the diabetic state, thereby favouring the highly toxic hydroxyl radical in the presence of free iron or copper (Cameron and Cotter, 1995, Cameron et al., 2001) [Figure 1.10.2]. This alteration in the homeostatic balance is further compounded by the recognition that glycated haemoglobin shows a reduced ability to scavenge free radicals (Vallejo and Manas, 2000). If unchecked, this will lead to an increased level of biologically active superoxide anions, setting up a positive feed back mechanism (Chan et al., 2000). Consequently, as hyperglycaemic conditions further promote the oxidation of proteins via the Maillard reaction, the continued generation of free radicals is amplified dramatically (Keaney and Loscalzo, 1999).

To appreciate the magnitude of these implications, it is vital that a holistic position is taken. Indeed, the unopposed dominance of this reactive oxygen species will become a catalyst for other pro-inflammatory reactions, such as platelet adhesion, neutrophil infiltration, mast cell degeneration and thereby,

localised inflammatory and proapoptic conditions (Masini et al., 1999, Armstrong, 2001). The role of superoxide in mediating neutrophil/macrophage-perpetrated injury to cells has been demonstrated by studies, which have monitored the effects of superoxide dismutase [SOD] (Tesfamariam and Cohen, 1992, Williamson et al., 1993, Curcio et al., 1995). The results of such intervention have borne out the proposed theories relating to superoxide's active participation within the inflammatory process and ischaemic reperfusion injury and simultaneously, have confirmed the protective property of SOD (Dileepan et al., 1993, Diederich et al., 1994, Ohishi and Carmines 1995, Craven et al., 2001).

The importance of an increased inflammatory state / cellular adhesion and the associated cytokines is also vital for the regulation of ecSOD (Stralin and Marklund, 2000). The results of a study conducted by Stralin and Marklund (2000) found that ecSOD was regulated by inflammatory cytokines, such as TNFα, leading to a decrease in ecSOD expression. In the diabetic state, where NO bioavailability is reduced, whilst hyperglycaemia, leukocyte / endothelial cellular adhesion and nitric oxide production increases, the disadvantaged ecSOD levels can only serve to expedite an environment ripe for diabetic long-term complications (Chibber et al., 2000, Sytze van Dam, 2002, Dickinson et al., 2002, Maritim et al., 2003).

Recent investigation by Fukai et al., (2000) has further established the interplay between ecSOD and NO, demonstrating that exogenous NO actually up regulates ecSOD expression in human aortic smooth muscle cells. A study conducted by the same group found that eNOS-deficient mice displayed a

notable reduction in the expression of ecSOD, when compared to normal mice (Fukai et al., 1998). This work strongly suggests that NO exerts a control over ecSOD expression. As atherosclerosis and hyperglycaemia have both been found to correlate with a reduced bioavailability of NO (Kelm et al., 1997, Honing et al., 1998, Kojda and Harrison, 1999, Ritter and Chowienczyk, 2001), factors frequently coincident within the diabetic state, the redox state of the vessels may be periodically subject to this biochemical imbalance and ultimately, tissue injury. This has been established to an extent in a study by Fukai et al., (1998), which found that in apo(E)-deficient mice, vascular smooth muscle cell expression of ecSOD was reduced, against a background of progressive atherosclerosis.

The variability from patient to patient in the development of certain diabetic complications, such as ulceration, may also be partially answered by the NO / SOD relationship. For example, physical exercise has been positively associated with a reduction in cardiovascular morbidity and mortality (Paffenbarger et al., 1993, Blair et al., 1995). Indeed, its beneficial effects have also been demonstrated in the treatment of diabetes (Boulton et al., 1994). However, whilst the surrounding metabolic and biochemical consequences of exercise cannot be refuted, general consensus has failed to consider the effect on ROS production and plasma LDL oxidation (Shern-Brewer et al., 1998, Ji, 1999). Hypothetically, exercise will serve to stimulate the synthesis of eNOS and thereby, ecSOD (Sessa et al., 1994, Horning et al., 1996, Higashi et al., 1999, Hambrecht et al., 2000). This lends some level of support to previous studies, which have identified that both lifestyle and socio-economic status are

important in determining the risk of developing diabetic complications, notably foot ulceration (Weng et al., 2000, Abbott et al., 2001).

Superoxide and other ROS have been implicated as important mediators of ischaemia-reperfusion injury (Dhalla et al., 2000). This is noteworthy in the pathogenesis of conditions, which promote ulceration in the diabetic foot. It can be stated with relative safety that increased plantar pressures and temporal loading will undoubtedly affect vessel occlusion during gait. Healthy tissues subjected to ischaemia-reperfusion are equipped with antioxidant mechanisms, such as superoxide dismutase, glutathione peroxidase, vitamin C and vitamin E (Melhem et al., 2001). Unfortunately, these protective mechanisms have been shown in both human and animal models of diabetes to be greatly diminished (Cunningham et al., 1991, Asayama et al., 1993, Braunlich et al., 1994, Mukherjee et al., 1994, Paolisso et al., 1994, Sandaram et al., 1996). The particular relevance of superoxide in ischaemia-reperfusion has been demonstrated by Hatori et al., (1992) and Sjoquist et al., (1991), who demonstrated that ecSOD preserved cardiac function and notably reduced infarct size, following ischaemia-reperfusion. This work has been further developed by Chen et al., (1996, 1998) and cited by Fukai et al., (2002), where it was shown that increased levels of human ecSOD in transgenic mice also improved cardiac function, following global normothermic ischaemia-reperfusion.

Whilst this form of investigation has been adapted by Li et al., (1998), incorporating a rabbit model and by Schachinger et al., (2000) on human coronary circulation studies into the diabetic foot, the precise role of ecSOD has yet to be determined. However, as a reduction in NO bioavailability is one of

the key postulated events in the pathogenesis of diabetic complications, the import of ecSOD to prevent NO / O_{2^-} interaction becomes evermore pertinent.

Furthermore, it has been observed that superoxide possesses the ability to cause the release of ferritin (Harris, Cake and Macey, 1994). This event occurs by the reduction of Fe^{+3} to Fe^{+2}, the effects of which, render the molecule into a state, where it is no longer possible to be bound by the protein, so invoking an iron redox (Aruoma et al., 1989). The manifest results of this altered state have been documented in other conditions such as thalassaemia, leading to iron overload, which itself can effect both neuropathy and tissue destruction (Porth, 1998). Recent studies in this area by Salonen (1998) have demonstrated that first generation relatives of individuals with Type II diabetes display altered trans-ferritin levels prior to diabetic onset and more disconcertingly, in its total absence. Notably, long-term complications arising from the redox state can lead to DNA mutations and oxidative stress, further increasing the scope for on-going free radical production (Salonen, 1998).

1.13.2 **Superoxide and Nitric Oxide Interaction Theory**

Given the reciprocal relationship between these two species, this second theory of free radical mediated tissue damage is currently more widely accepted (Xie and Wolin, 1996). Both animal and human diabetic models have revealed an alteration in the observed levels of nitric oxide (Calver et al., 1992, McVeigh et al., 1992, Johnstone et al., 1993, Ting et al., 1996, Watts, et al., 1996, Williams et al., 1996). However, there is some controversy, as to whether levels are indeed raised, as found by Cosentino et al., (1997) or actually reduced, as

demonstrated in studies by Tooke and Goh (1999). Irrespective of the change in direction, sufficient evidence has been recorded to suggest that hyperglycaemia causes NO to exist outside the expected normal bounds (Mayhan and Patel, 1998). In conjunction, another well-documented feature of diabetes is the manifestation of endothelial dysfunction (McVeigh et al., 1992, Malik, 1998, Pinkney et al., 1999). This has been attributed to the marred or complete inability of NO to act as a vaso-relaxant, in response to specific stimuli (Feener and King, 1997, Perreault et al., 2000).

The loss of vaso-tone and its subsequent effects are profound, as diabetic animal models have shown an up regulation of both ET-I and angiotensin II, both powerful vasoconstrictors affecting vessel resistance (Hopfner and Gopalakrishnan, 1999, Stefanec, 2000). An environment favouring hypertension is further exacerbated by the fact that a reduction of bioavailable nitric oxide removes smooth muscle cell proliferation inhibition (Chan et al., 2000). Consequently, increased smooth muscle production coupled with hypertension within the peripheral vessels act as strong factors for the onset of atherosclerotic lesion development (Beckman and Koppenol, 1996). The presence of hypertension causes the characteristic feature of capillary rarification and in turn, imparts endothelial dysfunction (Stefanec, 2000). This, in itself, has been demonstrated to increase the production of superoxide, further compounding the scope for hypertension (Gazis et al., 1999).

The combination of hyperglycaemia, increased superoxide, hypertension and hyperlipidaemia (a condition frequently associated with Type II diabetes) favour cellular apoptosis (Higami and Shimokawa, 2000). Indeed, many of the

associated features of diabetes are factors, which have been shown, when outside normal ranges, to contribute to cellular apoptosis (Table 1.13.2.i).

Table 1.13.2.i **Factors influencing cell survival**

PROAPOPTIC	ANTI-APOPTIC
AGE	Antioxidants
Angiotensin II	Endothelian –1
Calcium – increased intracellular concentration	Extracellular matrix components
Hyperglycaemia	Insulin-like growth factor
Hypoxia	Iron chelators
Interleukin-1 (beta)	Nitric oxide
Nitric oxide in high doses	Shear stress
Ischaemia/reperfusion	Vitamin C
Nitric oxide inhibition	Vitamin E
Oxidised cholesterol	
Oxidised low density lipoproteins	
Proinsulin	
Peroxynitrite	

Adapted from Stefanec, T. (2000) Endothelial apoptosis. Chest 117: 841-854

Whilst the nature of apoptosis in these conditions is rarely generalised, a complicating factor is potentially introduced into this equation. This occurs in response to the apoptic vascular endothelial cells losing their ability to produce normal anti-coagulant factors, thereby leading to proapoptic and procoagulant conditions (Matteucci, 2000). An increased expression of thrombin formation by adherent detached apoptic endothelial cells subsequently occurs, so activating metalloproteinase and protein kinase C (PKC) derived from interleukin-1 and TNFα (Rickard and Gowen, 1993). Prostacyclin production is

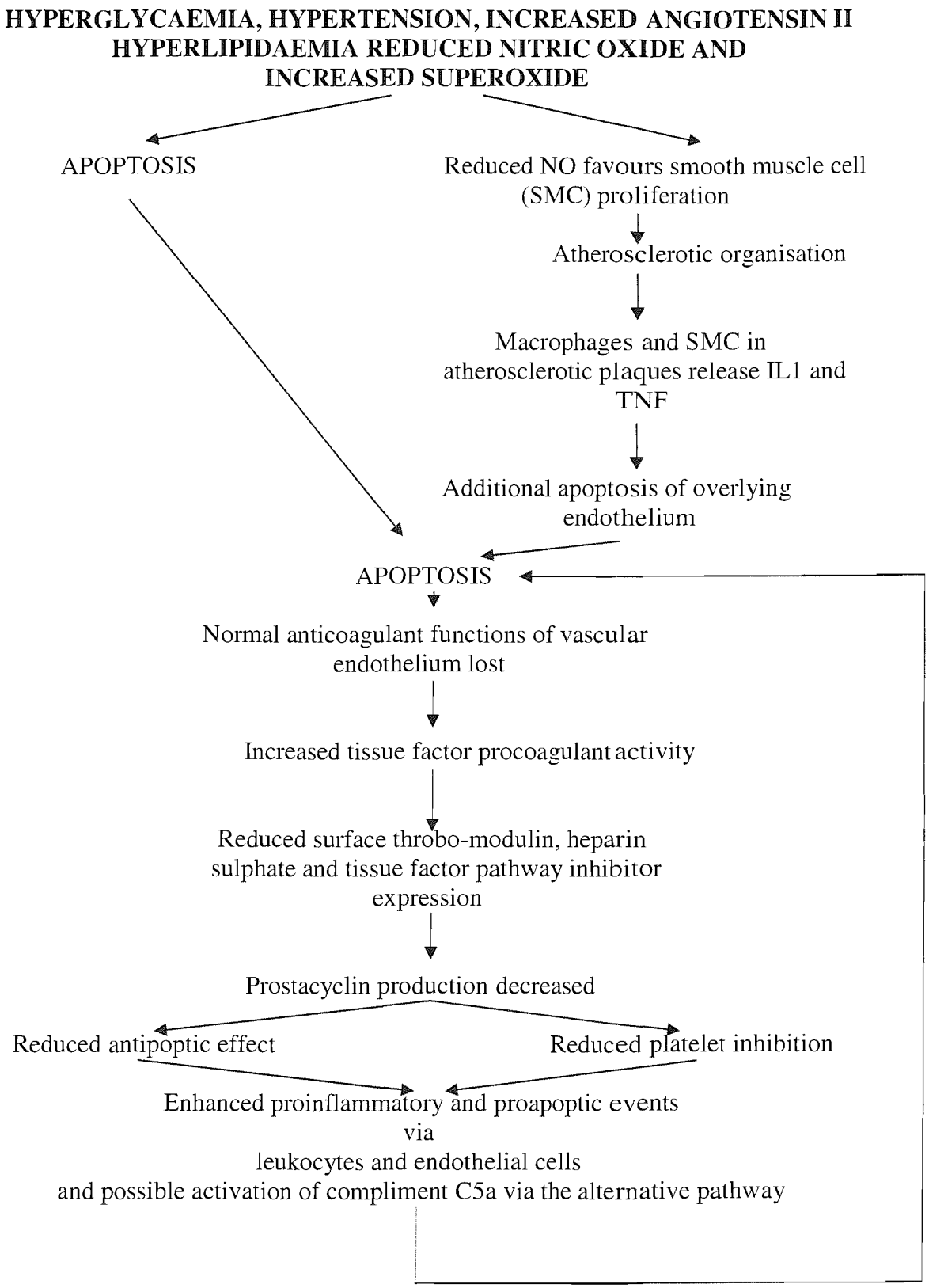
also decreased, culminating in a diminished antipoptic effect and reduced platelet inhibition (Mayhhan and Patel, 1998). Consequently, this form of "closed loop" mechanism actually enhances cellular death (Figure 1.13.2.ii).

Additional studies have also shown that the adherence and subsequent diapedesis movement of macrophages into the atherosclerotic lesion, together with the proliferating smooth muscle cells, produces TNFα (Baowan et al., 1998, Guha et al., 2000). This finding corroborates previous cytokine experiment material, which has commented that TNFα and interleukin-1 (IL1), synergistically linked as TNF and IL1, induce mutual synthesis (Rickard and Gowen, 1993). The importance of these cytokines could prove to be extremely enlightening in the field of rheumatic disease research, as both IL1 and TNF have been recently reported as the primary destructive catalyst in Rheumatoid Arthritis (Mukaida et al., 1998, Starkebaum, 1998). As they initially stimulate the synthesis of metalloproteinases in invading macrophages, fibroblasts, neutrophils and chondrocytes, the metalloproteinases go on to systematically degrade structures such as cartilage, collagen and proteoglycans, thus potentially creating an environment of tissue destruction and poor wound resolution (Murphy and Hembry, 1992). Furthermore, both cytokines stimulate the release / activation of neighbouring cells, resulting in inflammation and the recruitment of additional macrophages to the atherosclerotic lesion via cellular adhesion molecules [CAM's] (Weaver et al., 1998).

Associated complications of diabetes have traditionally had important links to cytokine production, as osteoblast derived IL1 and TNF have also been suggested as primary factors in the active osteophyte remodelling and

subchondral bone absorption (Starkebaum, 1998). The characteristic features of these cytokines in RA closely mirror the diabetic complication Charcot Foot, particularly during the active and late phases of this condition (Boulton et al., 1994). The pathological process involved within the Charcot Foot is still relatively unknown, although it has been proposed that an initial fracture / trauma may lead to the subsequent active re-absorption and remodelling of bone (Edmonds and Foster, 2000). Consequently, future study may provide interesting insight into this particular complication and the implications of IL1 and TNF as the mediators responsible for triggering excessive osteoclast activity.

Figure 1.13.2.ii **Proposed route to apoptosis**



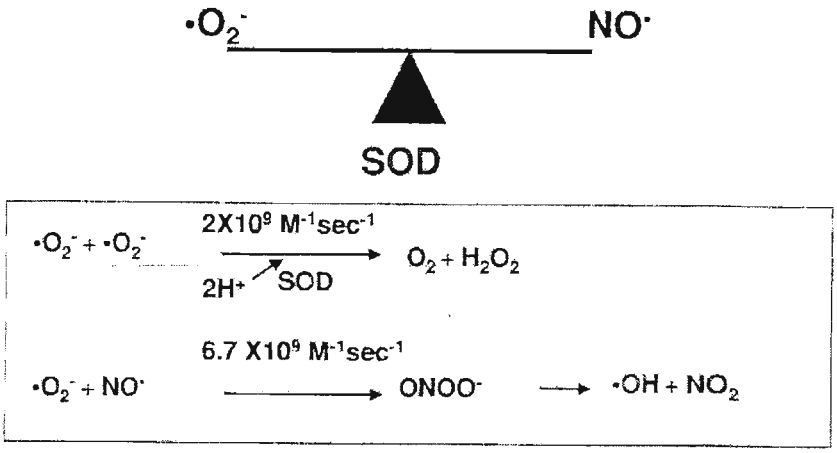
1.14 Peroxynitrite (ONOO -)

The theory of reduced NO production has been called into question by Wang and Zweier (1996), who intimated that NO synthesis may actually be increased. At first glance, these findings appear to completely contradict the majority of contemporary information regarding the suspected mechanisms for cellular apoptosis and damage. However, this may not be the case, as a theory is now developing which proposes that, it is the bioavailability of NO, which is reduced, not the absolute levels (Casentino et al., 1997, Honing et al., 1998, Li and Forstermann, 2000). The basis for this supposition is that normal or potentially increased levels of NO are overwhelmed by unchecked amounts of O_{2}-. due to reduced levels of superoxide dismutase (Weaver et al., 1998). Consequently, there is an increased probability of NO and O_{2}- reacting to form the toxic radical, peroxynitrite [ONOO-] (Beckman and Koppenol 1996).

This hypothesis is not entirely unfounded, as the interaction of NO and O_2 was first demonstrated in 1986 (Halliwell, 1991). As both O_{2}- and NO. contain unpaired electrons in their outer orbitals, they can undergo an extremely rapid diffusion-limited radical-to-radical interaction (Beckman and Koppenol, 1996). Investigation by Beckman et al., (1990) also suggests that ONOO- is strongly implicated in oxygen radical mediated injury towards a variety of molecules. The inevitability of this interaction may be placed into context, when considering that the reaction potential is three times faster ($6.7 \times 10^{9} M^{-1}s^{-1}$) than the catabolism of superoxide via superoxide dismutase (Beckman and Koppenol, 1996). Furthermore, O_{2}- displays the ability to interact with NO.

10,000 times faster than with endogenous antioxidant enzymes, such as vitamin A, C and E (Darley-Usmar et al., 1995).

Figure 1.14.1 **Superoxide / NO. interaction**



Taken from Fukai et al., Cardiovascular Research 55: 240.
The rapid interaction between the superoxide and nitric oxide radicals leading to the formation of nitrite, nitrate and peroxynitrite anion.

Consequently, through the rapid interaction of the two oxygen reactive species, NO. and O_{2}-, the generated ONOO- forms a potent oxidant and proapoptic radical, whilst simultaneously reducing the bioavailability of NO (Radi et al., 1991, Beckman and Anderson, 1994, Kelm et al., 1997). The forging of a "cycle of cytotoxicity" is further underpinned by peroxynitrite's ability to inhibit superoxide dismutase and other antioxidant systems (Ischiropoulos et al., 1992, Yamakuri et al., 1998 and Szabo, 2003). Indeed, work conducted by Cuzzocrea et al., (1998) discovered that ONNO- leads to the depletion of GSH, indicating a likely feedback mechanism for cellular injury and ROS formation.

This theory is further substantiated by Wang and Zweier (1996), who have shown that during the early phase of post ischaemic reperfusion, elevated levels of NO. were generated simultaneously with the reperfusion associated burst of

O_{2^-}, thus leading to the formation of ONOO-, coupled with reperfusion injury. The implications of these observations for diabetes is potentially profound, as hypercholesterolaemia, a condition often coincident with diabetes has been shown to diminish the glutathione (GSH) detoxification system responsible for the handling of peroxynitrite (Muchova et al., 1999). This issue is further exacerbated by the fact that atherosclerotic plaques contribute to the additional production of superoxide in both the plaque itself and migrating leukocytes, promoting an environment for further vascular dysfunction and damage (Leuwenburgh et al., 1997).

1.14.1 **ONOO- Mediated Cell Injury**

Oxidative stress has previously been implicated in the mediation of tissue injury via the initiation of precursors of matrix metalloproteinase [proMMPs] (Szabo, 2003). However, recent work has now determined that ONOO- generation may have a key role in starting this chain of events, by acting as a catalyst for a substantial S-glutathiolation reaction (Okamoto et al., 2001). Consequently, it has been proposed that an inhibition of this reaction via ONOO- decomposition catalysts may simultaneously reduce proMMP activation (Okamoto et al., 2001). The ability to modulate this form of activity in inflammatory and reperfusion states would clearly hold profound implications for tissue injury resolution.

The pathological range of ONOO- is further extended, when considering its ability to trigger a pro-inflammatory state. A clear example of this is Zingarelli's et al. (1998) work, which highlighted that ONOO- increased the

expression of P-selectin and ICAM-1 in human endothelial cells. Additionally, the chemotaxtic cytokine IL-8 was shown to be up regulated by ONOO- in human leukocytes, coupled with the down regulation of L-selectin (Zouki et al., 2001). All the above data, coupled with the ability of ONOO- to promote and increase the expression of NF-κB, leads to a pro-inflammatory state, favouring increased cellular adhesion and dysfunctional effects on cellular signalling pathways, i.e. NO synthesis enhancement / ROS formation (Marui et al., 1993, Matata and Galinanes, 2001, Maritim et al., 2003). However, it is noted that whilst direct links have been established between ONOO- and the up-regulation of P-selectin, the actual interaction between P-selectin and increased leukocyte endothelial adhesion remains elusive.

ONOO- has also been shown by Muriel and Sandoval (2000) to inhibit Na+-K+- ATP-ase activity. This holds particular relevance to diabetic complications, as alterations to this essential component of cellular function have been observed in diabetic peripheral neuropathy (Borghini et al., 1994, Stevens et al., 1996, Cameron et al., 1999. Additionally, it has been recorded that ONOO- readily oxidizes BH_4, thus reducing its bioavailability (Szabo, 2003). Therefore, in cells demonstrating BH_4 levels outside nominal homeostatic balance, such a deficit will propagate a cycle of self-destruction, driven by NO synthase-dependent formation of peroxynitrite (Milstien and Katusic, 1999).

A possible further pathophysiological role of ONOO- in diabetic peripheral neuropathy has been mooted by Liu and Martin (2001), where motor neurones exposed to ONOO- lead to an accumulation of DNA single strand breaks. This

denigration, as a consequence of endogenous or exogenous ONOO-, activates the nuclear enzyme poly-ADP ribosyl synthetase or polymerase (PARP), which in conditions of protracted oxidative stress, acts as a terminal mediator of cellular necrosis (Virag and Szabo, 2002).

Similarly, high concentrations of ONOO- were observed to promulgate expeditious cell death via extreme alterations to vital mechanisms providing energy within the cell (Evans et al., 1996, Szabo, 1996, Leuwenburg et al., 1997, Moriel and Abdalla, 1997). On the other hand, lower levels of exposure, over a period of hours, caused the release of cytochrome c from the mitochondria, promoting apoptic cell death (Zhuang and Simon, 2000).

1.14.2 **ONOO- Interactions**

A review by Weaver et al., (1998) has discussed the possibility of nitric oxide synthase III (NOS III) acting in a dual manner. In the normal physiological environment, NOS III generates NO and normal levels of peroxynitrite, the latter molecule having demonstrated anti-atherosclerotic properties at low levels. However, in the presence of hyperlipidaemia or atherosclerosis, conditions both seen in Type II diabetes mellitus, Pritchard et al., (1995) as cited by Weaver, et al., (1998) found that NOS III was responsible for the in vitro formation of superoxide, inferring directly that NOS III has a peroxynitrite inducing function.

It has been hypothesised that peroxynitrite is a key mediator in post-ischaemic or reperfusion injury through lipid peroxidation (Weiss et al., 1996).

Additionally, its cytotoxic properties towards protein and non-protein thiols and deoxyribose have also been forwarded (Halliwell, 1991). The effects of lipid peroxidation or cytokine stimulation, as the result of activated phagocytes, leads to membrane disruption and potential collapse, possible altered cell signalling and the activation of the cyclooxygenase catalyst (Mukaida et al., 1998).

The stimulation of cyclooxygenase (COX) activity, namely COX-2 via arachadonic acid, may have important implications on the mediation of pain and the potentiation of inflammation due to the generation of prostaglandins (Kujubu et al., 1991, Misko et al., 1995). Indeed, accumulating evidence now suggests that an interaction between NO and COX-2 may form a key role in the mediation of neuro-toxicity following cerebral ischaemia (Baowan et al., 1998, Kawasaki, et al., 2001). Animal models with manifest focal and global cerebral ischaemia further support this theory. Following the use of a highly selective COX-2 inhibitor, a reduced level of cerebral ischaemic damage was observed (Schwab, et al., 2000).

The further relevance of peroxynitrite in reperfusion injury has been substantiated by work, which has studied the beneficial effects of peroxynitrite decomposition catalysts (Schulz et al., 1995, Cuzzocrea et al., 1998, Bianchi et al., 2002). Administration of a specific decomposition catalyst to ischaemia-reperfusion injury of the bowel reduced lipid peroxidation and the production of ONOO- (Cuzzocrea et al., 1998). Furthermore, work by the same authors demonstrated that the administration of a decomposition catalyst reduced P-selectin and ICAM-1 expression (Cuzzocrea et al., 1998). Animal models of myocardial ischaemia and reperfusion have also recently demonstrated the

protective benefits of peroxynitrite decomposition catalysts. This was highlighted in a study conducted by Bianchi et al., (2002), where the infarct size was reduced by ≈ 40% in comparison to a control group.

Whilst human studies confirming the presence of a direct NO and COX-2 interaction are currently absent, Iadecola et al., (1999) have demonstrated COX-2 up regulation in the brains, neutrophils and vascular endothelium of patients, who died following cerebral infarction. The mechanism of toxicity unleashed by COX appears to act in a pincer-like fashion; firstly, by the postulated NO-COX-2 interaction. Secondly, by the amplificatory relationship that NO exerts over COX-2 to produce prostaglandins and ROS, further promoting oxidative stress and its subsequent chain reactions (Koide et al., 1993, Imai et al., 1994, Nagoshi et al., 1998).

Observations have also been made that peroxynitrite can subsequently be converted to peroxynitrite acid and ultimately, to hydroxyl radicals [.OH] (Masini et al., 1999). The latter is the most reactive known to chemistry with the potential to attack and damage almost every molecule found in living cells (Halliwell, 1991). However, its half-life within the body is extremely brief, persisting at intervals of less than 1 μs, as it rapidly combines with molecules in its immediate vicinity (Halliwell, 1991). Interactions with non-radicals may also set up chain reactions, as .OH can affect both the purine and pyrimidine bases of DNA (Beckman et al., 1990, Cameron et al., 2001). Furthermore, interaction with thiols leads to the formation of sulphur radicals, which, in the presence of oxygen, can cause the additional generation of O_{2^-} and .OH, favouring lipid peroxidation (Miles et al., 1996).

1.15 Antioxidant Intervention

The role of lipid peroxidation in oxygen radical mediated tissue damage and mitochondrial dysfunction is pivotal (Majima, et al., 1998). However, in normal conditions, the preceding chain of events leading to lipid peroxidation can be broken by alpha-tocopherol (vitamin E) [Pinkney et al., 1999]. Alpha-tocopherol is incorporated into cell membranes and plasma lipoproteins (Asayama et al., 1993, Braunlich, et al., 1994, Mukherjee et al., 1994). The chain breaking function is achieved by an .OH sited on the hydrophobic structure (Packer et al., 1995). This .OH group interacts readily with products generated from lipid peroxidation, i.e. peroxyl and alkoxyl species (Packer et al., 1995). Consequently, tocopherol-O is produced, which has a lower reactivity and is unable to potentiate further attacks on the fatty acid chains (Halliwell, 1991).

Moreover, vitamin E has shown some success in stimulating perfusion and improving nerve condition velocity (Cotter et al., 1995, Ruhnaut et al., 1999). However, a caveat must be expressed regarding these findings, as elevated levels of vitamin E can also act as an oxidant, thus removing its protective benefits (Manzella et al., 2001). The antioxidant effects of vitamin C have also been explored with relatively limited success, as its therapeutic values can only be achieved via intravenous administration (Ting et al., 1996, Lindsay et al., 1998). However, despite the current efforts to achieve therapeutic levels of these antioxidants, there is growing evidence to suggest that diabetes mellitus displays a reduction in key antioxidants, namely, vitamins E and C, superoxide dismutase and glutathione (Cross et al., 1987, Collier et al., 1990, Garg et al.,

1996, Will and Byers, 1996, Palmer, 1998, Sargeant et al., 2000, Cinar et al., 2001).

1.16 Protein Kinase C

Protein Kinase C (PKC) is a serine / threonine kinase, which exists in twelve known isoforms and has been implicated in specific hormonal, neuronal and growth factor stimuli (Nishizuka 1992, Way, Katai and King, 2001) [Table 1.16.1]. Each isoform is distributed in specific tissues and requires varying co-factors dependent upon its location (Idris et al., 2001). The PKC isoform family can be broadly categorised into three sub-groups. Firstly, the "classical" type, which are both calcium and phospholipid-dependent (Chang and Tepperman, 2001). Similarly, they are activated by phosphatidylserine (PS), Ca^{2+} and diacylglycerol [DAG] (Newton, 1995). Secondly, the "novel" group are calcium independent, but phospholipid dependent, requiring PS and DAG for activation (Newton, 1995). Lastly, the "atypical" class are calcium and phospholipid independent. However, whilst they require PS to initiate their activity, these enzymes do not require DAG or Ca^{2+} (Idris et al., 2001).

Irrespective of which isoform is activated, there are profound resultant cellular effects. These include cell proliferation, lipid metabolism, gene expression, smooth muscle contraction, alteration to the redox state and glucose transport and metabolism (Nishizuka 1984, Nishizuka 1992, Nishizuka 1995). Whilst cloning of the isoforms has been relatively successful, there still remains considerable scope for investigation into the specific functional activity of the individual isoforms (Xia et al., 1996). However, it has been repeatedly demonstrated that PKCβ is preferentially expressed in hyperglycaemia induced

pathological states within macro vascular tissues, endothelial cells and smooth muscle cells (Inoguchi et al., 1992, Kunisaki et al., 1994).

Table 1.16.1 **PKC isoforms and co-factors**

Isoform	**Tissue**	**Co-factors required**		
		DAG	**Ca^{2+}**	**PS**
Classical cPKCs α	Widespread	✓	✓	✓
β_I	Widespread	✓	✓	✓
β_{II}	Widespread	✓	✓	✓
γ	Brain	✓	✓	✓
Novel nPKCs δ	Widespread	_	✓	✓
ε	Brain, heart	_	✓	✓
η	Heart, skin, lung	_	✓	✓
θ	Muscle, brain, blood cells	_	✓	✓
μ	Lung epithelial cells	_	✓	✓
Atypical aPCKs ζ	Widespread	_	_	✓
ι	Kidney, brain, pancreas	_	_	✓
λ	Kidney, brain, pancreas	_	_	✓

Adapted from Way, K., Katai, N., King, G. L. (2001) Protein kinase C and the development of diabetic vascular complications. Diabetic Medicine 18: 945-959

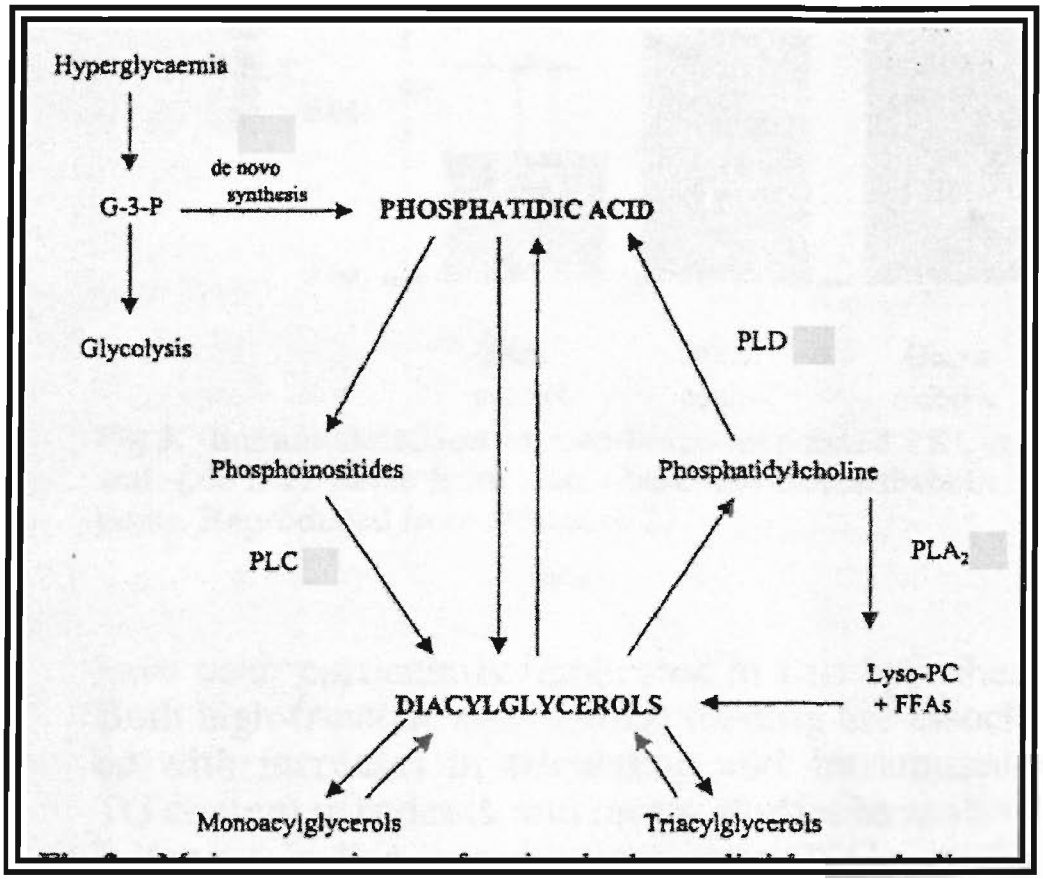
1.16.1 **DAG / PKC Mutuality**

Whilst DAG acts as the principle foundation block for the initiation and translocation of PKC, four potential pathways to DAG formation have been proposed (Porte and Schwartz 1996, Lee et al., 1989). First, is the hyperglycaemic route, whereby de novo synthesis of DAG is achieved via glyceraldehyde-3-phosphate (G-3-P), a glycolytic intermediate of phosphatidic acid (Morigi et al., 1998). It is through this source that PKC has previously been implicated in the development of diabetic cardiovascular complications (Xia et al., 1994). Secondly, DAG can be mediated through phospholipase C (PLC), which augments the hydrolysis of phosphoinositides (Rhee et al., 1989, Nishizuka 1992). Thirdly, phospholipase D (PLD) causes the hydrolysis of phosphatidylcholine (PC) via phosphatidic acid, stimulating DAG formation (Okumura et al., 1991). Finally, as a consequence of phospholipase A_2 (PLA_2) action, non-esterified fatty acids (NEFA) also promote DAG activation (Diaz-Guerra et al., 1991, Lu et al., 2000).

Whilst the activation of DAG, as a consequence of hyperglycaemia, has previously been purported as a key route to diabetic vascular disease (Kunisaki et al., 1994, Ganz and Seftel 2000, Assert et al., 2001), the liberation of NEFAs could prove to be particularly poignant with regard to diabetic complications. Given observations that NEFA's may be elevated in diabetes (Lu et al., 2000), it is therefore reasonable to suppose that PKC activity could be heightened independently of de novo synthesis of DAG (Idris et al., 2001, Way et al., 2001).

Studies have been successfully conducted to identify PKC and DAG levels within the various tissue types and associated isoforms (West, 2001). However, it should be recognised that many of these focused upon measuring levels after the selected cells / tissues had been exposed to high levels of glucose. Whilst this is an important practice in the study of diabetes per se, it potentially undermines the true relationship between PKC and DAG levels following oxidative stress or the release of NEFA's. Measurement of PKC within the peripheral nerve may still hold further pertinent information, as previous studies have tended to focus strictly upon nerve activity (Gabbay et al., 1990, Kim et al., 1991, Ido et al., 1994, Kowluru et al., 1998, Cameron et al., 1999, Kishi et al., 1999). Of note, it has been suggested by Cameron and Cotter (2002), that the success of the specific PKCβ inhibitor LY333531 in experimental diabetic neuropathy, is due to its apparent effect upon the vasa nervosum, augmenting blood flow and improving nerve conduction velocity (NCV).

Figure 1.16.1.i **Pathways to DAG mediated PKC activation**



Taken from Idris, I., Gray, S., Donnelly, R. (2001) Protein kinase C activation: isozyme effects on metabolism and cardiovascular complications in diabetes. Diabetologia 44:659-673.

1.16.2 PKC Co-factors and Effects

Lee et al. (1989) have also found increased glucose concentrations alone are sufficient to trigger PKC activity in cultured capillary endothelial cells. This is further corroborated by Hempel et al. (1997) and Williams et al., (1992), who have demonstrated long-term elevated PKC activation in endothelial, mesangial and vascular smooth muscle cells exposed to increased glucose levels. They and others have observed that glucose-induced PKC activation interferes with agonist-induced intracellular calcium signalling (Williams and Schrier, 1992,

Williams et al., 1992, Haller et al., 1995). Indeed, increased PKC activation in diabetes has been associated with vascular cell alterations, noted particularly as increased basement membrane thickening, cell contraction and cellular proliferation (Hempel et al., 1997). However, given the metabolic disruption with diabetes, it is unclear whether these events occur directly as a consequence of PKC activation or are in fact, a downstream product of PKC, vessel occlusion, oxidative stress and VEGF interaction (Xia et al., 1996).

Historically, PKC activation has also been implicated as both a potential mediator and inhibitor of insulin action (Considine and Caro 1993, Berti et al., 1994, Busch et al., 1998). However, recent studies have indicated that specific tissue dependent isoforms promote varying effects (Cotter et al., 2002). This has been implied by work, which demonstrated that both PKCβI and PKCβII have a notable inhibitory effect on the insulin receptor, tyrosine kinase, in terms of autophosphorylation (Muller et al., 1991, Bassenmaier et al., 1997). Development of this line of investigation by Strack et al., (2000), has further mooted that serine residues 994 and 1023/25 are targeted by PKC-θ and PKC-β mediated inhibition. The import of this study within diabetic foot complications gathers impetus, as decreased tyrosine kinase activity and a reduction in skeletal muscle will ultimately lead to muscle atrophy (Itani et al., 2000). Notably, this form of clinical wasting to the anterior compartment of the diabetic leg is a common clinical observation (Boulton et al., 1994).

PKC activation will potentially stimulate PLC to increase catabolism of the lipid membrane fraction and so form DAG (Xia et al., 1996). As DAG is a progenitor of arachadonic acid, this leads to prostaglandin formation (Bohlen

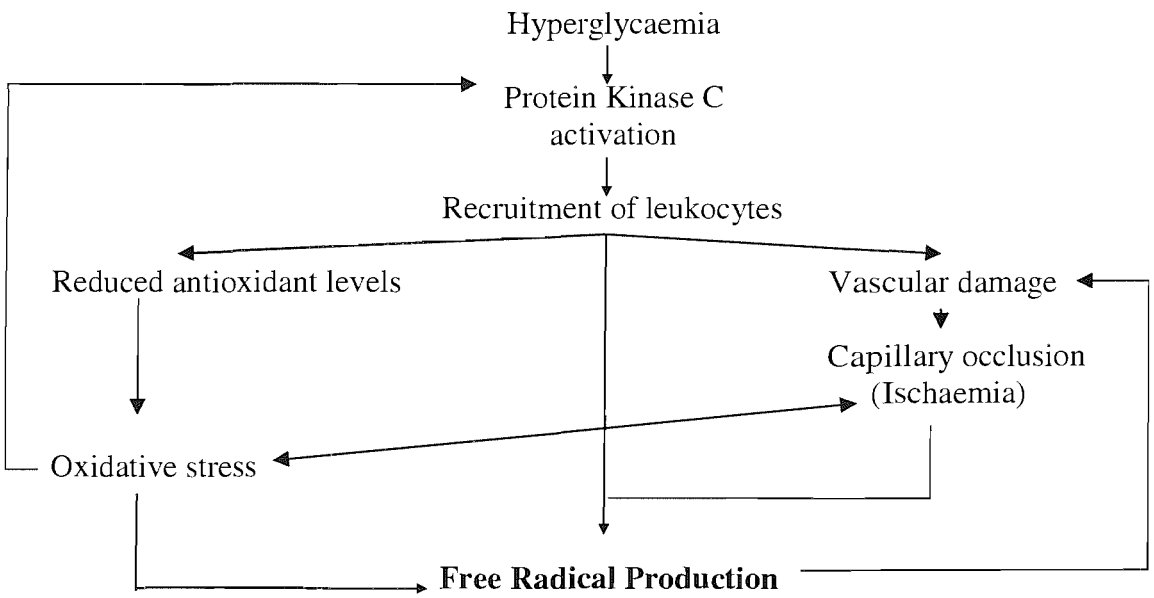
and Nase, 2001). This process causes an increase in ROS, which can interact with NO, thus reducing its bioavailability and increasing the potential for the generation of highly toxic radicals, such as peroxynitrite (Ishii et al., 1996, Chakravarty et al., 1998, Nakamura et al., 1999). Additionally, the derived prostaglandins (PGE_2) and arachadonic metabolites can lead to hyperalgesia and influence potassium channel function in certain neurones (Rang et al., 1996). This may prove a novel insight into the potential synergistic link between the pathogenesis of painful and insensate neuropathy.

1.16.3 **PKC Vascular Theory**

At its nominal ratio, PKC regulates the suppression of the nitric oxide syntheses (NOS) gene, thus maintaining correct vascular tone (Ohara et al., 1995). Hence, should the level of PKC exceed its accepted limits, the delicate balance of this mutual relationship becomes disturbed. As a result, there is an increased smooth muscle cell contractile response, promoting a hypertensive, atherosclerotic environment in the vasa nervosum and thereby, a reduced nerve conduction velocity [NCV] (Cameron et al, 1999). The natural effect of this condition will be a marred nervous transmission, which could have a number of manifestations for the individual, not least, numbness and a loss of sensory feedback, as typically noted within diabetic neuropathy (Cameron and Cotter, 1994, 1997, Tesfaye et al., 1994, Hotta et al., 1996). However, the precise role of PKC activation in peripheral neuropathy remains unresolved, as various studies have shown it to be either reduced or unaltered (Kim et al., 1991, Borghini et al., 1994, Mathew et al., 1997, Roberts and McLean, 1997).

The PKC circle is partially closed, as the relative reduction in NOS effects a superoxide imbalance, allowing excessive molecular oxidisation and corruption into free radical elements (Hirata et al., 1995, Ohara et al., 1995). As previously stated, it is this very environment, which accelerates oxidative stress and the formation of PKC abnormality. Hence, the association between oxidative stress and PKC activation is paramount, as antioxidants modulate these actions and it is this inhibition, which actually prevents tissue damage (Govers and Rabelink, 2001).

Figure 1.16.3.i **PKC-mediated vascular damage**



Studies examining PKC effects on nerve conduction in streptozotocin-induced diabetes within rats have further highlighted the potential importance of altered PKC activity within the field of diabetic neuropathy (Tesfaye et al., 1994, Maxfield, et al., 1995). These studies have shown that increased vascular PKC is associated with elevated contractile smooth muscle responses and diminished endothelial dependent relaxation.

1.17 Capillary Occlusion Theory

Advances within the sphere of diabetic retinopathy and microangiopathies have suggested that leukocytes play an important role in these complications (Davis, 1993, Olsson et al., 1999, Chibber et al., 2000, 2003). Studies have shown that leukocytes in both individuals with diabetes and animal models display an increased leukocyte / endothelial adhesion activity (Chang and Tepperman, 2001, Snapp et al., 2001, Sperandio et al., 2001, Norman et al., 2000). Recently, Morigi et al. (1998), demonstrated that acute exposure of cultured human endothelial cells to high glucose concentrations promotes leukocyte / endothelial cell adhesion, partially supporting the role of hyperglycaemia in the pathogenesis of microvascular disease (Cameron et al., 2001). This activity appears to be in response to an up-regulated cell surface expression of adhesive proteins through the NF-κB and PKC intracellular pathways (Park et al., 2000b). Indeed, Miyamoto et al. (1998) and Harris et al. (1994) have also shown capillary occlusion to be increased in streptozotocin induced diabetic rats.

Similarly, Tesfaye et al. (1994) have also recorded tortuous capillaries with associated angiogenic structural changes occurring to the vasa nervosum, with a consequential increase in capillary occlusion in diabetic individuals. This would possibly substantiate the theory of leukocyte involvement, as increased leukocyte / endothelial activity would tend to promote blockage (Schroder et al., 1991, Lutty et al., 1997, Barouch et al., 2000, Kaplar et al., 2001, Chibber et al., 2003). Arguably, vessel distension would also occur in structures prior to the occlusion, whilst the resulting ischaemia to the capillary would follow

downstream. Consequently, arteriovenous shunting would be invoked, prompting the "capillary steal" effect and exacerbating post occlusion ischaemia (Tesfaye et al., 1994). Notably, Sunderkotter et al. (1991) have demonstrated that accumulated neutrophils release proangiogenic factors in response to hypoxia and oxygen derived free radicals. This observation has the potential to evolve into a cyclic mechanism, as Wierusz-Wysocki et al., (1987) and Freedman et al., (1992) have shown that trapped leukocytes will respond by producing increased levels of the superoxide radical.

The potentially devastating results of this form of alteration were also demonstrated by Schroder et al. (1991), who showed that increased leukocyte / endothelial activity in rats lead to capillary occlusion, ischaemia and subsequent destruction of the capillary bed. Furthermore, it has been demonstrated by Pecsvarady et al. (1994) that leukocytes show reduced membrane deformability, resulting in impaired diapedesis and deterioration in the immune response.

1.17.1 **Leukocyte / Endothelial Adhesion**

In normal haemodynamics, during an inflammatory response, leukocytes adhere to the vascular endothelium through selectins and integrins, following a process of margination, capture, rolling, activation and firm adhesion (Kunkel and Ley, 1996, 1998, Hempel et al., 1997). The process of rolling has two primary purposes, both of which involve the leukocytes being slowed in their progression through the vessels (Lawrence et al., 1997). The primary function of this mechanism is to increase the exposure time of the leukocytes, the concentration of cytokines acting in a chemo attractant manner to aid leukocyte

stimulation, activation and adhesion (Lawrence and Springer, 1991). The second function appears to promote the attraction of the leukocytes from the normal blood velocity, in order to assist adhesion (Kunkel and Ley, 1996).

The mediation of this trafficking is achieved by o-linked oligosacchardies (glycoproteins/glycolipids), which are found on the surface of the leukocytes, platelets and the endothelium (Chibber et al., 2000). These structures are a family of selectin receptors (L-, P- and E-selectin), tethered to the cell membrane and are responsive to their respective carbohydrate abundant ligands (Alon et al., 1995, Berlin et al., 1995, Park et al., 2002b). P-selectin is stored in Weibel-Palade bodies in endothelial cells and can be rapidly translocated to the luminal surface upon endothelial cell activation via histamine or thrombin (Geng et al., 1990), the latter being often elevated in diabetes (Standeven et al., 2002, Arjomard et al., 2003). The mediating factors for E-selectin expression are inflammatory cytokines, such as TNFα and IL1, both of which are released by stimulated leukocytes and are frequently present following cellular injury (Bevilacqua et al., 1987, Sanders et al., 1992). These particular cytokines have also been observed to provide a dual action in the increased expression of selectins, as they synergistically increase P-selectin (Hahne et al., 1993, Bussollino and Camussi, 1995, Vestweber and Blanks, 1999). Of the three selectins, P-selectin has demonstrated the highest specificity for a ligand, acting preferentially with P-selectin glycoprotein ligand-1 (PSGL-1) both in vitro and in vivo (Moore et al., 1995 and Yang et al., 2001).

This particular ligand is expressed on leukocytes and is a disulphide-bonded dimeric mucin with a subunit mass of approximately 120 kDa (Leppanen et al.,

1999). PSGL-1 is located on microvilli projecting 0.2-0.3 μm from the surface of leukocytes (Moore et al., 1995). It has also been suggested that this form of microvilli extension assists in P-selectin association (Berlin et al., 1995). This is particularly relevant, when considering the effects of shear flow in leukocyte binding, as it has been proposed that the microvilli extend to increase cell contact and thus exposure time during increased flow velocity (Schmidtke and Diamond, 2000). This theory has been partially supported by the findings of Park et al., (2002), which indicate that elasticity during PSGL-1 / P-selectin interaction can achieve a range of 152 -1340 pN/μm. Consequently, even under increased strain, this form of binding can be extremely robust.

1.17.2 **Inhibition of Selectins**

The role of selectins in the inflammatory process has been observed using mice deficient for individual selectins, the results of which show notable inflammatory defects and an often combined dysfunction (Mayadas et al., 1993, Arbones et al., 1994, Labow et al., 1994, Subramaniam et al., 1995, Tedder et al., 1995). Indeed, studies involving the use of P-selectin deficient mice have demonstrated that trauma-induced leukocyte rolling onset is significantly slowed and that upon rolling activation, the number of rolling cells is only 10-15% of those observed in wild mice (Ley et al., 1995, Kunkel et al., 1996). The degree of redundancy between P- and L-selectin in trauma-induced inflammation is further highlighted by the fact that in P-selectin deficient mice, L-selectin is wholly responsible for the rolling function (Ley et al., 1995). However, whilst some overlap may initially appear to be beneficial, it was also noted that rolling velocity was increased [approximately 5 times faster] in

comparison to wild mice (Jung et al., 1996). Consequently, this would reduce the expose time of the leukocytes to chemo attractant and thereby, the potential for firm adhesion.

Conversely, in L-selectin deficient mice, trauma induced rolling is entirely P-selectin mediated and is shown to be no different in terms of rolling velocity between the sample groups (Jung et al., 1996). However, whilst the role of L-selectin continues to be fully investigated, the importance of P-selectin in mounting a normal inflammatory response has been established as vital (Sperandio et al., 2001). This was demonstrated by Labow et al., (1994), who found that there are no obvious inflammatory deficits in E- and L-selectin deficient mice, unless P-selectin is blocked. The blocking of P-selectin was also seen to lead to a significant reduction in the number of rolling leukocytes (Kunkel and Ley, 1996).

1.17.3 **Velocity and Shear Force**

The velocity of the rolling leukocytes will have a marked effect on the process of adherence and it has been discovered that selectins display particular properties to address this variable (Ley et al., 1995, Kunkel and Ley 1996, Jung and Ley, 1999). This is particularly poignant in conditions such as diabetes, where the bioavailability of vasodilators, such as NO, maybe reduced (Honing et al., 1998, Li and Forstermann, 2000). Additionally, agents such as angiotensin II and ET-1 can be increased, favouring a hypertensive / vasoconstrictive environment and effectively elevating the shear forces exerted upon the vascular endothelium (Chen et al., 2000).

Notably, E-selectin displays superiority in mediating leukocyte rolling at velocities below 10 μm/s, in mice pre-treated with TNFα (Kunkel and Ley, 1996). The specificity of selectins upon shear rate in core 2 knockout (deficient) mice was further demonstrated, as E-selectin is greatly reduced in venules with shear rates above 300 s^{-1} (Sperandio et al., 2001). However, it was suggested that the rate of neutrophil transmigration is unaffected in core 2 deficient mice and that the dysfunction observed in migration is attributable to a decrease in rolling numbers (Sperandio et al., 2001).

P-selectin is able to facilitate leukocyte rolling at velocities up to 50 μm/s. This capacity differential during increased velocities could be a result of the presence of PSGL-1 on the microvilli of the leukocytes (Park et al., 2002). A study using a rolling leukocyte assay also found that the number of rolling leukocytes from core 2 deficient mice was reduced to 10% of that observed in wild specimens (Snapp et al., 2001).

1.17.4 **Adhesion Co-factors**

To date, there has been relatively little investigation into the specific structures of PSGL-1 within human leukocytes and the mediation of P-selectin binding (Leppanen, 1999). However, studies have indicated that many of the O-glycan structures of PSGL-1 appear to be core 2 β1,6-GlcNAcT based (Wilkins et al., 1996). Nevertheless, a body of evidence has now positively associated the role of core 2 β1,6-GlcNAcT (core 2 transferase) in PSGL-1 with subsequent P-selectin binding (Kumar et al., 1996, Li et al., 1996, Maly et al., 1996, Ellies et

al., 1998). This has been demonstrated in animal models, where core 2 transferase and α1,3-fucosyltransferase (FucT-VII) were necessary co-factors for PSGL-1 to successfully bind with P-selectin (Kumar et al., 1996, Li et al., 1996).

Further extension of this work has also revealed that a sialyl Lewis x (sLe^x) motif on core 2 transferase based O-glycan structures within PSGL-1 is necessary for P-selectin adhesion (Leppanen et al., 1999). Sialyl Lewis x are ligands for E-, P- and L-selectins and are located on the glycoproteins of human leukocytes (Nakamura et al., 1998). Furthermore, it has been established that core 2 transferase dependent O-glycans are required for sLe^x expression of PSGL-1 and binding to P- and E-selectins on the activated endothelium (Koya et al., 1999).

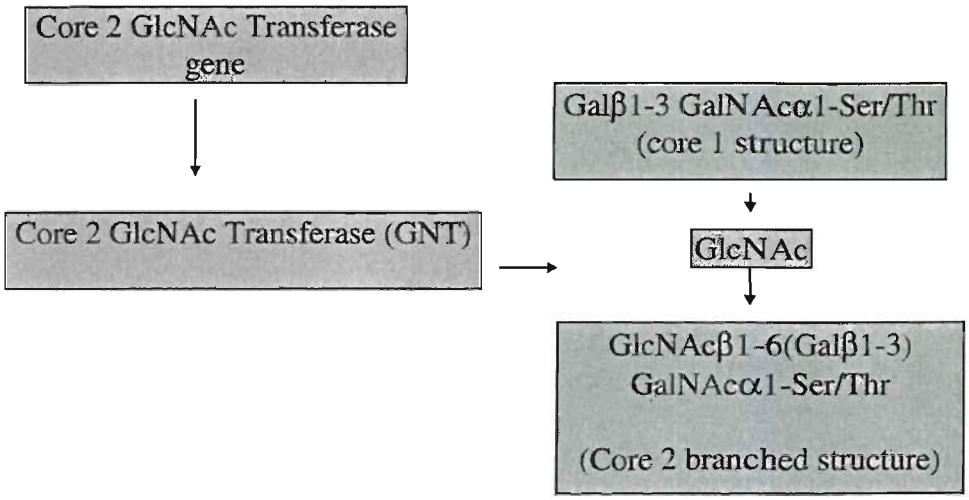
Additional support for the role of core 2 transferase in leukocyte-P-selectin binding was discovered by Ellies et al., (1998) and Maly et al., (1996), who both found that leukocytes from mice deficient in either core 2 transferase or FucT-VII only bind weakly to P-selectin (Ellies et al., 1998, Maly et al., 1996). These studies also found that leukocytes deficient in core 2 transferase were unable to successfully bind to E- or L-selectin.

1.18 Core 2 transferase [β1,6-GlcNAcT] - Function and Isoforms

It is proposed that core 2 transferase branching enzymes lead to changes in the expression of the o-linked oligosacchardies and hence, the adhesion to endothelial cells (Laskey, 1992). Core 2 transferase is an enzyme emanating

from the Golgi apparatus that substitutes core 1 o-linked glycans to produce core 2 structures (i.e. Galβ3{GlcNAcβ1-6}GalNAcα) [Figure 1.18.1].

Figure 1.18.1 **Core 2 transferase production**



Explanatory Notes: Core 2 GnT catalyses the transfer of β-GlcNAc to the core 1 structure, Galβ1-3 GalNAcα1-Ser/Thr of O-linked glycoproteins to make the core 2 branched structure GlcNAcβ1-6(Galβ1-3)GalNAcα1-Ser/Thr.

This enzymatic process is an important "curb" in the structuring of the o-linked oligosacchardies and influences their subsequent intercellular signalling, development and potential survival (Piller et al., 1988). To date, three types of core 2 transferase have been cloned and categorised as core 2 GnT1, 2 and 3. As yet, it has not been discovered which forms of core 2 transferase are responsible for the biosynthesis of glycoproteins (Schwientek et al., 1999, Yeh et al., 1999, Schwientek et al., 2000, Sekine et al., 2001).

Recent studies by Sperandio et al (2001) and Snapp et al., (2001) have demonstrated that in core 2 transferase deficient mice, there is an impaired P- and E-selectin mediated leukocyte rolling, causing reduced adherence. Consequently, it is tempting to propose that core 2 transferase may have a key role in mediating leukocyte / endothelial adhesion. Works by Chibber et al., (2000) appear to support this theory, as their studies have shown increased core 2 transferase activity in the leukocytes of individuals with diabetes, as well as a significant relationship between elevated core 2 transferase and increased leukocyte / endothelial adhesion generally.

In a recent study by Sperandio et al., (2001), core 2 transferase has been positively associated with endothelial-L-selectin binding. The results of this investigation demonstrated that L-selectin rolling of leukocytes was absent in core 2 knockout mice in TNFα treated and untreated venules, but not in control mice. This establishes positively that core 2 transferase modification is required for L-, P- and E-selectin ligand function (Jung et al., 1998, Knibbs et al., 1996, Melder et al., 2000). Furthermore, endothelial L-selectin ligands are completely dependent on core 2 transferase modification (Sperandio et al., 2001). Interestingly, it has now been intimated that leukocyte-leukocyte interactions are mediated by L-selectin ligands and therefore, are core 2 transferase dependent (Jung et al., 1998). Hence, increased up regulation of core 2 transferase would potentially not only lead to elevated leukocyte / endothelial rolling, but also a raised level of leukocyte-leukocyte adhesion. This would furthermore facilitate stasis, as it exercises its ability to act on all three selectin ligand functions simultaneously (Knibbs et al., 1996, Jung et al., 1998).

2.0 METHODOLOGY

2.1 Socio-Economic Bearing

The socio-economic relationship between secondary complications of diabetes has proved to be relatively elusive. However, study has shown that those living in lower socio-economic conditions are more likely to experience complications (Bott et al., 2000). This particular piece is primarily based upon establishing links between the sequencing of AGE products / free radical generation and ulceration within the same social / ethnic / geographic strata. However, if it could be accurately shown that a limited dietary intake of antioxidants may potentially place the diabetic individual at risk, this would dramatically elevate previous nutritional studies (Ford et al., 1994, Weng et al., 2000, Harris, 2001, Ceriello and Sechi, 2002, Gulliford et al., 2003). Consequently, diet may prove to be of major medical and political value in the future treatment of diabetic individuals.

Whilst this aspect of the study was considered to be relevant to diabetic ulceration per se, it was seen as impractical to study this particular dimension. This was due to the restricted geographical location of the observed study groups. Generally, participants were canvassed within the south Dorset region, which demonstrates very little ethnic diversity and may be regarded as prosperous. Hence, although the limitations of this study were recognised pan-culturally, it was felt that data obtained with regard to core 2 transferase activity would actually have greater validity, in light of the relatively high economic status of the sample population.

2.2 Free Radical Ischaemic Tissue Damage

Studies by Giugliano et al., (1995) and Cannon (1998) have demonstrated the potential role of reactive oxygen species in atherosclerosis, hypertension, hypercholesterolaemia and endothelial dysfunction (Nassar et al., 2002). However, the precise alteration to the redox balance still remains unclear, as the findings remain inconclusive (Way et al., 2001). This apparent confusion has been brought about due to the mixed results of initial project phases, some of which indicted that NO production / synthesis is reduced, whilst Mayhan and Patel (1998) found the contrary. Nevertheless, there remains a general consensus that one particular long-term effect of diabetes is to reduce the bioavailability and response to NO (Jude et al., 2001).

Consequently, investigations have revealed that other reactive oxygen species are potentially implicated in the destruction or reduced bioavailability of NO (Nassar et al., 2002). This has been substantiated by the beneficial effects of superoxide dismutase, catalase (a scavenger of hydrogen peroxide) and deferoxamine (a hydroxyl scavenger), which have demonstrated the ability to notably reverse endothelial dysfunction (Hattoria et al., 1991, Tesfamariam and Cohen, 1992). Alterations within the diabetic state have been further highlighted by investigations, which have recorded that antioxidants, such as vitamin E, glutathione and ascorbic acid were reduced in the blood and tissue of diabetic animals (Wohaieb and Godin, 1987, McLennan et al., 1988).

Hyperglycaemia has been implicated as a causal factor for the development of many diabetic complications through oxidative stress (Yagihashi, 1995). The

potential relationship between hyperglycaemia and diabetic complications is further strengthened by the high rate of neuropathy, retinopathy, peripheral vascular disease and nephropathy in Type II diabetes (DCCT Group, 1993, UKPDS Group, 2000, Clarke et al., 2003).

The oxidation of glucose leads to the liberation of ketoaldehydes, free radicals and superoxide (Gillery, Monboisse and Maquat, 1988). In the diabetic state, the disposal of these substances is impaired due to the reduced levels of endogenous antioxidants (Anggard, 1994). Consequently, the potential for a catalyst reaction is established, as the generated free radicals interact with and promote further reactive oxygen species (Dickinson et al., 2002, Sytze van Dam, 2002, Urata et al., 2002). This chain reaction has the further ability to interact in the formation of glycated proteins (terminally becoming Advanced Glycation End-stage products [AGE's]), thereby altering tissue structure (Singh et al., 2001). Moreover, glycated proteins themselves generate superoxide, thus setting up the mechanism for positive feedback (Forbes et al., 2001, King, 2001).

The catabolism of glucose via the polyol pathway leads to an increased conversion of sorbitol to fructose, which may correspondingly increase the cytosolic, NADH/NAD+ (Nassar et al., 2002). This condition is termed "hyperglycaemic pseudo-hypoxia", which in turn, favours the production of superoxide by hydroperoxidases (Williamson et al., 1993). This process is pivotal to the immune response of neutrophils. However, the potential switch to a pathological process is compounded by the discovery that leukocytes display an increased adherence to the vascular endothelium, so that the cytotoxic

contents of the cell could be delivered directly onto the vascular lining in a form of autoimmune attack (Chibber et al., 2003).

2.3 Nitric Oxide and Superoxide Measurement

Accurate capture and measurement of these reactive oxygen species is extremely complex and the selection of an appropriate method continues to provoke debate (Farias-Eisner et al., 1996). The primary limitation for the proposed measurement of absolute levels has been attributed to the rapid interaction between these two species, potentially causing a false negative (Mayhan and Patel, 1998). Moreover, access to facilities with the capability to conduct this level of testing has been barred due to financial implications.

Hence, this project will not incorporate testing of this nature, as sufficient documentation exists to support the theory of altered nitric oxide / superoxide balance within the diabetic state and the subsequent induction of oxidative stress (Graier et al., 1997, Schaeffer et al., 1999). Indeed, the benefit of such existing work allows the conceptualisation of an alternative thesis to develop, as it seeks to act as a bridge between previous islands of research.

2.4 Advance Glycated End-stage (AGE) Products

The accumulation of AGE products has been implicated in numerous studies in the pathogenesis of diabetic vascular complications and more recently, in the aetiology of diabetes (Hammes et al., 1999, Tooke and Goh, 1999, McEneny et al., 2000). However, the relationship between AGE products, cognitive

function and ulceration remains as yet, unclear. Previous studies have shown that hyperglycaemia and the polyol pathway lead to the formation of key AGE markers, i.e. pentosidine and N-(carboxymethyl)lysine, thus glycoxidation and glycation are implicated in their formation (Rumble et al., 1996, Farbound et al., 1999). Similarly, the body's apparent inability to remove these structures allows a slow accumulation to occur, adding to existing oxidative stress (Dickinson et al., 2002).

The relevance of vascular and tissue damage within the foot is also mirrored within the brain, as widespread AGE accumulation can occur, leading to potential atherosclerotic changes within the cerebral arteries (Shibata et al., 1999, Munch et al., 2003). Furthermore, recent research within the field of Alzheimer 's disease has revealed that AGE products are present within the amyloid plaques of affected individuals at post-mortem (Stewart and Liolitsa, 1999). Consequently, future works may explore methods to combat the oxidising effect of these structures, hopefully benefiting, albeit indirectly, diabetic research.

2.4.1 **AGE Product Measurement**

Laboratory facilities to measure AGE structures appear to be limited, with the website "Assay Finder" yielding only one institution in the UK, the Royal Liverpool Infirmary. Alas, investigation of this facility revealed the need for generous funding. Moreover, given the geographical distance between sample collection and assay testing site, the potential scope for delay and misadventure was adequate cause for concern. Hence, an alternative location was sought to

replicate the required assays. Communication with the Biochemistry Department at Poole Hospital NHS Trust confirmed that there was adequate potential to conduct the assays locally, given the availability of written protocols. Unfortunately, this line of enquiry was eventually exhausted, as the protocols exceeded the time and space available to the department.

Similarly, the nutritional laboratory at the University of Southampton was also capable of performing the relevant assay, but this facility was unavailable due to existing research commitments. Finally, this issue was resolved at a meeting with Dr. Chibber, St. Thomas's Hospital, London.

It was advised that blood serum AGE product levels could not directly predict tissue saturation levels. Consequently, direct extrapolation of AGE assay data was not sufficiently accurate to comment upon surrounding tissue levels in their promotion of an environment ripe for ulceration. Based upon this level of advice, the measurement of AGE products was rejected in favour of core 2 transferase, an emergent topic in diabetic medicine (Section 1.18).

2.5 Core 2 Transferase

Leukocytes adhere to the vascular endothelium through selectins and integrins (Kunkel and Ley, 1996, Hempel et al., 1997, Kunkel and Ley, 1998). Such trafficking is achieved by o-linked oligosaccharides (glycoproteins / glycolipids) found on the surface of the leukocytes themselves (Chibber et al., 2000). It is intimated that core 2 transferase leads to changes in the expression

of the o-linked oligosaccharides and accordingly, increased adhesion to endothelial cells (Lasky, 1992).

The core 2 transferase enzyme emanates from the Golgi apparatus, which substitutes core 1 o-linked glycans to produce core 2 structures, i.e. Galβ3{GlcNAcβ1-6}GalNAcα (Dalziel et al., 2000). This enzymatic process is an important "governor" to the structuring of the o-linked oligosaccharides, influencing their intercellular signalling, development and potential survival (Piller et al., 1988). Recent studies by Sperandio et al., (2001) and Snapp et al., (2001) have demonstrated that in core 2 transferase deficient mice, P- and E-selectin mediated leukocyte rolling is sufficiently impaired, so as to cause reduced adherence.

Seemingly, core 2 transferase is instrumental in mediating leukocyte / endothelial adhesion. Study by Chibber et al. (2000) support this theory, showing increased core 2 transferase activity in the leukocytes of individuals with diabetes, whilst positively linking elevated core 2 transferase levels to increased leukocyte / endothelial adhesion.

As yet, the mechanism responsible for the increased expression of core 2 transferase in diabetic leukocytes remains undetermined. Nishio et al., (1995) did, however, find that hyperglycaemia modulates core 2 activity in rat cardiomyocytes via the diacylglycerol-PKC pathway. PKC activation has also been highlighted in an additional study by Chibber et al., (2003), demonstrating that the administration of a PKCβII specific inhibitor LY379196 (0, 25, 50 nM: Eli Lilly, USA) attenuated endothelial cell adhesion. This work has furthermore

linked increased leukocyte / endothelial cell adhesion to capillary occlusion in diabetic retinopathy through the phosphorylation of core 2 enzymes by PKCβII.

2.5.1 **Core 2 Transferase Measurement**

The assay for the measurement of core 2 transferase has been successfully utilised by Yousefi et al., (1991) and Chibber et al., (2000) within the spheres of oncology, leukocyte adhesion and diabetic retinopathy research. However, the validity of this assay is difficult to question objectively, given the inherent absence of literature supporting a viable alternative. Nonetheless, the method was adjusted in accordance with Donovan et al., (1999) to achieve a coefficient of variance for positive controls of 9.4%. Moreover, paired groupings were not adopted in this phase, given that all blood samples were subjected to a positive / negative [absence / presence of acceptor] enzyme adhesion comparative protocol (Chibber et al., 2000). Accordingly, each subject sample underwent a double simultaneous analysis. Whilst it was recognised that intra-reliability ameliorations may have been preferential, it was deemed necessary to adhere to validated protocols for the purposes of this study.

The further employment of the Biorad assay (Biorad, Hertfordshire, UK) negated any concerns as to possible eccentricities within absolute protein concentrations, when compared to the nominal core 2 transferase value. Indeed, the assay automatically adjusts for leukocyte presence, producing a data-log of the key events. As shown in Table 2.5.1.i, whilst blood protein remains a prime variable, it is actually enzyme reactivity, which determines absolute core 2 transferase:

Table 2.5.1.i **Equation for Core 2 transferase**

PROTEIN (a)	ADJ. LOG (b)	BIORAD VALUE (c)	+ ENZYME (d)	- ENZYME (e)	VALUE (f) [d-e] . 200 . 56	VALUE (g) [f / c]	CORE 2 (g / 23)
2.2469	14.2692	214.0386	296	55.2	2696960	12600.34	547.84
2.6271	16.8672	253.0089	292	79	2385600	9428.92	409.95

Extracted from real-time data captured during instruction at Guy's Hospital, London on 09 September 2002

Of note, access to this assay test was obtained through Chibber and Mahmud of Guy's Hospital, Cardiovascular and Neurological Research Department, who further provided the additional benefits of personal training in laboratory techniques and associated research methods.

2.6 Design Considerations

2.6.1 Duration of Diabetes

The duration of Type II diabetes was recorded for each of the participants to ascertain the degree of association with core 2 transferase specificity. However, it should be noted that the official "duration" is not necessarily a true testament of diabetic onset, as cases may remain undetected for up to seven years (Zimmet, 2003).

2.6.2 **Geopolitical Bias**

Three distinct study groups were recruited from the electronic database of patients attending the Diabetes Centre at Poole Hospital NHS Trust. It was recognised that this method of single site recruitment would not give a directly representative selection of the UK diabetic population. Indeed, the hospital adopted as the data collection site serves a population of minimal socio-economic variation. Nevertheless, the influence of this factor over the variables being investigated was thought to assist in actually reducing the risk of bias. This is a particularly important issue, if socio-economic status and diet are considered, with specific reference to antioxidant bioavailability and the presence of diabetic ulceration (Lindquist et al., 2000).

2.6.3 **Pilot Study – Sample Size**

Although Study I (n=7) was accepted as being small, the rationale for this design was based upon two factors. Firstly, no previous studies into this area have been published, hence power calculations / statistical analyses were not feasible. Secondly, given the significant outcomes of parallel diabetic retinopathy studies (Chibber et al., 2000, 2003), a relatively small population would at least allow potential trends to be highlighted. With the resultant data, power calculations were retrospectively performed to determine the number of participants required in Study II for statistical merit.

2.6.4 **Retinopathy Screening**

Retinopathy is one of the commonest complications of diabetes and the biggest single cause of registered blindness in the UK, within the working population (Evans et al., 1990). Indeed, it has been reported by MacLeod et al., (1988) that up to 10% of diabetic individuals will have retinopathy at any one time. Consequently, the detection methods employed by the Diabetes Department, Poole Hospital NHS Trust are in line with the National Screening Committee and Health Technology Board for Scotland's recommended screening programme (Olson et al., 2003). Hospital guidelines further outline that all diabetic individuals receive annual retinopathy screening, unless abnormalities are detected, at which point, checking frequency is increased and/or treatment undertaken.

In view of existing work by Chibber et al., (2000), who demonstrated elevated core 2 transferase levels in diabetic individuals with retinopathy, a condition often concurrent with polyneuropathy, all participants with this particular complication had to be excluded, in order to produce a "sanitised" population. Therefore, all potential participants were required to have undergone slit-lamp biomicroscopy performed by a qualified optician within the last 6 months. To meet the inclusion criteria for this study, individuals had to score 0 on the grading scale, which was modified from the Early Treatment Diabetic Retinopathy Study (Olson et al., 2003). A copy of the grading sheet is included at Appendix A.

2.6.5 **Vascular Screening**

Diabetic individuals with recorded incidents of stroke were deliberately excluded from this project, as the presence of macrovascular disease may affect the manifestation of ulceration and/or the biochemical markers being investigated (Mantey et al., 2000, Murphie, 2001, Jeffcoate and Harding, 2003, Wrobel et al., 2003). The absence of Peripheral Vascular Disease was verified by the Ankle Brachial Pressure Index method (McLeod-Roberts, 1995).

Ankle Brachial Pressure Index (ABPI) was first described in the 1960's, as cited by McLeod-Roberts (1995). The ABPI provides a good indication of the presence of ischaemia in the lower limb (Ameli et al., 1989, Davies, 1992). Indices are derived, by recording the systolic pressure at both the brachial and posterior tibial artery. Thereafter, the ABPI is calculated thus: ankle ÷ brachial [expressed as a decimal] (Merriman and Tollerfield, 1996). The average values for healthy adults lie between 0.98 - 1.31 (Davies, 1992). It has been suggested that values of less than 0.75 are indicative of severe obstructive problems, whilst readings of 0.5 and below are critical, in terms of healing to lesions or ulcers (Faris, 1991). As Monckeberg's sclerosis can lead to the false elevation of readings, any apparent trophic change, i.e. absence of hair, skin pallor, temperature of skin and tissue vitality, was also acknowledged.

2.6.6 **Medication History**

Participants within both diabetic groups were observed to take augmentary medication, chiefly statins and aspirin, within their respective diabetes

management regimes. Whilst there was no attempt made to control for this particular variable, as such intervention would have been highly unethical, the various types, dosages and possible interactions were recorded (Table E-2). Accordingly, it was recognised that statins, in particular, might reduce the scope for potential co-effects between dyslipidaemia and core 2 transferase. However, the risk was accepted as being minimal within the confines of this study.

2.7 Metrology Standards

Although diabetic peripheral neuropathy remains a common complication of diabetes mellitus, there is no national or international standard for screening this degenerative condition (Young and Matthews, 1998, Booth, 2000). However, for this research piece, it was determined that existing clinical tools should be employed to ensure data hysteresis and moreover, to validate their effectiveness within a clinical setting.

2.7.1 Vibration Perception Thresholds (VPT's)

The neurothesiometer uses the principle of a battery operated oscillator set at a nominal frequency of 50 Hz inserted into a power amplifier. The amplifier is voltage adjustable by means of a rheostat, the resultant output terminating at a vibrating probe. As the probe is placed on the skin, the input voltage can be adjusted incrementally up to a maximum of 50 V or until vibration is detected by the patient. Of note, the unit is factory calibrated for both amplitude and excursion and cannot be manually overridden.

The reliability of this particular piece of equipment has rarely been reported on, with the notable exception of Fagius et al., (1981) and Bloom et al., (1984). Even so, both parties found no significant repeatability error, its popular field use being ready testament to its overall capability (Murray et al., 1996, Coppini et al., 1998). With this in mind, the same neurothesiometer was used throughout this study and fully charged overnight prior to each testing session, assuring reliable data acquisition.

Extensive use of aesthesiometry has been made to ascertain vibration perception thresholds (VPT's), namely the quantification of the subject's perception to vibratory sensation (Booth, 2001). The validity of VPT's has been demonstrated by several studies, which have successfully confirmed neuropathy against nerve conduction velocities in both laboratory and clinical environments (Boulton et al., 1986, Sosenko et al., 1999, Pham et al., 2000). The importance of VPT screening was further demonstrated by Young et al (1994), who were able to calculate that diabetic individuals displaying a VPT > 25 V were seven times more likely to develop ulceration than those with a VPT rating of ≤ 15 V.

The sensitivity of VPT's has been further enhanced by Coppini et al., (1998), after the data produced was placed contextually against "normal" age related deterioration in nerve function. The result of this study was the generation of an algorithm, which provides a standard deviation of the subject's VPT from the mean or "normal" reading. This dimension of age compensation was of potential use for this project, as the target population was increasingly over the age of 55 years. Consequently, the ability to discriminate between normal

physiological deterioration and that related to diabetic neuropathy was seen as extremely beneficial.

Figure 2.7.1.i

Algorithm to calculate age adjusted vibration perception thresholds

$$SD\ Score\ (age\ adjusted\ vpt) = \frac{(\log_{10} VPT_0) - (slope * age) + intercept}{SD^2}$$

Where:

SD score	=	age adjusted VPT
VPT_0	=	observed reading of VPT on neurothesiometer
log10 VPT0	=	logarithm of observed VPT reading
slope and intercept	=	constants derived from linear fit of VPT data to age, collected from normal subjects
SD	=	residual standard deviation of the normalised values
Age	=	age in years

(Coppini et al., 1998)

However, whilst this algorithm is valid within nerve deterioration profiling, it is not widely used in a diabetic clinical environment. Furthermore, this particular equation was configured using the biothesiometer and not the more commonly employed neurothesiometer (Bril and Perkins, 2002). Whilst Young et al., (1993) have demonstrated a positive correlation between the two devices ($p<0.01$), no additional research has since been conducted using the algorithm forwarded by Coppini et al., (1998) and the neurothesiometer. Consequently,

the > 25 V threshold for diminished sensation (Edmonds and Foster, 2000) ascertained by neurothesiometer was adopted. Accordingly, VPT's with both the neurothesiometer and biothesiometer have historically been tested at the pulp of the hallux and the mean value recorded (Deursen et al., 2001, Pham et al., 2000 and Fryberg et al., 1998, Young et al., 1993).

2.7.2 **10 g Monofilament Detection**

Monofilaments comprise a length of extruded polymer, usually polyamide, which is mounted in a handle and yields (buckles) under a linear force of a given value (Booth, 2001). In practised hands, monofilaments with a 10g resistance are capable of discriminating between subjects at low and high risk of ulceration (Birke and Rolfson, 1997, Litzelman et al., 1997). However, differences in crystalline structure, fibre length and elasticity amongst polyamide grades, can affect the "break away" force, at which the filament buckles (Timonshenko and Young, 1962). Moreover, polyamide is extremely hydroscopic and is therefore susceptible to changes in humidity, which may adversely affect its nominal mechanical properties (Brydson, 1975).

Nevertheless, the use of monofilaments as a "practical" measurement of neuropathy has traditionally yielded consistent results (Booth and Young, 2000). Various pieces of research maintain that the inability to detect a 5.07/10g monofilament is a sufficiently valid measure of neuropathy, presenting the opportunity to identify individuals at risk of ulceration (Kumar et al., 1991, Sosenko et al., 1999, McGill et al., 1999). However, previous studies have recorded a degree of variability in the number of sites and location tested (Padua

et al., 2002, Perkins et al., 2001, Lunetta et al., 1998, Rith-Najarian et al., 1992). Despite some disparity in the number of sites tested, the hallux and metatarsal heads (1^{st}, 2^{nd} 3^{rd} and 5^{th}) remain consistent themes, as they correlate with the areas most frequently associated with high pressure loading (Paisley et al., 2002, Veves et al., 1992, Holewski et al., 1988).

Given the theory of compromised cell structure, it was considered necessary to test all metatarsal heads, as well as the hallux. McGill et al., (1999) also viewed this as the method of choice with the 10g monofilament. However, in doing so, it was realised that this approach would potentially capture individuals previously regarded as non-neuropathic. In view of this increased risk of bias within the diabetic cohorts (cross-contamination), it was determined that a minimum 80% detection rate for non-neuropathic classification should be set, with VPT acting as a further control. Furthermore, this method of filtration also offered scope for the early detection and implementation of prevention strategies (Edmonds et al., 1986), as cited by McGill et al., (1999).

Alternative studies have proposed that an inability to detect the 10g monofilament at any callous-free site is indicative of "high risk", in terms of ulceration (Pham et al., 2000, Edmonds and Foster, 2000, McNeely et al., 1995). Similarly, it has been reported that abnormal perception can be indicated by a detection rate of < 8/10 [< 80% - 5 sites per foot] (McGill et al., 1999, Sosenko et al., 1999). Accordingly, a single undetected site per foot would be sufficient to indicate the presence of peripheral neuropathy. Therefore, given the differences in peer opinion, the selection of VPT measurement, harnessed to 10g

monofilament testing was regarded as the most satisfactory method for the effective diagnosis of diabetic polyneuropathy (Booth, 2001).

2.7.3 **Classification of Neuropathic Ulceration**

Classification of ulceration was based upon the Wagner ulcer system, which has been validated by Oyibo et al., (2001) and was in service within the Diabetes Department of Poole Hospital NHS Trust. However, a modification of this tool was made, in order that the integrity of the wound was not disturbed. Consequently, the depth of the lesion was not probed, classification being made on a purely observational basis for this dimension. This decision was taken in light of the fact that the actual depth of the lesion was not considered relevant for this study. Hence, undue disturbance of the wound was considered wholly unnecessary.

2.8 **Biometrics**

Biometric data was also recorded for each participant, so that observation of the "between group" and "between subject" design could be adhered to. The selection of these variables was supported by historical literature, implicating certain of these markers in diabetic neuropathy and ulceration (Boulton et al., 1983, Wiles et al., 1991, Unwin, 2000). Consequently, analysis of the key factors was undertaken to ensure that any deviation could be correctly attributed.

The following variables were recorded:

- Gender
- Height
- Weight

In particular, gender was seen as an important variable, following evidence that ulceration is more prevalent in males (Reiber, 1996). Thus said, height and weight bias was also considered, to avoid any possible eccentricity in the data. Notably, Bloom et al., (1984) and Wiles et al., (1991) demonstrated an increased VPT rating in taller neuropathic individuals relative to their age matched controls. Hence, the need to ascertain both height and weight for later comparison, particularly in light of the purported increased risk of ulceration, was acknowledged within the design (Boulton et al., 1983).

2.9 Blood Chemistry Analysis

In line with the recommendations of the Diabetes Control and Complications Trial [DCCT] (1993) and UK Prospective Diabetes Study (1996), glycated haemoglobin values, glucose levels, lipid profiles and creatinine function are utilised by the Diabetes Department, Poole Hospital NHS Trust, as potential markers for the associated complications of diabetes, i.e. neuropathy, nephropathy and retinopathy. Consequently, this form of blood and biochemical analysis is a Standard Operating Procedure (SOP) within the clinical treatment plan. Given these factors, it was considered that this apparently "free of charge" service should be exploited to determine whether any association between existing biochemical markers and core 2 transferase expression is present. Similarly, as qualified laboratory staff undertook the

analysis, it further negated the need for specialist training and moreover, assured non-biased results.

2.9.1 **Biochemical Assays**

i. Cholesterol Measurement

The test was performed using the Olympus System Reagent OSR 6116 assay to determine cholesterol levels after enzymatic hydrolysis and oxidation, as demonstrated by Trinder (1981) and Richmond (1973).

ii. HDL Measurement

HDL testing was performed using Olympus System Reagent OSR 6187 direct measurement. This test protocol has been shown to quantify HDL levels in the presence of the enzyme chromogen system (Warnick et al., 1995)

iii. LDL Measurement

The Olympus OSR 6516 sorbitol dehydrogenase assay directly measures the LDL content.

iv. HDL / Cholesterol Ratio Measurement

Measurement of the HDL / Cholesterol ratio is not a direct assay, but is performed using the values from the cholesterol measurement divided by the

HDL value and is expressed as a ratio.

v. Triglyceride Measurement

The Olympus System Reagent OSR 6133 assay was used to directly measure triglycerides after enzymatic hydrolysis with lipases (Jacobs and Van Demark, 1960, Koditschek and Umbreit, 1969, Trinder, 1981).

vi. Creatinine Measurement

The Olympus System Reagent OSR6178 assay used picric acid to form a creatinine picrate complex. Of note, the rate of change in absorbance is directly proportionate to the creatinine concentration (Cook, 1971, Larsen, 1972).

vii. Blood Glucose Measurement

The Olympus System Reagent OSR 6121 assay employs the principle that glucose is phosphorylated in the presence of hexokinase and Adenosine Triphosphate (ATP). The subsequent glucose-6-phosphate reaction in the presence of glucose-6-phosphate dehydrogenase forms gluconate-6-phosphate and NADH. Consequently, the increase in absorbance is proportional to the glucose concentration (Bonder and Mead, 1974).

viii. Glycated Haemoglobin (HbA1c) Measurement

This test utilises an automated ion-exchange chromatography technique in accordance with DCCT recommendations. Reproducibility is expressed by the coefficient of variation, i.e. the repeat measurement of the same sample 20 times and calculation of the mean and standard deviation. Results are expressed as a percentage of the final calculation (SD ÷ Mean). A cut-off point of < 4% has been selected by the Haematology Department at Poole Hospital NHS Trust, in accordance with the assay principles (Colman et al., 1997).

2.9.2 **Blood Chemistry Analysis Equipment**

Blood chemistry analysis for creatinine levels, HDL's, LDL's, HDL / Cholesterol ratio, blood glucose, triglycerides and cholesterol was performed using an Olympus AU640 analyser (Olympus Diagnostics GmbH, Hamburg). Glycated haemoglobin was tested by reversed-phase chromatography, using a Biomen 8160 (A. Menarini Diagnostics, Italy).

The Haematology and Biochemistry Departments within Poole Hospital NHS trust are aligned to the standards identified by the Diabetes Control and Complication Trial (DCCT). Similarly, all specialist equipment is accredited by the International Federation of Clinical Chemistry (IFCC). The selection of this resource was further regarded as necessary, as the initial "spinning down" prior to the core 2 transferase assay had to be undertaken within two hours of

collection, to ensure sample preservation. Therefore, if only from a logistical standpoint, the use of in-house laboratory facilities was a natural decision.

2.9.2.i **Olympus AU640 (Olympus Diagnostic GmbH, Hamburg)**

The following information was kindly supplied by Olympus Diagnostic GmbH (Ireland).

a. Standardisation

Olympus standardises chemistry immuno testing from 400 to 6,600 tests per hour.

b. Assay Timings

Up to 800 photometric tests per hour and up to 1200 tests per hour with electrolytes.

c. Walk Away Design / Capacity

- 150-sample rack loading for peak workloads
- 22-sample carousel for STAT
- Direct tube sampling for 3.0, 5.0, 7.0, 10.0 ml primary tubes and paediatric cups
- Mixed bar code capability
- Automated reflex and repeat testing

- Automated pre-dilution for urine and other specimens
- Easy connectivity for automation

d. Automated Reagent Handling

- All Olympus reagents are liquid and in a ready-to-use preparation
- Calibration system stores up to 5 calibration curves per test
- Automatic changeover from empty to fresh bottle

e. Review of Results

- Choice of QC trending: Westgard multi-rule procedure, twin plots, Levey-Jennings daily and day-to-day plots
- Search function to retrieve data by individual sample and type of test (up to 30,000 patient files)
- User-definable flags for abnormal results

f. Reliability and Accuracy

- Capacitance liquid level detection to ensure accurate dispensing of reagents and specimens
- Clot detection and crash prevention for probes
- Precision optics

g. Windows NT® Software

- Drop-down menus, icons, colour-keyed graphics
- User menu to customise operation
- Operator's guide with links, search, and bookmarks

h. Maintenance

- Programmable start-up
- Automated washes and checks

2.9.2.ii **Biomen 8160**

The Biomen 8160's key features were kindly supplied by A. Menarini Diagnostics, Italy:

a. 2.9 minutes per sample in "Diabetes" mode and 4.2 minutes per sample in "Thalassaemia" mode
b. Measures up to 100 samples, plus continuous loading
c. Highly accurate
d. Automatic calibration procedure
e. Level sensors prevent reagent shortages
f. Built-in bar code reader
g. HbA1c results not interfered with by carbamylated, acetylated haemoglobin or labile HbA1c
h. Automatic adjustment of dilution ratio to compensate for anaemia

A full specification is available at Appendix G.

Figure 2.9.2.ii.1 **Biomen 8160 analyser**

2.10 Linear Regression

Linear regression attempts to model the relationship between two variables by fitting a linear equation to the observed data. One variable is considered to be an explanatory variable, whilst the other is considered to be a dependent variable. Prior to fitting a linear model to the observed data, the relationship between the variables of interest must be determined. The numerical association between two variables is the Pearson "correlation coefficient".

A linear regression line has the equation ($Y = a + bX$), where X is the explanatory variable and Y is the dependent variable. The slope line is b, whilst a is the intercept (the value of y, when $x = 0$).

2.10.1 **Least-Squares Regression**

The most common method for fitting a regression line is the method of least-squares. This method calculates the appropriate line for the observed data by minimising the sum of the squares of the vertical deviations from each data point. As the deviations are initially squared and summed, there are no cancellations between positive and negative values.

To fit the model to the observed data, the computed regression line is plotted over the actual data points to evaluate the results. Points lying far from the main cluster are known as "outliers" and depending on their location, may have a major impact on the regression line.

2.10.2 **Outliers and Influential Observations**

Outliers may be representative of erroneous data or may indicate a poorly fitting regression line. If a point lies far from the other data in the horizontal direction, it is known as an "influential observation". This distinction is made, as these points may have a significant impact on the slope of the regression line.

2.10.3 **Residuals**

Once a regression model has been fitted to a group of data, examination of the residuals (the deviation from the observed values) allows investigation into the linear relationship. Plotting the residuals on the Y-axis against the explanatory variable on the X-axis reveals any possible non-linear relationship among the variables and/or any lurking variables.

2.10.4 **Lurking Variables**

If non-linear trends are visible in the relationship between an explanatory and dependent variable, there may be other influential variables. A lurking variable exists, when the relationship between two variables is significantly affected by the presence of a possible third variable not included in the initial modelling.

2.10.5 **Extrapolation**

Whenever a linear regression model is fitted to a group of data, the data range should be carefully observed. However, the use of a regression equation to predict values outside of this range is often inappropriate. This practice is known as extrapolation. Consider, for example, a linear model, which relates weight gain to age for young children. The carry-over of such a model to adults would be inappropriate, as the relationship is not consistent throughout. Hence, some degree of common sense must be applied.

2.10.6 **Benefits of Regression Modelling**

Regression modelling is a powerful tool for predicting the relationship between independent scores, involving a linear transformation of the predictor variable into the predicted variable. Parameters are selected, so that the least squares criterion is met, resulting in an "optimal" model. Appropriate use of this tool can facilitate the prediction of both exact scores (point estimates) and/or intervals of scores (interval estimates). In the context of this particular study, combinations of existing clinical measurements are subjectively analysed, thereby providing a statistical validation of their predictive efficacy for core 2 transferase.

3.0 METHOD - STUDY I (PILOT)

3.1 Ethical Approval

Approval was secured by East Dorset Local Research Ethics Committee on 23rd March 2001 and allocated the LREC NO: 28/01/S (Appendix B).

3.2 Study Design

A between subject, cross-sectional design was adopted for the study comprising:

- Individuals without diabetes (control group, n=2)
- Individuals with diabetes, but without diabetic complications (diabetic control group, n=2)
- Individuals with diabetes and neuropathy (diabetic neuropathic group, n=2)
- Individuals with diabetes, neuropathy and ulceration (diabetic neuropathic sub-group, n=1)

3.3 Participant Recruitment

The initial approach for recruitment was based upon a database filter of potential participants attending the Diabetes Department, Poole Hospital NHS Trust. Those responding to the request for assistance were entered onto the test programme following a detailed patient interview, review of personal records (age, gender and diabetic history) and explanation of the study. During this initial phase of contact, the individual was actively given the opportunity to ask

any questions. Finally, informed consent was successfully obtained and documented for each recruit. An example of the patient information sheet is included at Appendix C.

3.4 Selection Criteria

All individuals with Type II diabetes mellitus were considered appropriate for this study. However, those with learning difficulties or unable to comprehend the patient information sheet were not selected, as the nature of consent would have been inappropriate.

Table 3.4.1 **Inclusion / exclusion criteria**

CRITERION	CON	DNC	DN	DN (U)
Type II Diabetes	×	✓	✓	✓
Peripheral Sensory Neuropathy	×	×	✓	✓
Retinopathy	×	×	×	×
Plantar Ulceration	×	×	×	✓
Aged 30-75 years	✓	✓	✓	✓
> 8 sites with 10g Monofilament	✓	✓	×	×
VPT ≤ 25 V	✓	✓	×	×
APBI > 0.9	✓	✓	✓	✓
Vitamin E Supplement	×	×	×	×
Pernicious Anaemia	×	×	×	×
Established Neurological Disorder	×	×	×	×
Alcohol Intake > 20 units	×	×	×	×

3.5 Preliminary Examination

All data collection during this phase was taken at the Diabetes Department, Poole Hospital NHS Trust, within the Diabetic Foot Clinic treatment rooms. On

each occasion, the same treatment bay was utilised in an attempt to standardise environmental factors, i.e. room temperature. Furthermore, whilst the preliminary examination was conducted, curtains surrounding the treatment bay were drawn. This action was undertaken for two reasons. Firstly, to preserve the patient's dignity at all times, in line with ethical guidelines. Secondly, to prevent other participants viewing the examination process in advance. This was particularly important in the neurological testing phase, as an element of prior knowledge or "pre-emption" may have otherwise distorted the results. A copy of the data collection form is attached at Appendix D.

3.5.1 **Retinopathy Screening**

Prior to data collection, the retinopathy status was confirmed by the supervising consultant, using an ophthalmoscope. Moreover, the same consultant was made available throughout the study to avoid any potential investigator bias.

3.5.2 **Vibration Perception Threshold - Protocol**

1. The nature of the test was explained
2. The participant was asked to close his/her eyes
3. The probe of the neurothesiometer was placed against a bony prominence of the participant's wrist. The vibration was increased, in order to condition the subject as to what the sensation felt like
4. The participant's hosiery was removed and the probe placed against the apex of the hallux
5. The participant was asked to say "yes", when he/she felt vibration in the toe

6. The vibration was increased using the output dial
7. The VPT as seen on digital display was recorded, once the participant said "yes" to detecting vibration

Steps 5-7 were repeated a further 2 times.

The three readings were entered separately onto the data collection sheet and the mean VPT calculated and recorded.

3.5.3 **10g Monofilament - Protocol**

1. The nature of the test was explained
2. The monofilament was applied to one's own hand three times before use, thus displaying to the participant that the nylon probe was not a needle or noxious stimulus
3. The monofilament was placed of the dorsal surface of the participant's hand to condition him/her, as to what the pressure sensation felt like
4. The participant was asked to close his/her eyes, so that detection was based upon light pressure and not influenced by visual senses
5. The participant was asked to say "yes", when he/she can felt the monofilament applied to the foot
6. The monofilament was applied to the apex of the hallux, until seen to deflect by approximately 1 cm. The monofilament was re-applied on the same site after a delay of approximately 5 seconds
7. Steps 5 and 6 were repeated on the plantar aspect of the first, second, third, fourth and fifth metatarsophalangeal joints avoiding any areas of callus

8. Participants' responses (positive/negative) were recorded on the data collection sheet

3.5.4 **Vascular Screening (non-invasive) - Protocol**

For ease, a Doppler (Huntleigh Healthcare) was utilised throughout the trial, in preference to a stethoscope.

(a.) **Brachial Pulse**:

1. The procedure was explained to the participant
2. The participant was placed in a supine position (5 minutes prior to testing commencing)
3. The brachial pulse was palpated
4. Doppler media gel was applied to the site
5. The cuff of the sphygmomanometer was wrapped around the selected arm of the participant, clear of the brachial pulse point
6. The Doppler was switched on and applied to the brachial pulse site
7. The pressure cuff was inflated, until the brachial pulse was no longer audible using the Doppler
8. The value of this pressure was noted
9. The pressure was released slowly and steadily, watching the mercury level fall. As the pressure fell to the level of the participant's systolic blood pressure, a beat was heard. The reading of the manometer was noted; this corresponded to the value of systolic blood pressure
10. The cuff pressure continued to be released

11.The cuff was fully deflated and removed

(b.) **Ankle (Posterior Tibial) Pulse**:

The protocol was identical to the above, with the exception that the posterior tibial pulse was palpated.

3.5.5 Ulceration – History and Recording

During examination, any measurement of ulceration included a written description of its outline characteristics, i.e. width, depth and site. This was undertaken with a sterile Opsite grid scale and rated using the Wagner Ulceration scale (Oyibo et al., 2001). All measurements were non-invasive and followed a strict non-contact technique during both the measuring phase and redressing of the wound. The patient's synoptic record (demographic and complications summary) was also checked for any history of ulceration within the last 12 months and recorded accordingly. To ensure that any incidence of ulceration within that time frame had not been overlooked (treatment in a primary care setting), the patient was asked to confirm any wounds to his/her feet in the last 12 months.

3.5.6 Height and Weight

Participants' heights were recorded using a Seca 240 wall-mounted measuring rod, with a range of 60-240 cm. Individuals were asked to remove their footwear prior to measurement to achieve an accurate reading. All height data

was collected using the same piece of equipment, thus avoiding any potential variation.

Weights were recorded using Seca electronic chair scales. The scales have an inherent calibrating mechanism and the individual's weight is displayed in kilograms on a discreet digital panel. Of note, this instrument is serviced annually by the Bristol Scale Service Company, thus ensuring that the system is fully accredited.

Participants' weights were recorded in the following manner:

- It was explained that a recording of participants' weights was required
- Individuals were asked to remove their overcoat, if applicable
- Individuals were asked to sit on the chair scales and remain as still as possible
- Once the reading had stabilised, the final value was recorded and the individual informed that they could stand up
- Participants' normal clothing was included in the "as weighed" result

3.5.7 **Data Storage**

All data was held at the Diabetes Department, Poole Hospital NHS Trust, in a locked filing cabinet, the access to which, was restricted to the researcher. Participants were also encouraged to review their individual records. However, no requests were forthcoming.

3.6 Blood Chemistry

After the initial clinical examination, each participant underwent venipuncture for the following primary markers:

- HbA1c's - Glycated haemoglobin levels (5 ml)
- Core 2 transferase activity levels (10 ml)
- Plasma cholesterol levels (5 ml)

All blood samples were passed to the Haematology and Biochemistry Departments at Poole Hospital NHS Trust. In accordance with standard hospital procedure, as well as IFCC and DCCT guidelines, the samples were loaded into the Olympus AU640 (Olympus Diagnostics GmbH, Hamburg) and the Biomen 8160 (A. Menarini Diagnostics, Italy) automatic analysers. The principle of each assay is outlined at Section 2.9.

Whilst the additional information generated (Section 2.9.2) was not utilised within the pilot study, it was nevertheless recorded and maintained. The Ethics Committee was informed of this modification and confirmation obtained for the use of this supplementary data. However, it was stipulated that this was granted only within the strict confines of this particular study.

3.7 Logistics

Preparation of samples, i.e. centrifugation and freezing to -20 °C, was conducted by staff from Poole Hospital NHS Trust, whilst the assay testing had

to be carried out at Guy's Hospital, London. The transportation and identification of samples was undertaken personally, ensuring that all assays were both blinded and free from third party interference. Following completion of the laboratory phase, the blood samples were disposed of in accordance with Guy's standard operating procedures to prevent subsequent data corruption.

3.8 Preparation of Blood for Core 2 Transferase Enzyme Analysis

10 ml of whole blood was drawn and collected in ethylenediamine-tetraacetic acid (EDTA) tubes and labelled using a proprietary alpha-numerical code. Thereafter, samples were transported in the EDTA tubes to the Haematology Department at Poole Hospital NHS Trust for polymorphonuclear leukocyte separation.

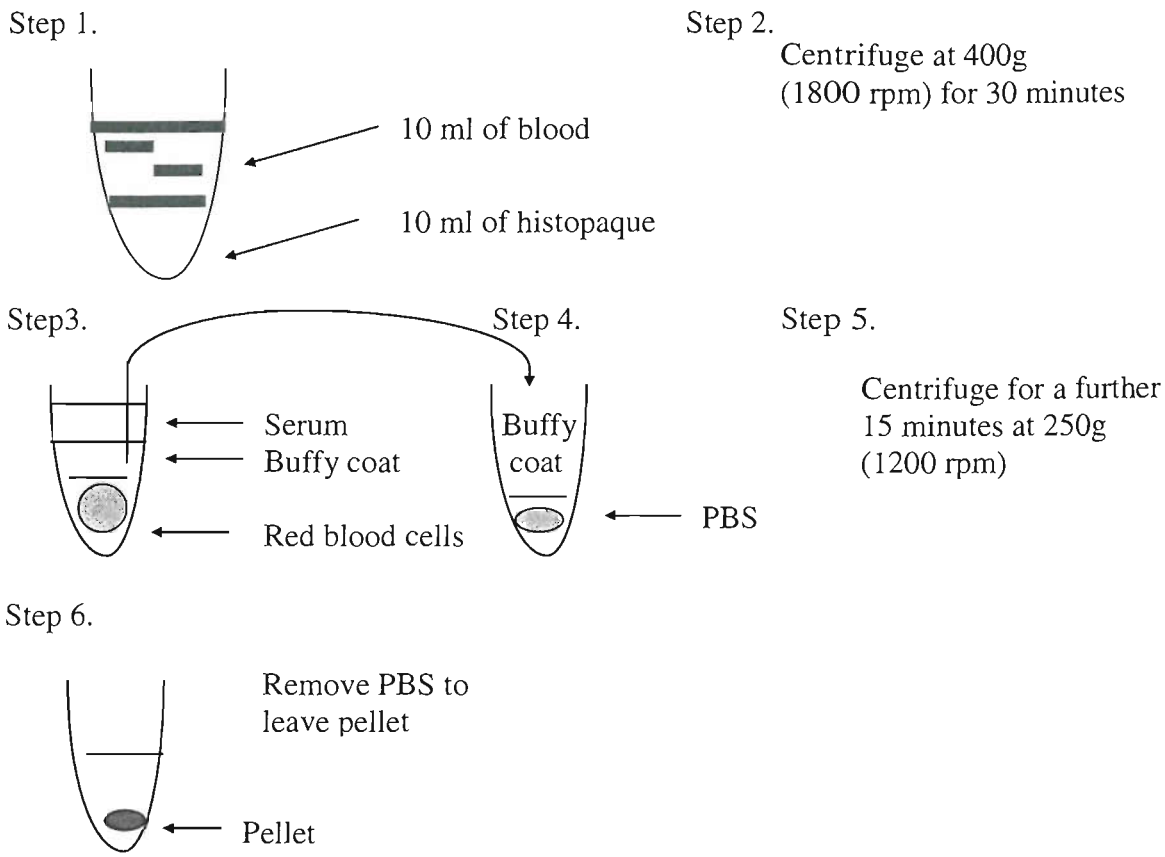
3.8.1 Polymorphonuclear Leukocyte Separation

The pure polymorphonuclear leukocyte (PMN) suspensions were prepared in the following manner:

10ml of whole blood was layered onto an equal volume of histopaque (Pharmacia) and centrifuged at 400g (1800 rpm) for 30 minutes at a nominal ambient temperature of 23 °C (RT). This resulted in a three layer strata of serum (top layer), buffy coat (middle layer) and red blood cells (base layer). The buffy coat was carefully extracted and re-suspended in 15ml phosphate-buffered saline (PBS) and centrifuged at 250g (1200 rpm) for a further 15 minutes @ RT. Finally, the PBS was carefully removed, leaving a PMN pellet,

which was individually marked and stored at -20°C until required in the assay for core 2 transferase.

Figure 3.8.1 **Protocol for the preparation of PMN pellet**



3.9 Core 2 Transferase Assay

3.9.1 Transportation

Given the need for specialist laboratory facilities, transportation to Guy's Hospital was required for the assays. The materials necessary for this phase included: a polystyrene box for insulation and a sandwich layer of dry ice to ensure a degree of temperature stability to avoid premature deterioration of the

PMN pellet. Prior to loading, all containers were checked for leaks and their suitability for transporting human fluids, in respect of health and safety guidelines.

3.9.2 **Materials for Core 2 Transferase Assay**

- 400 mM 2-(N-morpholino ethanesulfonic acid; MES Buffer) [Sigma-Aldrich, Dorset, UK]
- 8 mM UDP GlcNAc; [Donor] (Sigma-Aldrich, Dorset, UK)
- 0.5 μCi UDP-6[^{3}H]-N-acetylglucosamine; [Radioactive donor] (NEN, Life Science Products, Hounslow, UK)
- 8 mM Galβ1-3GalNAcα-PNP; [Acceptor] (Sigma-Aldrich, Dorset, UK)
- 0.8 M GlcNAc [Substrate] (Sigma-Aldrich, Dorset, UK)
- 100 mg C18 SEP Pak column (Waters-Millipore, Watford, UK)
- β-counter (LKB-Wallac, London, UK)
- Biorad protein assay (Biorad, Hertfordshire, UK)
- Leukocyte pellet, containing enzyme fraction
- Scintillation fluid
- Manifold (to create negative pressure)
- Absolute Methanol

3.9.3 **Preparation of the Core 2 GlcNAc-T Reaction Mixture**

i ***2-(N-morpholino) ethanesulfonic acid (400 mM MES, Buffer)***

MES (MW 262.3; 2.1 g) was dissolved in 40 ml of deionised H_2O, pH adjusted to 7.0 and stored at room temperature.

ii ***N-acetyl-$_D$-Glucosamine (0.8 M GlcNAc, Substrate)***

GlcNAc (MW 221.2; 0.35 g) was dissolved in 2 ml deionised H_2O and 40 µl aliquots, stored at - 20°C.

iii ***Uridine 5-diphospho-N-acetyl-$_D$-galactosamine (8 mM UDP-GlcNAc, Donor)***

UDP-GlcNAc (MW 651.3; 20.8 mg) was dissolved in 4 ml deionised H_2O and 40 µl aliquots, stored at - 20°C.

iv ***p-Nitrophenyl 2-Acetamido-2-Deoxy(β-$_D$-Galactopyranosyl) -α-$_D$-Galacto-Pyranoside [8 mM Galβ1-3Galα-PNP, Acceptor]***

Galβ1-3Galα-PNP (MW 504.4; 1 mg) was dissolved in 248 µl of deionised H_2O and 40 µl aliquots, stored at - 20°C.

v ***UDP-6[^{3}H]-N-acetylglucosamine (0.5 µCi Radioactive Donor)***

The radioactivity donor was purchased as 50 µCi (1850 kBq) in 500 µl ethanol.

3.10 Measurement of (β1-6)GlcNAc Transferase (Core 2 Transferase) Enzyme Activity

The PMN pellet was freeze-thawed and lysed with a lysis buffer (0.9% Nacl, Triton X –100) at 0°C for a minimum of 15 minutes. The measurement of core 2 transferase activity was performed in a reaction mixture containing the following: 400 mM 2-(n-morpholino) ethanesulfonic acid (MES) at pH 7.0 (Buffer), 8 mM UDP-GlcNAc (unlabelled donor), 8 mM Galβ1-3 Galα-PNP (Acceptor), 0.8 M GlcNAc (substrate; Sigma-Aldrich, Dorset, UK), and 0.5 μCi UDP-6 [^{3}H]-N-acetylglucosamine (donor; 16,000 dpm/nmol, NEN, Life Science Products, Hounslow, UK); and 25 μl cell lysate (100 - 200 μg protein) for a final volume of 50 μl.

After incubating the mixture for 1 hour at 37°C, the reaction was terminated with 5 ml of ice-cold water and processed on a C18 Sep-Pak column (Waters-Millipore, Watford, UK). Once complete, the column was washed with 20 ml of distilled water and the product eluted with 5 ml of methanol. Finally, the radioactivity of each sample was counted in a liquid scintillation β-counter (LKB-Wallac, London, UK), the endogenous activity of core 2 transferase measured in the absence of the added acceptor. For each result, the specific activity was expressed as picomoles per hour per milligram of cell protein. The method adopted was identical to that outlined by Chibber et al., (2000).

3.11 Bicinchoninic Acid Protein Assay (Biorad assay - Biorad, Hertfordshire, UK)

In each case, the protein concentration was determined with a modified Biorad assay, which is an alteration of the reagent originally proposed by Smith et al., (1985). Protein concentration in mg/ml was determined by measuring the optical density at 595 nm.

4.0 RESULTS - STUDY I

4.1 Scope

The pilot study was conducted over a three-week period with all participants attending the Diabetes Department, Poole Hospital NHS Trust for one visit only. Participants were loaded into three distinct study groups: control (C, n=2), diabetic with no complications (DNC, n=2) and diabetic with neuropathy, to include one sub-group member with discreet ulceration (DN, n=3).

The results are divided into the following subsections:

i. Participant demographics
ii. Absolute core 2 transferase levels
iii. Vibration Perception Thresholds
iv. Monofilament (10g) detection
v. Height and weight
vi. HbA1c levels
vii. Cholesterol levels
viii. Power Calculations resulting from Study I

4.2 Participant Demographics

Table 4.2.1 **Participant demographics**

PARTICIPANT	GENDER	AGE	DURATION OF DIABETES (Yr)
C1	F	30	N/A
C2	F	42	N/A
DNC 1	M	56	8
DNC 2	M	61	1
DN 1	F	61	4
DN 2*	M	55	9
DN 3	M	51	9

* plantar ulceration present

4.2.1 Gender

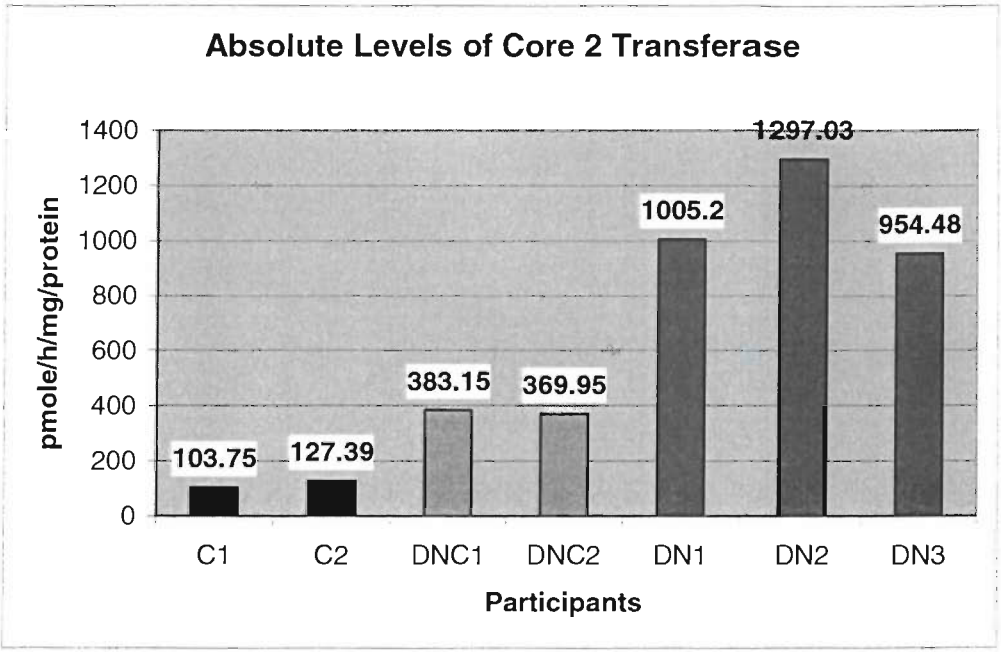
All participants within the diabetic no complications group in Study I were male (n=2). The control group was comprised of female participants (n=2), whilst the diabetic neuropathic group consisted of 2 males and 1 female (n=3). This was not an active decision, but a direct consequence of participant availability and the satisfaction of inclusion and exclusion criteria. However, the benefit of this selection allowed clinical variables, such as weight and lipid profile to be more readily matched, given the sample composition. Hence, if only serendipitous, certain biochemical factors were capable of being regarded in context.

4.3 Absolute Core 2 Transferase Levels

Clinically, this investigation demonstrated that neuropathic individuals displayed up to a ten-fold increase in the absolute levels of core 2 transferase, compared to the control group. Whilst it was impractical to perform statistical

analysis on the available sample population, the apparent gulf between the two groups supported the basic theory and reinforced the need to pursue Study II. Similarly, the results of this phase mirror the studies conducted by Chibber et al, (2000), who displayed a similar trend between diabetic and non-diabetic groups with specific regard to retinopathy.

Figure 4.3.1 **Absolute levels of core 2 transferase activity**



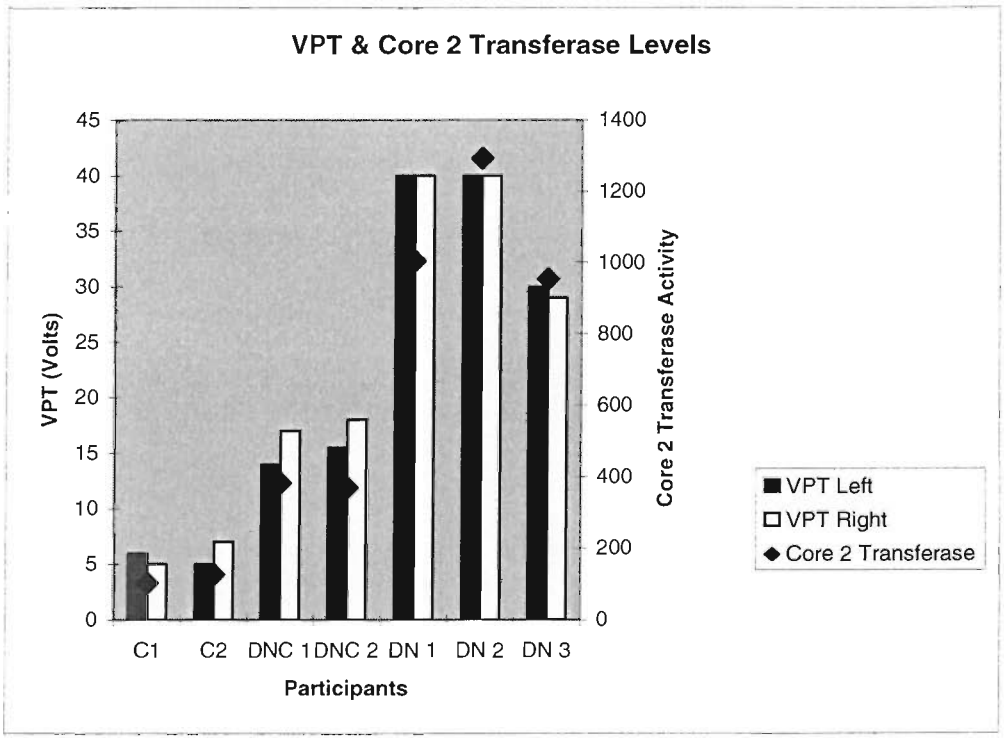
The results demonstrated a notable difference between diabetic individuals without complications and those with neuropathy, thus supporting the hypothesis that core 2 transferase may be elevated in neuropathic patients.

4.4 Vibration Perception Threshold (VPT's)

The preliminary data demonstrated that elevated VPT's correlated with the neuropathic study group. Moreover, those individuals showing higher VPT

readings also exhibited elevated levels of core 2 transferase. This observation was further strengthened by the fact that two individuals showing the highest yields of core 2 transferase had the correspondingly greatest VPT values. The VPT values for left and right feet are given in Figure 4.4.1, together with the corresponding core 2 transferase levels. A table of the raw data is shown at Appendix E.

Figure 4.4.1 **Comparison between VPT and core 2 transferase activity**



4.5 10g Monofilament Testing

The data collected using a 10g monofilament (Semmes Weinstein, North Cost©) was recorded over a maximum of 12 locations tested on both feet (apex of hallux and metatarsal phalangeal joints 1-5, per foot). Initial observation of

these findings confirm the work by Kumar et al., (1991), McGill et al. (1999) and Pham et al., (2000), which has indicated that a loss of peripheral sensory perception to light touch stimuli is a valid measure of neuropathy. Consequently, it was seen that the three individuals formally classified as diabetic neuropathic, showed the lowest scores for the detection of the 10g monofilament. The rates of 10g monofilament detection for individuals' feet and the total scores for the three groups are shown in Table 4.5.1.

Table 4.5.1 **Number of sites detected using a 10g monofilament**

PARTICIPANT	LEFT FOOT (Sites detected)	RIGHT FOOT (Sites detected)	TOTAL (Sites detected)
Control 1	6	6	12
Control 2	5	6	11
Diabetic No Comp. 1	3	4	7
Diabetic No Comp. 2	3	3	6
Diabetic Neuropathic 1	0	0	0
Diabetic Neuropathic 2	0	0	0
Diabetic Neuropathic 3	1	1	2

Notably, for those participants with diabetes and no complications, the results showed that, whilst not strictly meeting the classification criteria for non-neuropathic incidence (80% detection of 10g monofilament or ≥ 8/10 sites) [McGill et al., 1999, Sosenko et al., 1999], the overall performance was progressively lower than the control group.

4.6 Height and Weight

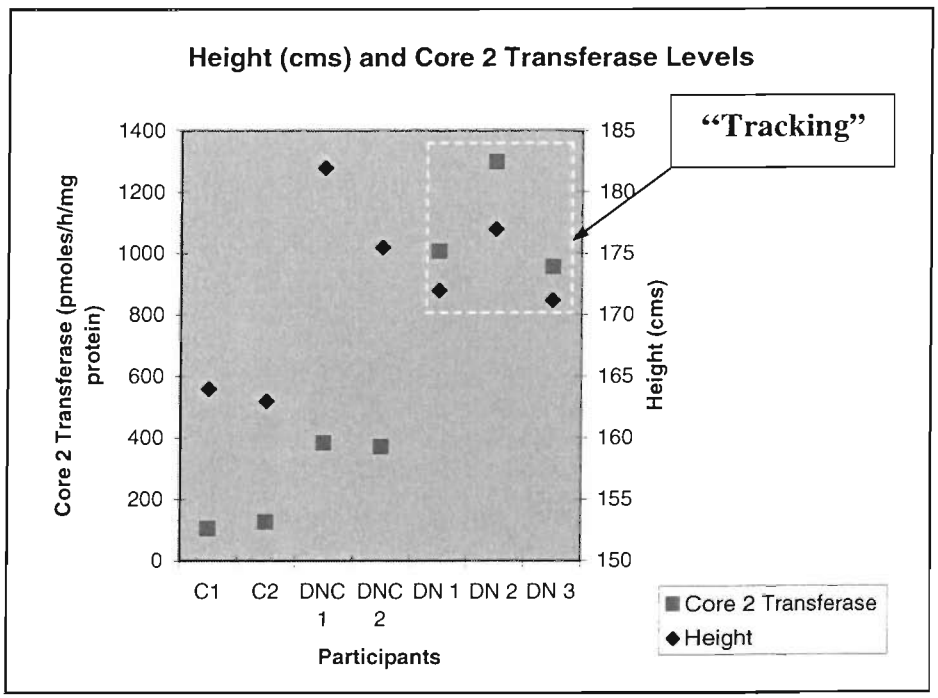
Participant height showed little deviation (mean 172.1 cm) between the groups. Table 4.6.1 shows the respective height for all participants:

Table 4.6.1 **Participants' heights**

PARTICIPANT	HEIGHT (CM)
Control 1	164
Control 2	163
Diabetic No Complications 1	182
Diabetic No Complications 2	175.5
Diabetic Neuropathy 1	172
Diabetic Neuropathy 2	177
Diabetic Neuropathy 3	171.2

The data illustrates that within the control and diabetic group without complications, there appears to be no notable trend or association between height and core 2 transferase. However, as Figure 4.6.2 demonstrates, there does appear to be some mirroring within the diabetic neuropathic group. Whilst the numerical sample size is small, this form of "tracking" (mimicry of the variables) is particularly noticeable and may have a pathogenic cause.

Figure 4.6.2 **Comparison of height and core 2 transferase activity**



The clinical data collected on weight was interpreted far more readily. This is a potential area for further investigation, as both diabetic groups were seen to be heavier than the control, as demonstrated in Table 4.6.3. Despite a gender mix between the groups, the data still yielded that diabetic females showed an increased body weight in comparison to controls. Furthermore, the diabetic neuropathic group was seen to be consistently heavier than the diabetic no complications group, irrespective of gender. Given the clinical data collected on height and weight, Study II combines these clinical factors to examine the potential influence of Body Mass Index (BMI) on core 2 transferase expression.

Table 4.6.3 **Participants' weight**

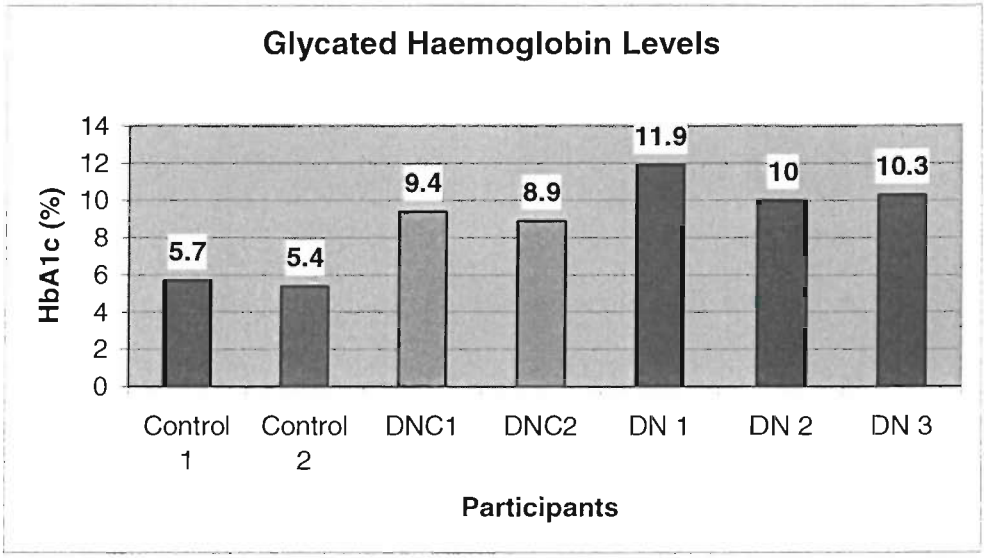
PARTICIPANTS	WEIGHT (Kg)
Control 1	48
Control 2	49
Diabetic No Complications 1	84.3
Diabetic No Complications 2	96
Diabetic Neuropathy 1	98.9
Diabetic Neuropathy 2	106.5
Diabetic Neuropathy 3	115.6

4.7 Glycated Haemoglobin - HbA1c's

As expected, HbA1c's were higher in both diabetic groups versus the control, seemingly despite strict guidelines and educational packages implemented by primary and secondary care in light of the Diabetes Complication Trial and the UK Prospective Diabetes Study (UKPDS). Notably, all participants involved within this study received three identical educational sessions, delivered by the same multidisciplinary team within the same hospital environment. Whilst

there is an absence of statistical analysis, observation of the data reveals that there is only slight variation in the HbA1c range between individuals with diabetes and no complications and those with diabetic neuropathy. These findings are supported by Chibber et al., (2000), who were unable to provide any significant correlation between elevated glycated haemoglobin and core 2 transferase in diabetic individuals with and without retinopathy.

Figure 4.7.1 **Absolute HbA1c levels**

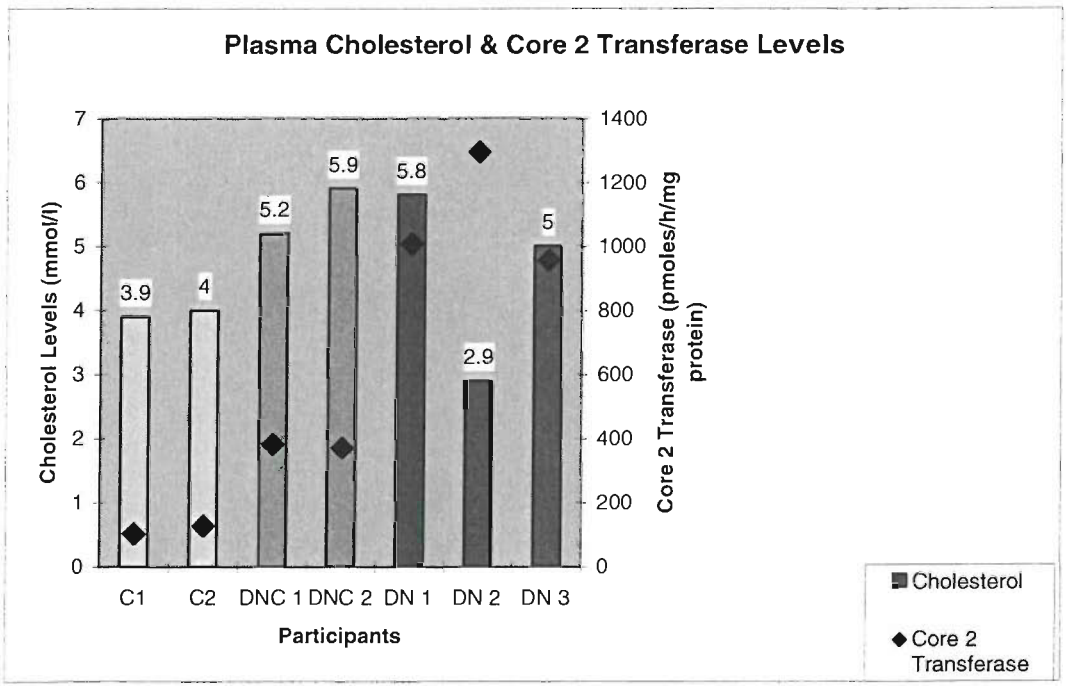


Generally, diabetic neuropathic individuals displayed higher HbA1c levels. However, given the size of the sample group for Study I, direct extrapolation would be inappropriate. In light of the 120-day life cycle of erythrocytes, the above "snapshot" does not necessarily allow for fluctuating glucose levels and as such, may well overshadow the intricacies occurring at a cellular level. This issue is addressed at Study II by incorporating serum glucose levels into the battery of clinical data collection tests.

4.8 Plasma Cholesterol Levels

The diabetic groups showed an increased level of plasma cholesterol over that of the control group (Figure 4.8.1). This finding is not entirely unexpected and correlates with the Framingham study (1977). However, the raw data from Study I indicates that two individuals from the diabetic no complications group had higher lipid levels, when matched to the diabetic neuropathic group. Whilst there appears to be no apparent association between cholesterol levels and core 2 transferase in any of the groups, possible links with other lipid profiles (HDL/cholesterol ratio, triglycerides and LDL's) are examined in Study II.

Figure 4.8.1 **Comparison between plasma cholesterol and core 2 transferase activity**



4.9 Power Calculations

Following Study I, it was essential to determine the necessary group compositions for Study II to attain statistical merit. By this stage, data from a further 8 participants (total n=15) had been obtained and stored. This "ring fencing" of potential subjects was considered vital, as individuals with diabetic peripheral neuropathy, but no retinopathy were shown to be extremely scarce. Consequently, all data would contribute towards Study II.

The summary values for core 2 transferase were submitted for SPSS calculation at the University of Southampton.

4.10 Statistics

Table 4.10.1 **Recorded levels of core 2 transferase activity prior to Study II**

	GROUP 1	GROUP 2	GROUP 3
	Control	Diabetic (DNC)	Diabetic (Neuropathic)
	103.75	383.15	1297.03
	127.39	369.95	1027.43
	160.00	473.41	954.48
		256.19	1005.20
			913.76
			873.73
			891.34
			1067.92
Σ Participants	3	4	8
Mean	130.45	370.68	1003.86
Std. Deviation	28.35	89.10	136.68

The information provided for each of the groups was paired, i.e. Group 1 versus Group 2, Group 1 versus Group 3, and Group 2 versus Group 3, to observe the sample size relative to each match. This technique was used to find the necessary sample population to detect a mean difference between groups at 5% significance level with a power of 80%.

4.11 Sample Sizes

4.11.1 Group 1 versus Group 2

A sample size of 3 in each group has 80% power to detect a difference in means of 240.23 (the difference between a Group 1 mean of 130.45 and a Group 2 mean of 370.68), assuming that the common standard deviation is 71.31, using a two group t-test with a 0.05 two-sided significance level.

4.11.2 Group 1 versus Group 3

A sample size of 2 in each group has 80% power to detect a difference in means of 873.41 (the difference between a Group 1 mean of 130.45 and a Group 3 mean of 1003.86), assuming that the common standard deviation is 121.28, using a two group t-test with a 0.05 two-sided significance level.

4.11.3 **Group 2 versus Group 3**

A sample size of 3 in each group has 80% power to detect a difference in means of 633.18 (the difference between a Group 2 mean of 370.68 and a Group 3 mean of 1003.68), assuming that the common standard deviation is 124.33, using a two group t-test with a 0.05 two-sided significance level.

4.12 **Final Group Size**

Whilst the sample sizes assembled by this stage would have statistically permitted a 0.05 significance level between the groups, a decision was made to augment the study to 25 participants in total. The rationale for this amendment was that further clinical markers may be highlighted and that a larger overall sample population would lend increased validity to the study.

5.0 METHOD - STUDY II

5.1 Scope

The second phase of this project replicated Study I, but with an increased sample population (n=25). However, in this arm of the investigation, additional biochemistry data was carried forward and analysed. Similarly, the group composition was also changed relative to Study I. This was due to the general non-availability of participants with manifest ulceration, notionally the diabetic neuropathic sub-group. Therefore, patients with a proven history of plantar ulceration within the last 12 months were loaded onto the test programme within the diabetic neuropathic group itself. This decision was founded upon the potential links between core 2 transferase and wound healing (Obrosova et al., 2001).

5.2 Statistical Methods

The statistical package utilised in the analysis of data was SPSS, Version 11 (Ohio). However, all data had to fulfil the four standard criteria for parametric testing:

- Interval / ratio level of measurement
- Randomly selected participants
- Normal distribution
- Variation within general tolerance

5.2.1 **Data Handling**

Standard statistical tools were employed during the initial data manipulation, to include:

- **Unrelated *t* test (2-tailed)**
  - This test was employed to determine the level of significance between two unmatched variables, i.e. core 2 transferase and neuropathic ulceration
- **One Way Analysis of Variance (ANOVA)**
  - This test was employed to compare the mean results from three or more conditions, within different, unmatched subject groups, i.e. 10g monofilament detection rates for left, right and both feet modalities for each cohort
- **Tukey Honest Statistical Difference (HSD)**
  - Whilst ANOVA identifies differences within a group, it cannot identify the precise area of significance. The Tukey HSD test was employed to perform pair-wise comparisons of the mean results, i.e. Control to DNC to DN for any one condition
- **Pearson Product Moment Correlation Coefficient (2-tailed)**
  - This test was employed to compare two sets of independent data to determine the degree of association, be it positive or negative. Accordingly, this test is able to show that as core 2 transferase increases, the detection rate for 10g monofilament is diminished

Detailed protocols and examples of each of the above tests are given at Appendix F. With these exceptions only, the method adopted for Study II follows the identical process outlined in Study I.

6.0 RESULTS – STUDY II

6.1 Scope

In total, the duration of Study II was 6 months. Whilst the data and sample collection from the participants only required a single visit, the process of performing the core 2 transferase assay required multiple laboratory sessions at Guy's Hospital, London.

The results are divided into the following subsections:

Participant Demographics

i. Gender

ii. Duration of diabetes

Core 2 transferase

i. Core 2 transferase between the groups

ii. Core 2 transferase and ulceration within a 12 month period

Neurological Data

i. Vibration Perception Thresholds between the groups

ii. Vibration Perception Thresholds in relation to core 2 transferase

iii. Monofilaments (10g) between the groups

iv. Monofilaments (10g) in relation to core 2 transferase

Biometric Data

i. Age between the groups

ii. Age in relation to core 2 transferase

iii. Height between the groups

iv. Height in relation to core 2 transferase

v. Weight between the groups

vi. Weight in relation to core 2 transferase

vii. Body Mass Index (BMI) between the groups

viii. BMI in relation to core 2 transferase

Blood Chemistry

i. Glycated haemoglobin correlations

ii. Glucose level correlations

iii. Cholesterol level correlations

iv. Cholesterol / HDL ratio correlations

v. HDL correlations

vi. LDL correlations

vii. Triglyceride correlations

viii. Creatinine correlations

Linear Regression Modelling

6.2 Participant Demographics

6.2.1 Gender

The study comprised the following gender mix:

Table 6.2.1.i **Gender**

SEX	CON	DNC	DN
M	2	6	8
F	3	4	2

6.2.2 **Duration of Type II Diabetes**

No significance in the duration of diabetes was evident between the diabetic study groups (Table F-4). Therefore, the recorded duration of Type II diabetes bears no relation to core 2 transferase activity within the bounds of this study.

Table 6.2.3 **Participant demographics**

CHARACTERISTIC	CONTROL MEAN / SD	DNC MEAN / SD	DN MEAN / SD	GROUP SIG. (*p*)
Age (Years)	41.40 ± 10.78	53.80 ± 6.09	59.60 ± 9.72	**p<0.01**
Duration of Diabetes (Years)	N/A	11.30 ± 9.63	11.60 ± 9.54	p>0.05
Core 2 Transferase (pmoles/h/mg protein)	144.53 ± 30.90	384.31 ± 77.15	1040.51 ± 188.78	**p<0.001**
VPT (Volts)	5.1 ± 1.29	9.45 ± 5.26	34.85 ± 4.73	**p<0.001**
10g Monofilament (Total Sites Detected)	11.80 ± 0.45	9.90 ± 1.10	1.40 ± 1.71	**p<0.001**
Height (cm)	170.40 ± 9.48	170.47 ± 6.89	178.52 ± 8.73	p>0.05
Weight (Kg)	62.80 ± 15.12	89.53 ± 22.77	103.66 ± 17.54	**p<0.01**
BMI (Kg / M^2)	21.37 ± 3.36	30.50 ± 6.33	33.48 ± 3.90	**p<0.001**
Glycated Haemoglobin (%)	5.46 ± 0.39	8.38 ± 1.64	9.42 ± 1.28	**p<0.001**
Glucose Levels (mmol /l)	4.66 ± 0.30	10.73 ± 6.44	14.85 ± 4.43	**p<0.01**
Cholesterol Levels (mmol / l)	4.32 ± 0.79	4.70 ± 0.95	4.68 ± 0.89	p>0.05
Cholesterol / HDL Ratio (*x : y*)	2.86 ± 0.70	3.90 ± 1.31	4.80 ± 1.35	p>0.05
HDL Levels (mmol / l)	1.61 ± 0.56	1.31 ± 0.44	1.25 ± 0.41	p>0.05
LDL Levels (mmol / l)	1.96 ± 0.60	2.46 ± 0.96	1.54 ± 1.19	p>0.05
Triglyceride Levels (mmol / l)	1.02 ± 0.42	2.03 ± 0.76	2.82 ± 1.16	**p<0.01**
Creatinine Levels (µmol / l)	83.40 ± 4.16	84.40 ± 9.07	105.80 ± 10.76	**p<0.001**

6.3 Core 2 Transferase between the Groups

Twenty-five absolute core 2 transferase values were secured (1 per participant). Using an Analysis Of Variance (ANOVA), the results demonstrated a significant difference between the groups (F ratio of 100.16, $p<0.001$, Table F-2). Of note, the diabetic neuropathic group showed the highest levels of core 2 transferase (mean 1040.51 SD ± 188.78), when compared with diabetic no complication (mean 384.31 SD ± 77.15) and control groups (mean 144.53 SD ± 30.90, $p<0.001$). Similarly, core 2 transferase values between controls and diabetic individuals with no complications were also seen to be significantly different ($p<0.01$, Table 6.3.2). Whilst the standard deviation for both the diabetic groups was higher than the control, this may be attributed to the degenerative process associated with the diabetic neuropathic condition, thereby creating greater variability around the mean value.

Figure 6.3.1 **Mean core 2 transferase values between the groups**

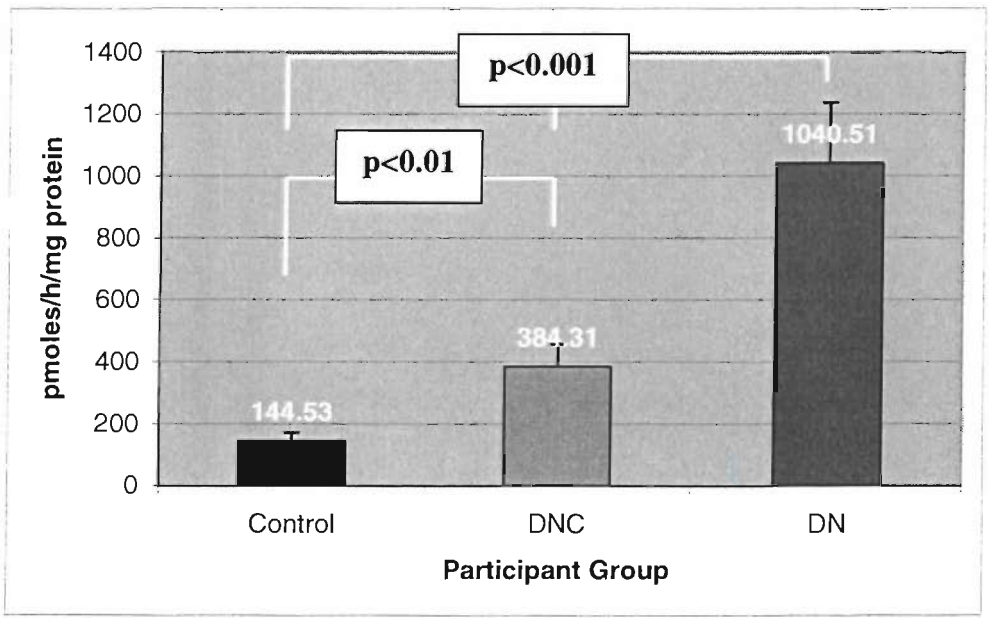


Table 6.3.2 **Core 2 transferase between the groups – multiple comparisons (Post Hoc Tukey HSD)**

(I) Grps of patients	(J) Grps of patients	Mean Difference	Std. Error	Sig. (*p*)
Control	Diabetic no complications	-239.78*	71.81	**.008**
	Diabetic neuropathic	-895.98*	71.81	**.000**
Diabetic no complications	Control	239.78*	71.81	**.008**
	Diabetic neuropathic	-656.20*	58.63	**.000**
Diabetic neuropathic	Control	895.98*	71.81	**.000**
	Diabetic no complications	656.20*	58.63	**.000**

* Mean difference significant at the 0.05 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

6.4 Core 2 Transferase and Ulceration within a 12 Month Period

Four participants were recorded as having had an incidence of plantar foot ulceration within the last 12 months. Generally, the diabetic neuropathic group was found to have significantly elevated levels of core 2 transferase over both the control and diabetic no complications groups (1040.51 SD ± 188.78 vs. 144.53 SD ± 30.90 vs. 384.31 SD ± 77.15 pmoles/h/mg protein, $p<0.001$), whilst detailed observation of the neuropathic ulceration sub-group revealed four of the five highest core 2 transferase values overall (Table 6.4.1). The magnitude of difference in the absolute values of core 2 transferase between the ulceration sub-group and the remaining diabetic individuals was demonstrated by the following respective means: 1194.44 SD ± 214.92 pmoles/h/mg protein versus 591.90 SD ± 286.00 pmoles/h/mg protein. Core 2 transferase was further demonstrated as being significantly elevated in the diabetic neuropathic ulceration sub-group using a 2-tailed 't' test ($p<0.01$, Table F-33).

Table 6.4.1 **Group statistics – core 2 transferase and ulceration**

History of Ulceration	N	Mean / SD	Sig. (p)
Ulcer present in the last 12 months	4	1194.44 ± 214.92	**0.01**
No ulcer present in the last 12 months	16	591.90 ± 286.00	-

6.5 Vibration Perception Thresholds between the Groups

VPT's between the three groups were significantly different for all modalities using a One Way ANOVA ($p<0.001$, Table F-6). However, additional data handling using a Post Hoc Tukey HSD test (mean difference significance set at the 0.05 level, see Appendix F) demonstrated a significant mean difference between the diabetic neuropathic group ($p<0.001$) and remaining sample population, when examining the VPT readings for left, right and both feet (Table 6.5.1.ii). Yet, no significant mean differences between the control and diabetic no complications groups were observed for the right foot ($p = 0.27$), left foot ($p = 0.29$) or both feet ($p = 0.21$). Whilst no significant difference was actually displayed between control and diabetic no complications groups, the modality utilising the "both feet" readings did give a lower p value overall.

6.5.1 Vibration Perception Thresholds in relation to Core 2 Transferase

The Pearson Correlation (2-tailed) test demonstrated a significant positive correlation between ascending VPT readings for the left foot ($r = 0.91$), right foot ($r = 0.89$) and both feet ($r = 0.91$) against core 2 transferase values, $p< 0.01$ (Table 6.5.1.i). Consequently, a worsening of neuropathic status (as indicated by the rising VPT score) is positively associated with elevated core 2 transferase levels. Notably, the r value for the "both feet" modality ($r = 0.91$) was observed as being highest, when compared to the left and right feet modalities for a positive correlation with core 2 transferase levels. This may be due to the effect of "averaging out" across the sample population.

Table 6.5.1.i **VPT correlations to Core 2 transferase – Pearson's Correlation (2-tailed)**

		Absolute values of core 2 transferase	VPT (Right)	VPT (Left)	VPT (Both)
Absolute values of core 2 transferase	Pearson Correlation	1	0.89**	0.91**	0.91**
	Sig. (2-tailed)	.	**0.00**	**0.00**	**0.00**
	N	25	25	25	25
VPT (Right)	Pearson Correlation	0.89**	1	0.96**	0.99**
	Sig. (2-tailed)	**0.00**	.	**0.00**	**0.00**
	N	25	25	25	25
VPT (Left)	Pearson Correlation	0.91**	0.96**	1	0.99**
	Sig. (2-tailed)	**0.00**	**0.00**	.	**0.00**
	N	25	25	25	25
VPT (Both)	Pearson Correlation	0.91**	0.99**	0.99**	1
	Sig. (2-tailed)	**0.00**	**0.00**	**0.00**	.
	N	25	25	25	25

** Correlation significant at the 0.01 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

Table 6.5.1.ii **VPT multiple comparisons (Post Hoc Tukey HSD)**

Dependent Variable	(I) Grps of patients	(J) Grps of patients	Mean Difference	Std. Error	Sig. (*p*)
VPT's (R)	Control	Diabetic no complications	-4.40	2.75	.267
		Diabetic neuropathic	-28.80*	2.75	**.000**
	Diabetic no complications	Control	4.40	2.75	.267
		Diabetic neuropathic	-24.40*	2.25	**.000**
	Diabetic neuropathic	Control	28.80*	2.75	**.000**
		Diabetic no complications	24.40*	2.25	**.000**
VPT 's (L)	Control	Diabetic no complications	-4.30	2.80	.293
		Diabetic neuropathic	-30.90*	2.80	**.000**
	Diabetic no complications	Control	4.30	2.80	.293
		Diabetic neuropathic	-26.60*	2.280	**.000**
	Diabetic neuropathic	Control	30.90*	2.80	**.000**
		Diabetic no complications	26.60*	2.28	**.000**
VPT (both feet)	Control	Diabetic no complications	-4.35	2.49	.212
		Diabetic neuropathic	-29.75*	2.49	**.000**
	Diabetic no complications	Control	4.35	2.49	.212
		Diabetic neuropathic	-25.40*	2.04	**.000**
	Diabetic neuropathic	Control	29.75*	2.49	**.000**
		Diabetics no complications	25.40*	2.04	**.000**

* Mean difference significant at the 0.05 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

6.5.2 **Reduced VPT Thresholds in relation to Core 2 Transferase**

Given the significant correlations observed during the above statistical analysis, it was decided to ascertain the apparent threshold at which, the association between VPT and core 2 transferase ceases to exist. In effect, this would establish an alternative VPT threshold for the classification of neuropathic status, based upon the purported link between core 2 transferase and cellular degradation (Schroder et al., 1991, Chibber et al., 2000, 2003). Consequently, a median value of 15 V was selected as the modified co-variable for remedial correlation testing.

Table 6.5.2.i **Modified VPT co-variate set at ≥ 15 V**

		Absolute values of core 2 transferase	**VPT (Both)**	**10g Mono. (Total)**
Absolute values of core 2 transferase	Pearson Correlation	1	0.85**	-0.80**
	Sig. (2-tailed)	.	**0.00**	**0.01**
	N	13	13	13
VPT (Both)	Pearson Correlation	0.85**	1	-0.95**
	Sig. (2-tailed)	**0.00**	.	**0.00**
	N	13	13	13
10g Monofilament (Total)	Pearson Correlation	-0.80**	-0.95**	1
	Sig. (2-tailed)	**0.00**	**0.00**	.
	N	13	13	13

**. Correlation significant at the 0.01 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

The outcome of this test confirms a negative correlation between VPT and 10g monofilament detection, whilst showing a significant positive correlation with core 2 transferase specificity ($p<0.01$). Hence, at previously accepted "reduced risk" VPT margins (< 25 V), core 2 transferase appears to be elevated within the confines of this study. Naturally, this finding requires further development to

ascertain the corresponding risk to diabetic patients in general and moreover, to confirm the efficacy of existing screening regimes.

6.6 Monofilaments (10g) between the Groups

The data produced from the 10g monofilament testing was divided into outcomes for the subjects' right foot, left foot and both feet (based upon the number of sites detected). The control group displayed the highest ability to detect the 10g monofilament for the right foot (mean 5.80 SD ± 0.45), when compared with the Diabetic No Complication group (mean 4.80 SD ± 0.79) and the Diabetic Neuropathic group (mean 1.00 SD ± 1.15) [Table F-7]. A similar pattern was displayed for 10g monofilament testing on the left foot between the respective groups: control 6.00 SD ± 0.00, DNC 5.10 SD ± 0.57 and DN 0.40 SD ± 0.7. The total number of sites detected on both feet was highest in the control group (mean 11.80 SD ± 0.45), followed by the DNC group (mean 9.90 SD ± 1.10) and finally, the DN group (mean 1.40 SD ± 1.71). Notably, the number of sites detected was significantly different between the groups using all three modalities ($p<0.001$, Table F-8).

Further pair-wise statistical handling (Table 6.6.1) revealed that the 10g monofilament testing to the right foot was significantly different between control and the DN group only ($p<0.001$). However, there remained a significant difference between the DN and DNC group ($p<0.001$). 10g monofilament testing for the left foot showed a significance difference between controls versus the DNC ($p<0.025$) and DN groups ($p<0.001$) respectively. Furthermore, a continued significant difference between DNC and DN was

established ($p<0.001$). The testing modality on both feet for controls versus DNC was also significant ($p<0.05$), as was the control versus the DN group ($p<0.001$). A further significant difference between the means of the DN and DNC groups was also demonstrated ($p<0.001$).

Table 6.6.1 **10g monofilament multiple comparisons (Post Hoc Tukey HSD)**

Dependent Variable	(I) Grps of patients	(J) Grps of patients	Mean Diff. (I-J)	Std. Error	Sig. (*p*)
10g Monofilament (Right)	Control	Diabetic no complications	1.00	.50	.137
		Diabetic neuropathic	4.80*	.50	**.000**
	Diabetic no complications	Control	-1.00	.50	.137
		Diabetic neuropathic	3.80*	.41	**.000**
	Diabetic neuropathic	Control	-4.80*	.50	**.000**
		Diabetic no complications	-3.80*	.41	**.000**
10g Monofilament (Left)	Control	Diabetic no complications	0.90*	.32	**.024**
		Diabetic neuropathic	5.60*	.32	**.000**
	Diabetic no complications	Control	-0.90*	.32	**.024**
		Diabetic neuropathic	4.70*	.26	**.000**
	Diabetic neuropathic	Control	-5.60*	.32	**.000**
		Diabetic no complications	-4.70*	.26	**.000**
10g Monofilament (Total)	Control	Diabetic no complications	1.90*	.72	**.039**
		Diabetic neuropathic	10.40*	.72	**.000**
	Diabetic no complications	Control	-1.90*	.72	**.039**
		Diabetic neuropathic	8.50*	.59	**.000**
	Diabetic neuropathic	Control	-10.40*	.72	**.000**
		Diabetics no complications	-8.50*	.59	**.000**

* Mean difference significant at the 0.05 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

6.6.1 **Monofilaments (10g) in relation to Core 2 Transferase**

The right foot testing modality compared against core 2 transferase produced a Pearson's value of (-0.83, $p<0.001$). Similarly, the subjects' left foot 10g displayed a value of (-0.91, $p<0.001$). Data compiled from the left, right and total detection rates revealed a further significant negative correlation with core 2 transferase (-0.89, $p<0.001$, Table 6.6.1.i). Consequently, it is seen that increased levels of core 2 transferase are inversely proportionate to 10g monofilament detection.

Table 6.6.1.i **10g monofilament correlations to Core 2 transferase - Pearson's Correlation (2-tailed)**

		Absolute values of core 2 transferase	10g Mono. (Right)	10g Mono. (Left)	10g Mono. (Total)
Absolute values of core 2 transferase	Pearson Correlation	1	-0.83**	-0.91**	-0.89**
	Sig. (2-tailed)	.	**0.00**	**0.00**	**0.00**
	N	25	25	25	25
10g Monofilament (Right)	Pearson Correlation	-0.83**	1	0.91**	0.97**
	Sig. (2-tailed)	**0.00**	.	**0.00**	**0.00**
	N	25	25	25	25
10g Monofilament (Left)	Pearson Correlation	-0.91**	0.91**	1	0.98**
	Sig. (2-tailed)	**0.00**	**0.00**	.	**0.00**
	N	25	25	25	25
10g Monofilament (Total)	Pearson Correlation	-0.89**	0.97**	0.98**	1
	Sig. (2-tailed)	**0.00**	**0.00**	**0.00**	.
	N	25	25	25	25

** Correlation significant at the 0.01 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

6.7 Age between the Groups

The control group had the lowest mean age, when compared to the DNC and DN groups (41.40 SD ± 10.78, 53.80 SD ± 6.09 and 59.60 SD ± 9.72, Table F-15). Consequently, a significant difference was observed between the groups for age ($p<0.01$, Table F-16). Further Post Hoc analysis for age displayed significant mean differences between the control group, when compared to the DN and DNC groups ($p<0.05$). However, no statistically significant difference was present between the DNC and DN groups ($p>0.05$, Table 6.11.2).

6.7.1 Age in relation to Core 2 Transferase

A Pearson Correlation (2-tailed) test confirmed a significant positive correlation between rising age and core 2 transferase specificity ($p<0.01$, Table 6.11.1). Positive correlations between age, weight and BMI were also exhibited ($p<0.05$).

6.8 Height between the Groups

Individuals from the DN group were taller than both the DNC and control groups respectively (mean 178.52 cm SD ± 8.73 vs. 170.47 cm SD ± 6.88 vs. 170.40 cm SD ± 9.48). However, height between the groups marginally failed to achieve significant statistical difference ($p=0.075$, Table F-10).

6.8.1 **Height in relation to Core 2 Transferase**

Height recordings within the study demonstrated a positive correlation with core 2 transferase values (0.52, $p<0.01$, Table 6.11.1). Similarly, height also correlated with weight and body mass index ($p<0.01$). This would seem to confirm the notion that taller individuals, most particularly males, present a higher incidence of ulceration (Boulton et al., 1983, Reiber, 1996).

6.9 **Weight between the Groups**

Participants from the DN group recorded a greater mean weight, when compared to both the controls and DNC groups respectively (mean 103.66 kg SD ± 17.54 vs. 62.80 kg SD ± 15.12 vs. 89.53 kg SD ± 22.77, Table F-11). Of note, a significant difference in weight was highlighted across the groups, when analysed using an ANOVA ($p<0.01$, Table F-12). Further Post Hoc investigation was conducted to reveal a significant difference between the control and DN groups ($p<0.01$, Table 6.11.2), whilst no such relationship was present between the diabetic study groups ($p>0.05$).

6.9.1 **Weight in relation to Core 2 Transferase**

Participants from the study displayed a significant positive correlation between increasing weight and elevated core 2 transferase using ($p<0.01$, Table 6.11.1). Weight also showed a positive correlation with age ($p<0.05$).

6.10 Body Mass Index (BMI) between the Groups

The mean values for BMI showed the control group to have the lowest values (21.37 SD ± 3.36), when compared to the DNC group (30.50 SD ± 6.33) and the DN group (33.48 SD ± 3.90). Consequently, a significant difference between the groups was observed ($p<0.001$, Table F-14). Multiple comparison testing revealed significant differences between the BMI of the control group and the DNC group ($p<0.01$), as well as the control group and DN group ($p<0.01$, Table 6.11.2). However, there was no significant difference between the BMI's of the DN and DNC groups ($p>0.05$).

6.10.1 BMI in relation to Core 2 Transferase

A significant positive correlation was found between BMI values and absolute levels of core 2 transferase ($p<0.01$, Table 6.11.1). Consequently, this agrees with the observation that BMI is highest in the DN group. Similarly, BMI positively correlated with factors such as age ($p<0.05$), as well as height ($p<0.01$) and weight ($p<0.01$) due to their mathematical association.

6.11 Controlling for Age, Weight and BMI

Biometric variables (age, weight and BMI) for all three groups were entered as co-variates into the ANOVA for core 2 transferase to control for any potential bias. Consequently, core 2 transferase activity remains different in each of the sample populations independent of these factors.

Table 6.11.1 **Biometric multiple correlations – Pearson's Correlation (2-tailed)**

		Absolute values of core 2 transferase	Height	Weight	BMI	Age
Absolute values of core 2 transferase	Pearson Correlation	1	.52**	.54**	.58**	.53**
	Sig. (2-tailed)	.	**.008**	**.005**	**.002**	**.006**
	N	25	25	25	25	25
Height	Pearson Correlation	.52**	1	.66**	.55**	.35
	Sig. (2-tailed)	**.008**	.	**.000**	**.005**	.083
	N	25	25	25	25	25
Weight	Pearson Correlation	.54**	.66**	1	.96**	.43*
	Sig. (2-tailed)	**.005**	**.000**	.	**.000**	**.032**
	N	25	25	25	25	25
BMI	Pearson Correlation	.58**	.55**	.96**	1	.48*
	Sig. (2-tailed)	**.002**	**.005**	**.000**	.	**.017**
	N	25	25	25	25	25
Age	Pearson Correlation	.53**	.35	.43*	.48*	1
	Sig. (2-tailed)	**.006**	.083	**.032**	**.017**	.
	N	25	25	25	25	25

** Correlation significant at the 0.01 level
* Correlation significant at the 0.05 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

Table 6.11.2 **Biometric multiple comparisons (Post Hoc Tukey HSD)**

Dependent Variable	(I) Grps of patients	(J) Grps of patients	Mean Difference	Std. Error	Sig. (*p*)
Height	Control	Diabetic no complications	-.07	4.48	1.00
		Diabetic neuropathic	-8.12	4.48	.189
	Diabetic no complications	Control	.07	4.48	1.00
		Diabetic neuropathic	-8.05	3.66	.093
	Diabetic neuropathic	Control	8.12	4.48	.189
		Diabetic no complications	8.05	3.66	.093
Weight	Control	Diabetic no complications	-26.73	10.67	.051
		Diabetic neuropathic	-40.86*	10.67	**.003**
	Diabetic no complications	Control	26.73	10.67	.051
		Diabetic neuropathic	-14.13	8.71	.258
	Diabetic neuropathic	Control	40.86*	10.67	**.003**
		Diabetic no complications	14.13	8.71	.258
BMI	Control	Diabetic no complications	-9.13*	2.72	**.008**
		Diabetic neuropathic	-12.11*	2.72	**.001**
	Diabetic no complications	Control	9.13*	2.72	**.008**
		Diabetic neuropathic	-2.98	2.22	.388
	Diabetic neuropathic	Control	12.11*	2.72	**.001**
		Diabetic no complications	2.98	2.22	.388
Age	Control	Diabetic no complications	-12.40*	4.74	**.040**
		Diabetic neuropathic	-18.20*	4.74	**.002**
	Diabetic no complications	Control	12.40*	4.74	**.040**
		Diabetic neuropathic	-5.80	3.87	.311
	Diabetic neuropathic	Control	18.20*	4.74	**.002**
		Diabetic no complications	5.80	3.87	.311

* Mean difference significant at the 0.05 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

6.12 Pearson's (2-tailed) Correlation Testing - Blood Chemistry Variables

Blood chemistry factors were analysed to provide potential correlations with core 2 transferase (Tables 6.12.9 and 6.12.10). Similarly, Post Hoc testing was carried out in light of the significant group differences recorded for glycated haemoglobin ($p<0.001$), blood glucose ($p<0.01$), creatinine ($p<0.001$) and triglyceride levels ($p<0.01$), in order to determine the precise inter-group relationship (Table 6.12.11).

6.12.1 Glycated Haemoglobin Correlations

A significant positive correlation between glycated haemoglobin (HbA1c) and core 2 transferase existed across the groups ($p<0.01$, Table 6.12.9).

6.12.2 Glucose Level Correlations

Blood glucose readings demonstrated a significant positive correlation with core 2 transferase ($p<0.01$, Table 6.12.9).

6.12.3 Cholesterol Level Correlations

The cholesterol reading for all subjects did not show any significant correlation with core 2 transferase ($p>0.05$, Table 6.12.9).

6.12.4 **Cholesterol / HDL Ratio Correlations**

Measured plasma cholesterol/HDL ratio produced from blood chemistry analysis showed no significant correlation with core 2 transferase levels ($p>0.05$, Table 6.12.9).

6.12.5 **HDL Correlations**

The HDL recordings demonstrated no significant correlation with core 2 transferase ($p>0.05$, Table 6.12.10).

6.12.6 **LDL Correlations**

The LDL profile showed no significant correlation with core 2 transferase ($p>0.05$, Table 6.12.10).

6.12.7 **Triglyceride Correlations**

The triglyceride data from the groups provided a significant positive correlation with core 2 transferase ($p<0.05$). Furthermore, a negative correlation between triglyceride and HDL levels was established ($p<0.01$, Table 6.12.10).

6.12.8 **Creatinine Correlations**

Plasma creatinine levels for the participants were found to be within the accepted laboratory range [60 -130 µmol/l] (Porth, 1998). However, a

significant positive correlation was present between creatinine and core 2 transferase levels ($p<0.01$, Table 6.12.10).

Table 6.12.9 **Blood chemistry multiple correlations (1) - Pearson's Correlation (2-tailed)**

		Absolute core 2 transferase values	HbA1c	Blood glucose levels	Cholesterol levels	Cholesterol / HDL ratio
Absolute core 2 transferase values	Pearson Correlation	1	.64**	.69**	.13	.21
	Sig. (2-tailed)	.	**.001**	**.000**	.548	.317
	N	25	25	25	25	25
Glycated haemoglobin levels (HbA1c)	Pearson Correlation	.64**	1	.79**	.35	.47*
	Sig. (2-tailed)	**.001**	.	**.000**	.090	**.017**
	N	25	25	25	25	25
Blood glucose levels	Pearson Correlation	.69**	.79**	1	.30	.29
	Sig. (2-tailed)	**.000**	**.000**	.	.140	.161
	N	25	25	25	25	25
Cholesterol levels	Pearson Correlation	.13	.35	.30	1	.34
	Sig. (2-tailed)	.548	.090	.140	.	.094
	N	25	25	25	25	25
Cholesterol / HDL ratio	Pearson Correlation	.21	.47*	.29	.34	1
	Sig. (2-tailed)	.317	**.017**	.161	.094	.
	N	25	25	25	25	25

** Correlation significant at the 0.01 level

* Correlation significant at the 0.05 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

Table 6.12.10 **Blood chemistry multiple correlations (2) - Pearson's Correlation (2-tailed)**

		Absolute core 2 transferase values	Creatinine levels	Triglycerides	HDL's	LDL's
Absolute core 2 transferase values	Pearson Correlation	1	.88**	.50*	-.15	.20
	Sig. (2-tailed)	.	**.000**	**.011**	.478	.331
	N	25	25	25	25	25
HDL's	Pearson Correlation	-.15	-.07	-.63**	1	.10
	Sig. (2-tailed)	.478	.756	**.001**	.	.646
	N	25	25	25	25	25
LDL's	Pearson Correlation	.20	.22	-.02	.10	1
	Sig. (2-tailed)	.331	.284	.918	.646	.
	N	25	25	25	25	25
Triglycerides	Pearson Correlation	.50*	.27	1	-.63**	-.02
	Sig. (2-tailed)	**.011**	.198	.	**.001**	.918
	N	25	25	25	25	25
Creatinine Levels	Pearson Correlation	.88**	1	.27	-.07	.22
	Sig. (2-tailed)	**.000**	.	.198	.756	.284
	N	25	25	25	25	25

** Correlation significant at the 0.01 level

* Correlation significant at the 0.05 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

Table 6.12.11 **Blood chemistry - multiple comparisons (Post Hoc Tukey HSD)**

Dependent Variable	(I) Grps of patients	(J) Grps of patients	Mean Difference	Std. Error	Sig. (*p*)
Glycated Haemoglobin	Control	Diabetic no complications	-2.92*	0.73	**.002**
		Diabetic neuropathic	-3.96*	0.73	**.000**
	Diabetic no complications	Control	2.92*	0.73	**.002**
		Diabetic neuropathic	-1.04	0.60	.215
	Diabetic neuropathic	Control	3.96*	0.73	**.000**
		Diabetic no complications	1.04	0.60	.215
Blood Glucose	Control	Diabetic no complications	-6.07	2.74	.090
		Diabetic neuropathic	-10.19*	2.74	**.003**
	Diabetic no complications	Control	6.07	2.74	.090
		Diabetic neuropathic	-4.12	2.24	.179
	Diabetic neuropathic	Control	10.19*	2.74	**.003**
		Diabetic no complications	4.12	2.24	.179
Triglycerides	Control	Diabetic no complications	-1.01	0.49	.126
		Diabetic neuropathic	-1.80*	0.49	**.004**
	Diabetic no complications	Control	1.01	0.49	.126
		Diabetic neuropathic	-0.79	0.40	.147
	Diabetic neuropathic	Control	1.80*	0.49	**.004**
		Diabetic no complications	0.79	0.40	.147
Creatinine	Control	Diabetic no complications	-1.00	5.02	.978
		Diabetic neuropathic	-22.40*	5.02	**.001**
	Diabetic no complications	Control	1.00	5.02	.978
		Diabetic neuropathic	-21.40*	4.10	**.000**
	Diabetic neuropathic	Control	22.40*	5.02	**.001**
		Diabetic no complications	21.40*	4.10	**.000**

* Mean difference is significant at the 0.05 level

Refer to pp 132 and/or Appendix F for a detailed explanation of this particular statistical test

i. Glycated Haemoglobin Comparisons

A significant difference ($p<0.01$) was found to exist between the control and both diabetic study groups. However, this relationship was not evident within the diabetic population itself.

ii. Blood Glucose Comparisons

A significant difference ($p<0.01$) was observed between the Control and DN group. All other modalities failed to reach significance ($p>0.05$).

iii. Triglyceride Comparisons

A significant difference ($p<0.01$) was observed between the Control and DN group only.

iv. Creatinine Comparisons

A significant difference ($p<0.001$) was present between the DN group and the remaining population. However, there was no such association between the Control and DNC group.

6.13 **Regression Modelling**

An ***Rsq*** value is a measure of the predictive ability for a given set of variables, ranging from 0 (poor) to 1 (perfection). In practice, however, an ***Rsq*** of 0.7 or above (70% predictability) is usually considered suitable for validation purposes (Hicks, 1999). Hence, models for each of the measured variables were calculated as outlined at Section 2.10:

Table 6.13.1 **Analysis of regression models**

VARIABLE	Rsq
VPT (both feet) versus core 2 transferase activity	**0.836***
10g monofilament (both feet) versus core 2 transferase activity	**0.800***
Triglyceride levels versus core 2 transferase activity	0.251
Creatinine levels versus core 2 transferase activity	**0.779***
Ratio b/w cholesterol and HDL's versus core 2 transferase activity	0.044
Cholesterol levels versus core 2 transferase activity	0.016
HDL levels versus core 2 transferase activity	0.022
LDL levels versus core 2 transferase activity	0.041
BMI versus core 2 transferase activity	0.340
HbA1c levels versus core 2 transferase activity	0.413
Glucose levels versus core 2 transferase activity	0.474

* predictive independent co-variate

From the above analysis, it was seen that VPT's, 10g monofilament and creatinine levels, in relation to core 2 transferase specificity, achieved robust predictive scores. Therefore, it was determined that these three variables should be further scrutinised to achieve an adjusted ***Rsq*** value, by means of preloading the calculation with a single dependent variable only, namely core 2 transferase activity. Consequently, the modified ***Rsq*** value (adjusted $\mathbf{R^2}$) is representative of the remaining co-variates, in terms of predictive efficacy.

6.13.1 **VPT (both feet)**

Table 6.13.1.i **VPT regression – adjusted outcome**

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.914[a]	.836	.829	165.041

a. Predictors: (Constant), vpt both feet

This model is indicative of 82.9% predictability for core 2 transferase.

6.13.2 **10g Monofilament (both feet)**

Table 6.13.2.i **Monofilament regression – adjusted outcome**

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.894[a]	.800	.791	182.233

a. Predictors: (Constant), Mono 10g total

This model is indicative of 79.1% predictability for core 2 transferase.

6.13.3 **VPT and Monofilament Combined**

Table 6.13.3.i **VPT and monofilament regression – adjusted outcome**

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.918[a]	.842	.827	165.725

a. Predictors: (Constant), Mono 10g total, vpt both feet

This model is indicative of 82.7% predictability for core 2 transferase.

6.13.4 **VPT and Creatinine Combined**

Table 6.13.4.i **VPT and creatinine regression – adjusted outcome**

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.959[a]	.919	.911	118.737

a. Predictors: (Constant), Creatinin levels (ind. of renal function), vpt both feet

This model is indicative of 91.1% predictability for core 2 transferase.

6.13.5 **Monofilament and Creatinine Combined**

Table 6.13.5.i **Monofilament and creatinine regression – adjusted outcome**

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.952[a]	.906	.898	127.562

a. Predictors: (Constant), Mono 10g total, Creatinin levels (ind. of renal function)

This model is indicative of 89.8% predictability for core 2 transferase.

7.0 DISCUSSION

7.1 Scope

The purpose of this study was to explore the intrinsic factors implicated in the sequencing of diabetic foot ulceration. Given the established view that diabetic peripheral neuropathy remains a definitive marker for the increased risk of plantar ulceration (Ctercteko et al., 1981, Cavanagh et al., 1993, Albert and Chen 1996, Raginarson et al., 2001), the principal objective was to investigate the relationship between diabetic neuropathy and core 2 transferase specificity. Consequently, any resultant correlation would offer a tangible explanation as to the observed structural and metabolic alterations and moreover, establish potential mutuality with parallel studies in the field of retinopathy.

To achieve this objective, it was essential to view diabetic foot ulceration contextually, not as a secondary effect of diabetic neuropathy, but a separate entity in its own right. Whilst other long-term diabetic complications, such as retinopathy, nephropathy and autonomic neuropathy have been positively associated with poor glycaemic control and an altered metabolic state (UKPDS Group, 1996), diabetic foot ulceration appears to be the exception to the rule. Indeed, the traditional sequence of events is founded upon increased plantar pressure and foot deformity (Cavanagh et al., 1996, Armstrong 1999, Lord and Hosein, 2000). Consequently, the acknowledged pathogenesis for this complication is restricted entirely to factors, which act externally on the foot. This appears to contrast research into parallel diabetic complications, which

have successfully identified various metabolic routes (Ceriello, 2000, Fitzgerald and Brands, 2000, Ohmori, et al., 2000, Maejima et al., 2001).

Whilst the "external forces" scenario helps to promote plantar pressure and foot deformity as potential triggers, it conclusively fails to explain the inherent asymmetry of diabetic foot ulceration in vivo (Lavery et al., 2003). Hence, investigation into possible intrinsic factors provides the necessary "bridge" between the two schools of thought. This is particularly poignant, if the altered redox state in diabetes and metabolic slide towards peripheral neuropathy is considered (Cunningham et al., 1991, Asayama et al., 1993, Braunlich et al., 1994, Mukherjee et al., 1994, Paolisso et al., 1994, Sandaram et al., 1996). Plausibly, this sequence is further exacerbated by variables such as diet, thereby potentially corroborating tangential studies into socio-economic status and ethnicity (Ford et al., 1994, Bott et al., 2000, Weng et al., 2000, Harris, 2001, Ceriello and Sechi, 2002, Gulliford et al., 2003). Moreover, it would also support the view that long-term diabetic complications are both coincident and co-terminous (Urata et al., 2002).

Capillary occlusion, as a result of metabolic disturbance would seem to correlate with the pathogenesis of diabetic peripheral neuropathy and foot ulceration (Tesfaye et al., 1994, Kihara et al., 1995, Hotta et al., 1996b, Nakamura et al., 1999, Cameron et al., 2001, Cameron and Cotter 2002, Cameron et al., 2002b). This supposition is further substantiated by the observations of Edmonds and Foster (2000), who stated that "pure" neuropathic ulceration does not exist without a degree of ischaemic involvement. Similarly, both Tooke (1999) and Tesfaye et al., (1994, 1996) have demonstrated an

altered microcirculation in the diabetic individual. However, it was through work by Chibber et al., (2000, 2003) that core 2 transferase was found to be elevated in diabetic individuals with retinopathy and implicated in the PKC-mediated process of capillary occlusion. Above all, it was demonstrated that by modulating the expression of core 2 transferase, leukocyte / endothelial adhesion was curbed, thereby interrupting the occlusion cycle. Hence, it would appear that the PKC / core 2 transferase paradox is intrinsically linked, prompting further speculation as to the extent of the relationship.

7.2 PKC / Core 2 Transferase Co-dependence

7.2.1 PKC Inhibition Theory

The importance of PKC activity in nerve conduction velocity has been strengthened by data, which has shown that low doses of PKC inhibitors in diabetes-induced rats actually corrected nervous deficits (Nakamura et al., 1999, Cameron and Cotter, 2002, Cotter et al., 2002, Cameron et al., 1999). The proposed method of this reversal is by improved perfusion of the vasa nervosum (Kamiya et al., 2003). Additionally, in this and previous studies, it has been demonstrated that non-diabetic rats, administered with a chronic NOS inhibitor treatment, displayed NCV reductions, further heightening the relationship between PKC, NOS and neuropathic development (Cotter et al., 1995, Cameron et al., 1997).

This was reinforced by Chakravarthy et al., (1998), who found that PKC inhibition was able to partially restore NO production. Other animal studies

have also demonstrated that a hypoxic environment within the microcirculation stimulates the release of vascular endothelial growth factor (VEGF), leading to neovascularisation and increased capillary permeability, as observed in neuropathy and retinopathy (Tsujikawa, 2000, Obrosova et al., 2001). Moreover, Koya and King (1998) recorded a strong positive correlation between the presence of VEGF in a hypoxic environment and the increased activation of PKC, establishing the potential for mutual interaction. A likely mechanism for this particular interplay centres on the principle that, as VEGF Flk-1 receptors (found micro and macro vascular endothelial cells) are activated, they associate with PLCγ (Waltenberger et al., 1994, Guo et al., 1995). This, in turn, will effect the production of DAG and IP_3 (inositol triphosphate) to activate PKC via Ca^2 (Rhee et al., 1989, Nishizuka, 1992). This particular form of PKC activation is noteworthy, as it is initiated through a wholly non-hyperglycaemic route (Idris, et al., 2001).

The manifestation of endothelial dysfunction is an intrinsic feature of experimental diabetes and more recently, it has been reported by Tooke and Goh (1999) in first generation children of individuals with Type II diabetes, all of whom showed no previous evidence of glucose intolerance. Consequently, this raises the question as to whether oxidative stress, reduced antioxidant levels (glutathion and vitamin E) and the subsequent activation of PKC can act as indicators for the early development of vascular abnormality. The role of vitamin E has been partially explored by Keaney et al. (1996), who illustrated that vitamin E inhibits PKC activity via the DAG kinase pathway, offering protection against oxidised low density lipoprotein (LDL) triggered PKC within vascular endothelial cells.

Kunisaki et al., (1996) also recorded that an intraperitoneal injection of D-α-tocopherol (vitamin E) was able to significantly reduce the levels of DAG found in the aortas of diabetic rats, whilst simultaneously normalising retinal blood flow and glomerular filtration rates. Further studies have also shown that glucose initiated PKC activation is inhibited by administering vitamin E (Boscoboinik et al., 1991, Bursell and King, 1999, Lee et al., 1999). Moreover, the pathological effects of PKC stimulation, as displayed in the kidney and retina, were reversed following its administration (Kunisaki et al., 1995, Koya et al., 1997).

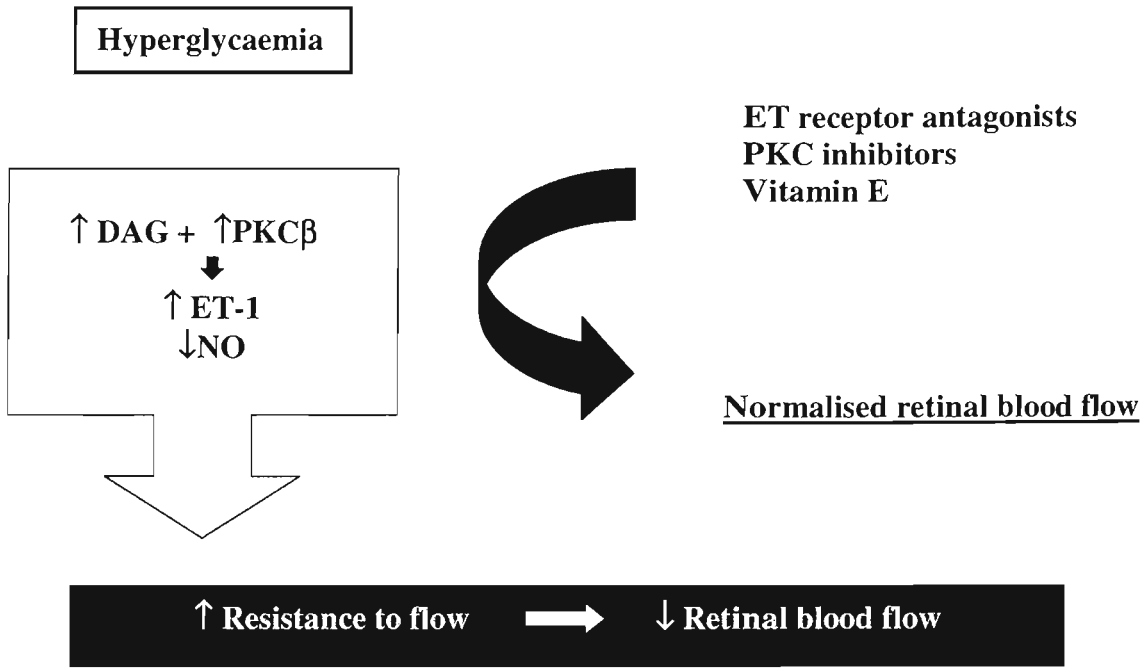
The role of vitamin E as an antioxidant and its ability to modulate the PKC signalling pathway has been further expounded by Ganz and Seftel (2000), who demonstrated that vitamin E was able to decrease the activated levels of PKCβII in the corpus cavernosal smooth muscle cells (invoked by a hyperglycaemic environment), whilst increasing the measured production of NO. These findings are in accordance with Keaney et al., (1996), who reported that D-α-tocopherol prevented endothelial dysfunction due to the impairment of PKC stimulation.

7.2.2 **PKC Microcirculation Theory**

Disturbance of the PKC / NOS balance could have potential detrimental effects on the vasa nervosum, as PKC activation causes the phosphorylation of smooth muscle contractile proteins (Cameron et al., 1999). Furthermore, it is indirectly implicated in the cascade sequence for the vasoconstrictors, endothelin-1 and

angiotensin II (Takagi et al., 1996, Park et al., 2000c, Stefanec, 2000). The latter may hold serious consequences for cell viability, as angiotensin can facilitate calcium ion entry into cells and is normally opposed by cyclic 3'5-adenosine monophosphate [cGMP] (Rang et al., 1996).

Figure 7.2.2.i **Proposed mechanism for decreased blood flow to the vasa nervosum**



Adapted from Way, K J., Katai, N., King, G. L. Protein kinase C and the development of diabetic vascular complications. Diabetic Medicine 18: 945-959

Given the down regulation or reduced activity of NOS and the subsequent reduced nitric oxide availability, cGMP is similarly restricted, so that the antagonistic effects of the dihydropyride sensitive channels are lost (Rang et al., 1996). This effectively renders the cell / neurone vulnerable to the cytotoxic effects of a calcium ion influx (Robertson et al., 1992, Kapelle et al., 1993).

Therefore, it would appear that the sequence for cellular damage / death follows a route identical to that accepted in the development of neuropathy. However, it is supposed that the latter is actually a by-product of the same process. If correct, this theory would corroborate the various clinical studies, which have cited neuropathy as a primary indicator for those individuals most at risk from diabetic foot ulceration (Murray et al., 1996, Mantey et al., 1999, Pham, 2000).

Hyperactivity of the polyol pathway and its interaction with PKC has also been implicated in the pathogenesis of diabetic neuropathy (Xia et al., 1995). Increased polyol pathway activity leads to an overall reduction in the phosphorylation of Na+-K+-ATPase, creating a deficit in nerve conduction and simultaneous accelerated nerve degeneration (Greene et al., 1992, Cameron et al., 1994, Cameron et al., 1996, Stevens et al., 1996, Cameron et al., 1997, Nakamura et al., 1999). Na+-K+-ATPase is an integral component of the Sodium Pump, which is vital for the maintenance of cellular excitability, cytoplasmic enzyme activity, cell volume and contractility (Vasilets and Schwarz, 1993).

Compromised Na+-K+-ATPase activity has been implicated in the prelude to diabetic neuropathy (Cameron and Cotter, 2002). This theory is founded upon the increased action of the polyol pathway leading to myo-inositol deficiency, causing alterations to phosphoinositide [PI] turnover (Kamiya et al., 2003). The consequent diminished levels of PI cause a reduction in DAG availability, so curbing PKC activity to effect a subsequent decline in the phosphorylation of the Na+-K+-ATPase α-subunit (Borghini et al., 1994b). However, whilst studies using aldose reductase inhibitors have corrected Na+-K+-ATPase

activity, they also appear to improve blood flow (Cameron et al., 1994, Cotter et al., 1995b, Cameron et al., 1996, Cameron et al., 1997). This is an important factor, as PKC inhibitors have also been shown to improve both nerve conduction velocity and blood flow, effectively rivalling the above (Jack et al., 1999, Nakamura et al., 1999).

Consequently, Cameron and Cotter (2002) proposed that the benefits observed in NCV and blood flow through PKC inhibition are separate from those of Na+-K+-ATPase activity. This alternative proposal for the PKC / Na+-K+-ATPase relationship has been further substantiated by studies, which have shown that the redress of Na+-K+-ATPase activity via acetyl-L-carnitine displayed no effect on NCV (Cameron et al., 1991, Stevens et al., 1996). In fact, the administration of evening primrose oil reduced Na+-K+-ATPase activity in diabetic rats, but failed to promote NCV dysfunction (Lockett and Tomlinson, 1992). Investigations using other antioxidants have also confirmed a clear boundary between PKC activity and Na+-K+-ATPase reductions (Cotter et al., 1995, Cameron and Cotter, 1995, Love et al., 1996). The outcome of these studies demonstrated that vitamin E, trientine and N-acetyl-L-cysteine increased nerve perfusion and NCV, but did not ameliorate Na+-K+-ATPase reductions. Consequently, there is no current evidence to suggest that PKC inhibitors achieve their beneficial effects by directly modulating Na+-K+-ATPase activity (Cameron and Cotter, 2002). Therefore, improvement following PKC inhibition may actually arise from the blocking of downstream events leading to vessel occlusion, a reduction in oxygen tension and the stimulation of factors such as VEGF (Xia et al., 1996).

One alternative explanation for reduced Na+-K+-ATPase activity is that it is, in fact, a compensatory reaction to conserve energy in relation to nerve hypoxia (Cameron et al., 1999). In the normal resting nerve, Na+-K+-ATPase activity accounts for approximately 70% of ATP consumption (Cameron et al., 1999). However, the observed 40% reduction in Na+-K+-ATPase activity within diabetes effectively "retards" hypoxic damage to other energy demanding aspects of the nerve (Ritchie, 1985). Consequently, this may provide an answer as to why overall concentrations of ATP in the diabetic peripheral nerve remain normal, whilst mitochondrial NADH/NAD^+ suggest endoneurial ischaemia (Low et al., 1989, Cameron et al., 1994b, Maxfield et al., 1995, Obrosova et al., 1997).

Hyperglycaemia-induced DAG and PKC availability has been seen to promote heightened levels of phospholipase A_2 (Way et al., 2001). This, in turn, unleashes arachadonic acid, stimulating the production of prostaglandins (prostaglandin E2 [PGE_2]) and inhibiting Na+-K+-ATPase (Clark et al., 1991, Xia et al., 1995). Hence, inhibition of PKC prevents reduced Na+-K+-ATPase activity in vascular smooth cells exposed to elevated glucose concentrations (Xia et al., 1995). Similarly, the administration of a phospholipase A_2 inhibitor was observed to have equal efficacy (Oskarsson et al., 1999).

Of note, phospholipase A_2 is metabolised by one of two fatty acid cyclo-oxygenases, (COX-1 and COX-2), which in turn initiate the biosynthesis of the prostaglandins and thromboxanes responsible for the inhibition of platelet aggregation, vasoconstriction and inflammatory processes (Rang et al., 1996). Consequently, this may substantiate the apparent success of aspirin in halting

the long-term complications of diabetes. Traditionally, the proponents of aspirin have tended to concentrate on apparent reductions in hypertension (Paulson and Eversole, 1977). Whilst its anti-hypertensive properties may undoubtedly be effective, the primary function would seem to be its ability to modify the biochemical steps occurring in relation to the activation of PKC.

Nakamura et al., (1999) further investigated the link between the polyol pathway and PKC in neuropathy, where a specific PKCβ inhibitor (LY333531) and an aldose reductase inhibitor (NZ-314) were able to ameliorate nerve deficits and increase blood flow. This effectively assists the supposition that these two previously isolated factors may work in disfigured harmony to produce pathological results. Early studies by Cameron et al., (1999) using low doses of the PKC inhibitor WY151003, successfully restored a 50% reduction in sciatic blood flow and was further able to correct Na+-K+-ATPase activity. Investigation utilising animal models and LY333531 has yielded promising results, as it appears to reduce PKC tissue activity, whilst providing functional improvements, most particularly, in glomerular filtration rate (Inoguchi et al., 1994, Ishii et al., 1996, Koya and King, 1998, Nakamura et al., 2001). Experimental diabetic models in relation to the eye have also displayed that PKC inhibition improved blood flow, reduced retinal permeability and neovascularisation, whilst correcting reduced Na+-K+-ATPase and Ca^{2+}-ATPase activity (Aiello et al., 1997, Danis et al., 1998, Kowluru et al., 1998).

The role of PKCβ in the pathogenesis of microangiopathy was confirmed by the treatment of PKCβ over-expressing transgenic mice with the inhibitor LY333531, which improved functional histological alterations in the kidney and

heart (Koya et al., 1997b, Wakasaki et al., 1997, Koya and King, 1998). Work on human mesangial cells conducted by Koya et al., (1997) revealed that LY333531 attenuated PKC activation and stabilised arachadonic acid release, PGE_2 synthesis and Na+-K+-ATPase activity. However, whilst this supports the existing studies linking PKC with VEGF, oxidative stress and reduced blood flow, the step(s) between causal factor and end result still remain unclear.

7.2.3 **PKC and Neuropathy Theory**

Endothelial dysfunction and increased cellular adhesion has also been linked to the activation of PKC specific isoforms (Idris, et al., 2001, Chibber et al., 2003). Evidence has shown that impaired NO-mediated vasodilation and the up regulation of ET-1 in the retina are downstream effects of the PKC-modulated inhibition of constitutive nitric oxide synthesase (cNOS) and induction of ET-1 gene expression (Takagi et al., 1996, Chakravarthy et al., 1998, Chen et al., 2000, Park et al., 2000c). The extent of ET-1 modulation has been shown as an over-expression of PKCβ and increased ET-1 mRNA levels in basal and glucose stimulated conditions (Park et al., 2000). Additionally, diabetes has been shown to enhance the activity of ET-1 and angiotensin II in the vasa nervosum, with nerve conduction being corrected by ET-1 and AT-1 antagonists (Maxfield et al., 1995, Cameron et al., 2001b).

Thus, PKC acts as a catalyst for vasoconstriction, corroborating studies using vasodilatory agents, which have corrected NCV and blood flow (Cameron et al., 1999, Jack et al., 1999, Nakamura et al., 1999). Furthermore, it is linked appreciably with the pathological increase of leukocyte adhesion and capillary

occlusion, which appears to be initiated by PKCβ (Murigi et al., 1998, Puente et al., 2001, Chibber et al., 2003). Seemingly, vasodilatory agents assist in reducing the level of ischaemia post occlusion by dilating the lumen of the vessel at the site of constriction. This is important, as occlusion would rarely exist in a total state, rather, it would be initially partial, increasing unless halted. This mirrors the progressive, degenerative process of peripheral diabetic neuropathy precisely (Thomas and Lascelles, 1965, Dyck et al., 1986, Cameron et al., 2001).

Monocyte binding to endothelial cells has been implicated as an early determinate of atherosclerosis (Ikeda et al., 1998). The potential for PKC activation in this process has been suggested, as the hyperglycaemic induced PKCβII isoform is notably increased in monocytes from diabetic individuals (Ceolotto et al., 1999). Conversely, a 40% reduction of the PKCβII isoform was observed in euglycaemic conditions (Ceolotto et al., 1999). The adhesion process itself requires specific molecules to orchestrate the monocyte-endothelial interaction and these have primarily been identified as P- and E-selectins, intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule [VCAM] (Li et al., 1996, Park et al., 2000b, Lowe, 2002, Leppanen et al., 2003).

A high glucose environment will lead to the increased expression of ICAM-1 via the PKC-NFκβ-dependent source (Park et al., 2000b). Increased leukocyte adhesion has also been attributed to retinal microcirculatory disturbances within diabetic rats (Nonaka et al., 2000). In this study, it was found that the administration of LY333531 attenuated this abnormality, further corroborating

the role of PKCβII in increased leukocyte / endothelial adhesion, capillary occlusion and a subsequent reduction in blood flow. Oral dosing of LY333531 in diabetic rats also confirmed that it was able to prevent a reduction in motor conduction velocity and sciatic nerve blood flow (Nakamura et al., 1999). This lead the authors of the study to conclude that the beneficial functional corrections were due to the properties of LY333531 acting upon apparent ischaemia in the endoneurial microcirculation.

Ischaemia to the microcirculation is now providing a probable route to diabetic peripheral neuropathy (Cameron and Cotter 2002). This theory has been grounded in studies, which have investigated oxygen tension in diabetes using both human participants and animal models (Cameron et al., 2001, Kihara et al., 1995, Hotta et al., 1996b). The findings of these studies have confirmed that ischaemia plays a key role in the development of neuropathy. It has similarly been forwarded that PKCβ activity is increased in the vasa nervosum, as a result of hyperglycaemia and/or oxidative stress (Cameron and Cotter, 2002). Consequently, nerve perfusion and function is marred, given the ability of PKC to easily quench NOS via phosphorylation (Hirata et al., 1995). This would mirror the aetiological processes involved in diabetic retinopathy and furthermore, explains the success of PKCβ specific inhibitors generally.

7.2.4 **Leukocyte Stasis**

The pathological implications of the adhesion process should also be considered, as any abnormal increase in P-selectin expression will lead to leukocyte stasis, causing either partial or total occlusion of the vessel (Nash et al., 2001). Indeed,

the increased stasis of activated leukocytes adhered to the endothelium may also inhibit them from leaving the vasculature altogether (Djaldetti et al., 2002). Consequently, the ROS released by these phagocytes are free to cause sustained tissue injury (Wagner and Roth, 1999).

7.2.5 **Core 2 Transferase Up regulation**

The alteration of core 2 transferase expression has been historically associated with pathological conditions, such as leukaemia and Wiskott-Aldrich syndrome (Brockhausen et al., 1991, Higgins et al., 1991). The implications of core 2 transferase over-expression have also been seen in animal models (Tsuboi and Fukuda, 1997). Transgenic mice were observed to exhibit a T cell over-expression and a reduced / delayed hypersensitivity immune response. A further study conducted by Tsuboi and Fukuda (1998) extended research into this area and found that an over-expression of T cells lead to signalling dysfunction between T and B cells, thus impairing the immune response. The association between core 2 transferase and increased P-selectin binding has even been recorded in Chinese hamster ovary cells (Kumar et al., 1996). This has been further supported by an additional study, which found that core 2 transferase was essential for PSGL-1 maximal binding (Li et al., 1996).

Investigation into core 2 transferase is therefore vital, as it appears to be elevated in diabetic patients with retinopathy and appreciably linked to cell death (Chibber et al., 2000). Furthermore, given the association between over-expression in animal models and a reduced immune response, this could help

explain why diabetic individuals show a predilection for infection (Djaldetti et al., 2002).

Unfortunately, the precise mechanisms responsible for the increased expression of core 2 transferase are presently unknown. However, Nisho et al., (1995) found that hyperglycaemia modulates core 2 transferase activity in rat cardiomyocytes via the diacylglycerol-PKC pathway. The importance of PKC activation has been further highlighted through an additional study by Chibber et al., (2003), which demonstrated that the administration of a PKCβII-specific inhibitor LY379196 (0, 25, 50 nM: Eli Lilly, USA) attenuated endothelial cell adhesion. Through this work, the role of PKCβII in the phosphorylation of core 2 enzymes in increased leukocyte / endothelial cell adhesion has been positively linked to capillary occlusion in diabetic retinopathy. Indeed, it is this very environment, which is thought to promote further ROS released by adherent cytokines, thereby establishing a positive feedback for increased oxidative stress, catabolism of NOS by PKC and resultant core 2 up regulation (Hahne et al., 1993, Bussollino and Camussi, 1995, Hirata et al., 1995, Glowacka et al., 2002, Chibber et al., 2003).

Prior to the undertaking of this study, retinopathy was the only area within diabetic medicine to have benefited from investigation into this enzyme. However, given the close parallel between retinopathy and neuropathy, the role of core 2 transferase may well prove to be the Rosetta Stone needed to further the understanding of the metabolic factors implicated in the increased risk of neuropathic foot ulceration.

7.3 Core 2 Transferase between the Groups

The results from this study demonstrated that individuals with diabetes displayed a significantly higher expression of core 2 transferase compared to the control group ($p<0.001$). Furthermore, the results also demonstrated that individuals with diabetic peripheral neuropathy displayed the highest levels of core 2 transferase, when compared to the diabetic and non-diabetic control groups respectively ($p<0.001$), thus confirming the original hypothesis. Whilst this is the first time that core 2 transferase has been investigated in diabetic neuropathic individuals, these findings mirror the work conducted by Chibber et al., (2000) in relation to retinopathy. However, Chibber's et al., (2000) work displayed notably higher readings of core 2 transferase than found within this study.

One potential explanation for this occurrence is participant profile. As this study deliberately excluded diabetic individuals with retinopathy, it allowed peripheral neuropathy to be effectively isolated. Yet, as the two conditions are often coincident, a possible synergistic effect between the two complications may well exist. Indeed, peripheral neuropathy could even be regarded as an incremental step towards retinopathy itself, befitting the "distal to proximal" model.

However, the geopolitical limitation of this study provides a more viable proposition as to the apparent difference in core 2 transferase yield. Given positive associations between core 2 transferase up regulation, hyperglycaemia-

mediated PKCβII activation (Porte and Schwartz, 1996, Hempel et al., 1997, Ceolotto et al., 1999, Chibber et al, 2003) and the known inhibitory effects of antioxidants (Chow, 1991, Cotter et al., 1995, Keegan et al., 1995, Baynes, 1991, Laight, et al., 2000, Cameron et al., 2001), nutritional condition is therefore imperative. Hence, a small, relatively affluent population may have unduly stunted the expression of core 2 transferase, whereas the findings of Chibber et al., (2000) remain more consistent with a target population drawn from a largely central London location. If true, this would elevate the observed prevalence of neuropathic ulceration within certain socio-economic bandings (Weng et al., 2000) to a political level.

7.4 Core 2 Transferase and Ulceration

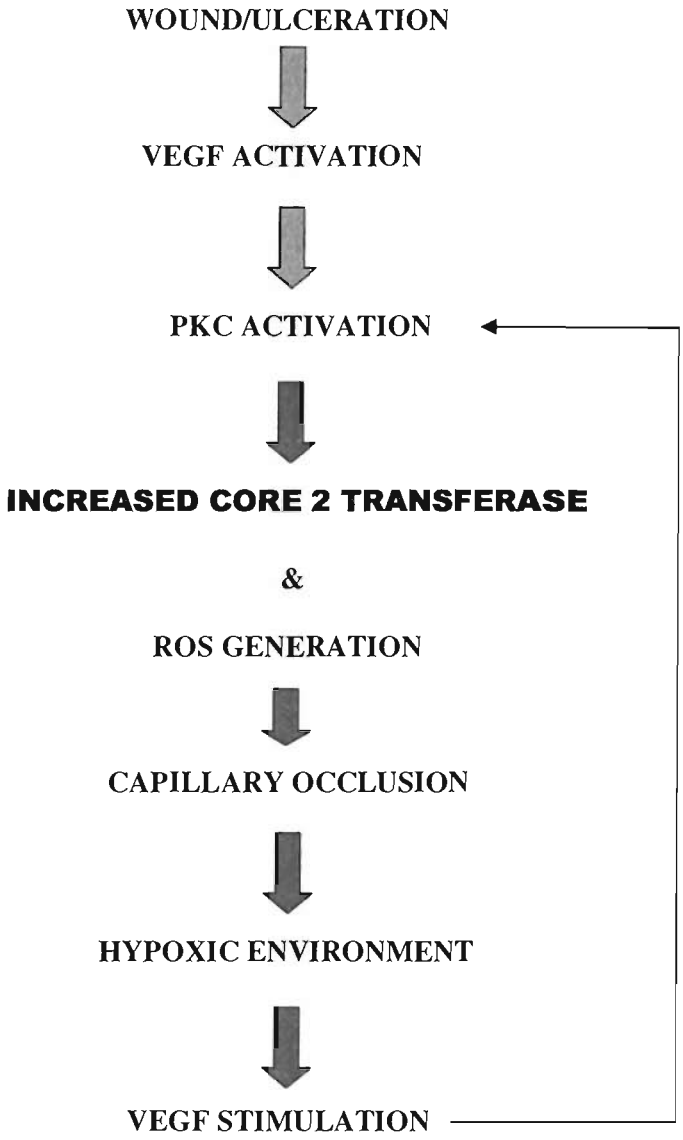
The findings of this study indicated that neuropathic individuals with a history of ulceration (within a 12 period) had a significantly higher level of core 2 transferase compared to those without ($p< 0.01$). In particular, it was shown that 4 of the 5 highest core 2 transferase readings belonged to individuals, who had experienced recent ulceration.

As core 2 transferase is significantly elevated in the neuropathic group versus the two controls and moreover, the recorded incidences of ulceration are all DN group specific, it could be argued that the process of diabetic foot ulceration is a by-product of the same pathological process. Indeed, increased leukocyte / endothelial adhesion, capillary occlusion and a shift in the redox state will potentially facilitate the development of neuropathy and concurrently, provide an environment ripe for ulceration. Similarly, this form of microcirculatory /

endothelial dysfunction would fit in with existing theories regarding the pathogenesis of other diabetic complications, such as diabetic nephropathy and retinopathy (Ceriello, 2000, Fitzgerald and Brands, 2000, Ohmori et al., 2000, Maejima et al., 2001).

The protracted up regulation of core 2 transferase observed amongst neuropathic individuals with a history of ulceration may be explained by the sustained presence of VEGF. Normal wound resolution requires VEGF to promote both angiogenesis and the coagulation cascade (Verheul et al., 2002). However, capillary occlusion within the microcirculation actually promotes VEGF expression due to the resultant pseudo-hypoxia (Gasmi et al., 2002). Such excursions beyond baseline levels will, in turn, potentiate PKCβII, core 2 transferase and further reactive oxygen species (Chibber et al., 2000, 2003). Moreover, this route would help address the apparent increased risk of re-ulceration (Figure 7.4.1). Similarly, given the pro-thrombotic properties of VEGF on platelets, its imbalance may offer an insight into increased blood viscosity in diabetes generally (Verheul et al., 2002).

Figure 7.4.1 **Potential route for sustained core 2 transferase up regulation**



Key:

= Normal

= Proposed pathological mechanism

Note: The above figure would generate an environment ripe for re-ulceration, when the appropriate established catalysts are applied, i.e. elevated plantar pressure and external trauma

7.5 Duration of Diabetes between the Groups

'T' test analysis of this variable showed that no significant difference existed between the two diabetic groups (p=0.945). This finding was considered of particular relevance, as it established a datum, from which core 2 transferase could be examined. Whilst it is accepted policy to consider this dimension, it should be recognised that diagnosis and duration are unlikely to coincide. Indeed, it has been reported by Zimmet (2003) that diabetes may go undiagnosed in individuals for up to seven years. Consequently, insidious metabolic damage and disarrangement may occur subversively within this timescale. Hence, whilst it is still considered appropriate to acknowledge the date of diagnosis, this should not be confused with the actual duration.

7.5.1 Duration of Diabetes and Core 2 Transferase

Analysis of this variable was negated by the findings of the previous section. However, it should be emphasised that core 2 transferase up regulation is not necessarily dependent upon time, as the key routes for PKC translocation (hyperglycaemia, oxidative stress and NEFA's) are inherently non-linear. Hence, studies linking the duration of diabetes to associated complications (Tesfaye et al., 1996, UKPDS Group, 1996, UKPDS Group, 2000) have, in all likelihood, acted simply as a formal chronology of ensuing metabolic events.

7.6 VPT between the Groups

Vibration Perception Threshold measurement is a commonly used, validated tool to monitor the potential loss of vibratory sensation in diabetic individuals (Boulton et al., 1986, Young et al., 1994, Coppini et al., 1998, Sosenko et al., 1999, Pham et al., 2000). As a result of this study, a significant difference between the groups was identified and further verified through Post Hoc analysis ($p<0.001$). During the data collection phase, VPT's for left foot, right foot and both feet were recorded and ANOVA's conducted for each variable ($p<0.001$). However, it was decided that only the VPT (both feet) modality should be carried into regression modelling, given its higher 'F' value over the left and right foot respectively ($F=105.76$ vs. $F=91.59$ vs. $F=80.80$). Moreover, it is current doctrine to obtain a mean VPT, when conducting neurological testing in individuals with diabetes (Coppini et al., 1998). Of note, this is consistent with existing procedure at Poole Hospital NHS Trust.

These findings echo a previous study, which has validated VPT measurement in comparison to nerve conduction velocities [NCV] (Coppini et al., 1997). Hence, it is proposed that elevated VPT's in the diabetic neuropathic group occurred as a result of microcirculatory occlusion, leading to reduced blood flow and a corresponding NCV abatement. Indeed, this supposition builds upon the recognised association between reduced nerve blood flow and nerve condition (Cameron et al., 1999). Of note, studies have further demonstrated a restoration of nerve blood flow following the administration of PKC inhibitors and/or antioxidant therapies (Chow, 1991, Cotter et al., 1995, Keegan et al., 1995, Baynes, 1991, Laight, et al., 2000, Cameron et al., 2001).

The generation of a pro-ischaemic environment via capillary occlusion would seem to provide a pathological process, which facilitates peripheral neuropathy and foot ulceration as secondary events. This theory is, in part, substantiated by work conducted by Young et al., (1994), who found that individuals with a VPT ≥ 25 V were seven times more likely to ulcerate than those with a VPT ≤ 15 V. Whilst this work has undoubtedly provided vital clinical indicators, the 25 V VPT threshold does not actually predict neuropathy, rather an increased risk of ulceration. Consequently, clinicians have tended, albeit unwittingly, to confuse the boundaries between neuropathic onset and ulceration risk. As a result of this study, it is supposed that the latter is a natural consequence of the former.

As expected, Pearson's correlation testing showed a negative correlation between VPT values and 10g monofilament detection, in keeping with previous study (Armstrong et al., 1998, Paisley et al., 2002). This particular finding not only validates the selection methods employed within the composition of the participant groups, but moreover, confirms the combined use of VPT and 10g monofilament as clinical tools.

7.6.1 **VPT and Core 2 Transferase**

This study has provided the first ever, positive correlation between VPT and core 2 transferase ($p<0.001$). Therefore, the notion that core 2 transferase is elevated in diabetic neuropathic individuals has been fulfilled. Moreover, given that the diabetic neuropathic group displayed significantly higher VPT's throughout, this particular experimental hypothesis has been demonstrated.

Further analysis of this variable also revealed that participants with a VPT ≥ 15 V displayed a significant positive correlation with core 2 transferase ($p<0.01$). Consequently, whilst core 2 transferase remains highest in the diabetic neuropathic group, significant changes appear to be occurring prior to the currently accepted VPT risk threshold of 25 V. This is particularly relevant, as an ability to detect factors influencing the microcirculation prematurely, would allow early prevention strategies to be developed, to include antioxidant intervention therapies and human trials of PKC inhibitors. Hence, the VPT / core 2 transferase correlation offers a vital clinical link using an established, low-cost method. Indeed, the singular employment of VPT as a co-variate during regression modelling suggested an adjusted predictability of 82.9% for core 2 transferase.

7.7 10g Monofilament between the Groups

This study revealed highly significant differences between the groups for all three modalities of 10g monofilament detection (left foot, right foot and both feet), with the diabetic neuropathic group displaying the most reduced rates ($p<0.001$), thus confirming the experimental hypothesis. This data is consistent with other findings, which have demonstrated a positive correlation between 10g monofilament insensitivity and diabetic peripheral neuropathy (Kumar et al., 1991, Sosenko et al., 1999, McGill et al., 1999, Booth and Young, 2000). Similarly, the methods employed for both participant selection and classification of neuropathic status are validated (Boulton et al., 1986, McGill et al., 1999, Sosenko et al., 1999, Pham et al., 2000, Paisley et al., 2002). .

Previous work by both Birke and Rolfson, (1997) and Litzelman et al., (1997) implies that an inability to detect a 10g monofilament is indicative of an increased risk of foot ulceration. However, it is argued that this assumption is entirely circumstantial and is based largely upon the individual's neuropathic status. That is not to say that it is irrelevant, as it clearly offers an important clinical marker, but nevertheless, it categorically fails to identify which particular individual will ulcerate. Consequently, the acknowledgement of other co-factors, chiefly core 2 transferase, will potentially facilitate this process.

The "degenerative process" hypothesis is supported by the results of this study, as controls were found to have the highest rate of 10g monofilament detection, followed by diabetic individuals without complications and diabetic neuropathic individuals respectively. The common factor involved within this process would appear to be Type II diabetes mellitus, with core 2 transferase acting as the principle antagonist. Hyperglycaemia is known to promulgate a host of metabolic responses, such as PKC activation and AGE product stimulation (Porte and Schwartz, 1996, Hempel et al., 1997, Ceolotto et al., 1999, Ceriello, 1999, Wautier et al., 2001, Dickinson et al., 2002). Indeed, the latter has been observed to react readily with collagen to cause stiffening of the dermal layers (Melling et al., 2000). Therefore, it would seem logical that 10g monofilament detection rates could be unduly affected by this so-called "leathering". Whilst it is not predicted as linear event, such occurrences may well contribute to the generally sporadic distribution of neuropathy within a given diabetic population (Boulton and Ward, 1986).

7.7.1 **10g Monofilament and Core 2 Transferase**

This study indicated a significant negative correlation between 10g monofilaments and core 2 transferase ($p<0.001$). The identification of this particular association is extremely valuable, as it establishes a sound basis for further research into this area. Indeed, it is suggested that current diabetic management strategies tend to follow a "scattergun" approach, lacking any definitive markers for foot ulceration and neuropathic development. Hence, given expansion of the proven relationship between 10g monofilament detection, VPT $\leq$ 15 volts and core 2 transferase specificity, a more accurate and economical intervention plan may be attainable.

7.8 **Age between the Groups**

Age between the groups was seen to be significantly different, when comparing control participants to both diabetic study groups ($p<0.05$). However, no such difference existed between the diabetic groups themselves. Hence, it can be stated categorically that the age factor did not influence core 2 transferase specificity.

The study also revealed a positive correlation between age and weight ($p<0.05$). A further positive correlation between age and BMI was also observed ($p<0.05$). Sedentary lifestyle offers one explanation for this particular event, although reduced wealth in retirement should also be considered within this context. Due to the associated cost and travel required, recreational activities are becoming increasingly denied to the less affluent.

7.8.1 **Age and Core 2 Transferase**

Surprisingly, this study demonstrated a positive correlation between age and core 2 transferase ($p<0.01$), despite acceptable matching within the diabetic study groups ($p>0.05$). Arguably, this outcome is due to the extraneous influence of the control group, which was significantly younger ($p<0.05$) and displayed significantly less core 2 transferase ($p<0.001$), in comparison to the diabetic groups.

However, the role of age with specific regard to core 2 transferase may actually be influenced by a reduced endogenous and/or exogenous antioxidant uptake, leading to PKC translocation. Antioxidant absorption and metabolism is thought to be affected by the process of ageing, consequently reducing the body's inherent resistance to oxidation (Nieves, 2003). If true, this would correlate with the greater mean ages observed amongst the diabetic individuals generally (control mean 41.40 SD ± 10.78, DNC mean 53.80 SD ± 6.09 and DN mean 59.60 SD ± 9.72). As a result, increased age would become consistent with the development of Type II diabetes and its associated long-term complications.

7.9 **Height between the Groups**

Whilst the diabetic neuropathic group displayed a generally increased height over the other groups (DN mean 178.52 cm SD ± 8.73 vs. DNC mean 170.47 cm SD ± 6.89 vs. Control mean 170.40 cm SD ± 9.48), the difference did not achieve statistical significance ($p>0.05$). However, the inherent characteristics

would nevertheless appear to mimic the findings of Sorensen et al., (2002), who found a positive correlation between height and peripheral neuropathy. Potentially, the relatively small sample population employed within this study might explain this particular outcome, although the numbers comfortably exceeded the power calculations. A more probable cause, however, is founded upon the homogeneity of ethnic origin within the participant banding. Given very similar socio-economic backgrounds and locale, the height consistency between the groups may have been unduly tilted, as contributory factors on growth, i.e. diet and ethnicity, would tend to predominate.

7.9.1 **Height and Core 2 Transferase**

The results from this study displayed a significant positive correlation between height and core 2 transferase ($p<0.01$), further corroborating Sorensen et al., (2002). However, the height and neuropathy theory, coupled with the acknowledgement of core 2 transferase activity, highlights a further potential event. Feasibly, the up regulation of core 2 transferase will facilitate leukocyte stasis and thereby, peripheral nerve and tissue damage (Kumar et al., 1996, Li et al., 1996, Maly et al., 1996, Ellies et al., 1998, Chibber et al., 2000, 2003). Hence, given a corresponding positive relationship between height and weight ($p<0.01$), increased load translation onto a compromised cellular structure would undoubtedly exacerbate tissue breakdown.

Accordingly, this supposition would call previous investigations into the pressure variables of gait and associated ulceration risk into immediate question, as anthropometric factors have been largely ignored throughout (Ctercteko et

al., 1981, Duckworth, Boulton and Betts, 1985, Veves et al., 1992, Armstrong et al., 1998). Similarly, the height / neuropathy parallel may be subject to core 2 transferase interference via alternative routes. Given a hypothetically increased nerve length, the accompanying vasa nervosum would provide a correspondingly greater area to interlock with core 2 transferase structures. Consequently, as the vessels progress distally, those with the smallest lumen would become occluded first. This would cause hypoxia and ultimately cell death in a "dying back" distribution (Said et al., 1992), manifesting the distal to proximal progression of diabetic peripheral neuropathy precisely (Allen et al., 1997, Thomas, 1997).

Alternatively, longer nerve fibres associated with taller individuals may actually lack an increased vasa nervosum network to compliment the essential nutrient requirement. Whilst these theories are, as yet wholly unsubstantiated, due to the invasive procedures necessary, they should not be readily dismissed. As height is relatively stable upon reaching maturity, other co-existing factors must be present to predispose taller individuals to neuropathy.

7.10 Weight between the Groups

Participants in the diabetic neuropathic group were seen to be significantly heavier than those in the control and diabetic no complications groups ($p<0.01$). Yet, diabetic individuals without complications were, in strict terms, not significantly different from control participants ($p=0.051$). Whilst this difference only marginally failed the significance threshold, the two diabetic groups nevertheless displayed consistently higher mean weights over the control

group (DN mean 103.66 kg SD ± 17.54, versus the DNC mean 89.53 kg SD ± 22.77, versus the Control mean 62.80 kg SD ± 15.12). Notably, obesity / excess weight has been positively associated with the increased risk of diabetic onset (Pan et al., 1997, Aguilar-Salinas et al., 2002, Hu et al., 2003).

Age was also observed to positively correlate with weight ($p<0.05$). Indeed, it could be suggested that older individuals generally take less exercise and/or lead a more sedentary lifestyle, so promoting weight gain. This supposition is possibly confirmed by virtue of the age difference between the groups. Of note, the control group was shown to be significantly younger ($p<0.05$), whilst both diabetic groups were generally well matched ($p>0.05$). When further analysing weight across the groups, a positive correlation between height and weight was also apparent ($p<0.01$). However, a comparison of BMI between the groups perhaps serves as a better measure of this particular variable.

Theoretically, excessive body mass will exaggerate the point loading of structures during gait. Indeed, kinetic energy and pressure translation onto the plantar aspect of the foot will be simultaneously enhanced. Hence, given the background of a compromised cell structure due to core 2 transferase interaction, the foot will effectively be rendered incapable of resisting daily stress. Consequently, it would logically follow that the favoured site for neuropathic ulceration would tend to correlate with the most apparent "wear pattern".

7.10.1 **Weight and Core 2 Transferase**

Weight demonstrated a positive correlation with core 2 transferase ($p<0.05$). This factor is especially noteworthy, given the anecdotal role of poor diet upon exogenous antioxidant availability. As a result, non-esterified fatty acids (NEFA's) may become elevated in Type II diabetes (Riemens et al., 2000), leading to the direct stimulation of DAG and PKC (Diaz-Guerra et al., 1991, Lu et al., 2000). This apparent "domino effect" represents an extremely hazardous and unseen threat to the diabetic individual, as no hyperglycaemic stimulus is actually required. This particular route to PKC stimulation is potentially enhanced by the significant correlation observed between triglycerides and core 2 transferase ($p<0.05$). Notably, increased triglyceride saturations help promote hydrolysis, so facilitating NEFA generation (Riemens et al., 2000).

On a more reactionary level, it could be stated with relative security that an inappropriate dietary intake amongst diabetic individuals will naturally affect both body weight and euglycaemia, so stimulating the PKC / core 2 transferase cascade. Moreover, this theory parallels the findings of the UKPDS (1996) and DCCT (1993), regarding glycaemic control and diabetic complications generally.

However, further recent research has suggested that adipose cells are not passive entities, but can function as an independent endocrine organ (Gaudiot et al., 1998, Mick et al., 2002). In obesity, there is notable growth and hypertrophy of adipose tissue, potentially producing both the NOS I and NOS II isozymes (Mick et al., 2002). In turn, this may expedite a tilt in the redox state,

so aiding the promotion of peroxynitrite and the further activation of PKC (Ishii et al., 1996, Szabo, 2003).

The insulin-mediated release of VEGF can also be initiated by adipose cells (Mick et al., 2002), triggering the PKCβII / core 2 transferase cascade. In Type II diabetes, insulin is often present, but at an insufficient level to achieve glucose transport (Anderson et al., 1990). However, this "dripping tap" allows certain pathogenic events to ensue, including increased vascular smooth muscle cell growth and VEGF release from adipose tissue (Mick et al., 2002). Nevertheless, despite the potential increase in basal VEGF availability, Borsheim et al., (2000) found that fat tissue blood flow was actually attenuated in obesity, suggesting the possible diversion of VEGF into the PKC / capillary occlusion cycle.

7.11 BMI between the Groups

The data produced from this study revealed that BMI was significantly different between the groups ($p<0.01$). Further analysis revealed a significant difference between the control and diabetic groups ($p<0.01$), although there was no such inter-group association within the diabetic population. Whilst diabetic neuropathic individuals were seen to be consistently heavier, the factor of height appears to annul this event.

The lowest BMI mean values were exhibited by the control group (21.37 SD ± 3.36), compared to the diabetic no complications group (30.50 SD ± 6.33) and diabetic neuropathic group (33.48 SD ± 3.90) respectively. One primary

explanation for this disparity has been previously discussed, i.e. activity level. Similarly, the apparent lack of statistical difference between the diabetic groups may be attributed to the apparent dominance of height over weight.

As expected, the BMI values positively correlated with weight ($p<0.01$). However, height also positively correlated with BMI ($p<0.01$). This may be attributed to the mean heights recorded for the diabetic neuropathic group, although this particular variable was initially seen as insignificant ($p>0.05$). Regardless, it is maintained that the BMI measurement offers greater balance and remains less susceptible to possible eccentricities.

7.11.1 **BMI and Core 2 Transferase**

This study showed that BMI positively correlated with core 2 transferase ($p<0.01$), thus satisfying the experimental hypothesis. The pathogenesis for this variable follows the two theories already outlined in Sections 7.8.1 and 7.9.1. Furthermore, it would support the work conducted by Abouaesha et al., (2001), who demonstrated that plantar tissue thickness is significantly reduced in diabetic individuals with elevated BMI's. Consequently, this would potentially unite the existing work on tissue atrophy (Landsman et al., 1995) with accumulating evidence for inflammation, cellular death and their possible role in diabetic foot ulceration (Koide et al., 1993, Imai et al., 1994, Nagoshi et al., 1998, Higami and Shimokawa, 2000).

Throughout the biometric analysis, core 2 transferase has shown a consistent relationship with all modalities. However, BMI has proved to be the most

statistically significant method in determining the level of association. The use of this method is considered to be particularly relevant, as it incorporates routine clinical data. Expansion of this practice would, it is envisaged, not only assist further research into core 2 transferase, but patient health and loading of the foot generally.

7.12 Biochemical Markers for Core 2 Transferase

All the biochemical variables selected and discussed are routinely taken in primary and secondary care, thus future consideration of these factors would incur no additional discomfort to the participant or financial burden to the NHS. The importance of finding such markers for core 2 transferase may have profound implications for future research, as the assay itself is highly specialised and relatively costly. Consequently, the identification of individuals with elevated core 2 transferase, by means of a biochemical and/or clinical screening process, would be extremely beneficial. Of note, the assays adopted within this phase followed existing validated protocols and were performed in an accredited laboratory.

7.12.1 Glycated Haemoglobin and Core 2 Transferase

This study demonstrated that HbA1c's showed a highly significant positive correlation with core 2 transferase ($p<0.01$). These findings correspond with previous work, implicating hyperglycaemia in the translocation of PKCβ and subsequent core 2 transferase up regulation (Morigi et al., 1998, Snapp et al., 2001, Sperandio et al., 2001). Furthermore, this would agree with the UKPDS

(1996) and DCCT (1993), implicating the role of hyperglycaemia in diabetic complications generally. However, it is essential that HbA1c's are observed contextually. Instead of being a simple indicator of glycaemic control, they should also be considered as a robust predictor of AGE specificity and the glycation process (Boel et al., 1995, Ceriello, 1999 and Singh et al., 2001).

As discussed in Section 1.9, AGE products have the potential to generate ROS and in turn, stimulate PKC. Consequently, a positive correlation between HbA1c's and core 2 transferase would seem to support the tenet of mutuality. Given that AGE products have a propensity towards quenching NO, the vasodilatory response during oxidative stress is severely impaired, so promoting VEGF, PKC / core 2 transferase and ultimately, capillary occlusion. Indeed, this supposition indirectly correlates with that of Schmidt et al., (1995) and Sengoelge et al., (1995), who both found that AGE products appeared to up regulate ICAM molecules. This theoretical "undermining" of the cellular matrix facilitates the process of ulceration, as external stresses may be readily translated into wounds due to a restricted nutrient supply.

This study also revealed a positive correlation between HbA1c's and blood glucose ($p<0.01$). Whilst this direct correlation has no immediate bearing on core 2 transferase, it does act in accordance with previous work, which has demonstrated associations between these two variables (Nisho et al., 1995, Chibber et al., 2000). Consequently, this further supports the validity of the HbA1c data, allowing certain concepts to be developed.

HbA1c's also positively correlated with the cholesterol / HDL ratio. However,

cholesterol failed to show any significant correlation with HbA1c's, when analysed separately (p=0.090), indicating that it is the HDL component, which undergoes alteration. This may have interesting applications, as hyperglycaemia and AGE products have already been implicated in foam cell pathogenesis and atherosclerosis (Hoff et al., 1992, Stitt and Friedman, 1997). The glycation of HDL's leads to substantial modification, reducing the cholesterol ester transfer protein [CETP] availability (Lemkadem et al., 1999). As a consequence, cardiovascular protective factors are negatively affected, permitting increased ROS generation (Dickinson et al., 2002). Indeed, it has previously been recognised that low HDL levels act as a predictive marker for diabetic foot lesions (Litzelman et al., 1997).

7.12.2 **Glucose and Core 2 Transferase**

This study found that glucose positively correlated with core 2 transferase ($p<0.001$) and remains consistent with previous work by Chibber et al., (2000). Of note, this particular data signature provides the immediate glycaemic condition of the individual at the point of venipuncture. It was further observed that participants with historically high HbA1c readings also demonstrated higher blood glucose levels ($p<0.01$), implicating protracted glycaemic irregularity.

However, it is not known whether there is a proportionate down-regulation, in terms of the core transferase / blood glucose relationship. Should core 2 transferase be directly mediated by blood glucose saturations, appropriate modulation strategies for core 2 transferase could be readily implemented.

Nevertheless, until evidence is made available to substantiate these proposals, the more likely event remains hyperglycaemia and its effect on the redox state.

7.12.3 **Total Cholesterol and Core 2 Transferase**

Whist epidemiological studies have identified dyslipidaemia as an identifiable risk factor in the development of diabetic neuropathy (Cameron et al., 2001b, Tesfaye et al., 1996), this study failed to find any significant correlation between plasma cholesterol and core 2 transferase ($p>0.05$). Yet, this remains in accordance with previous findings by Steiner (2001), as cited by Nangle et al., (2003), who found that total cholesterol levels in Type II diabetic individuals were comparable to a non-diabetic population.

In practice, the study's findings may be explained by the participants' use of anti-lipid agents, although this variable was not initially considered. The influence of statins on core 2 transferase functionality is entirely feasible, as they have been shown to protect cultured bovine endothelial cells from adverse changes in NOS and ET-1 expression, when exposed to LDL's (Hernandez-Perera et al., 1998). Accordingly, the resultant vasodilation would naturally diminish the extent of ischaemia caused by core 2 transferase up regulation.

Further proposed beneficial metabolic effects of statins include the inhibition of superoxide production and reduced LDL oxidation (Kalinowski et al., 2002, Sumi et al., 2001). Consequently, the rapid NO / ONOO- interaction due to unopposed superoxide is effectively barred, removing the oxidative stress

"trigger" for PKC activation and the subsequent stimulation of core 2 transferase.

Whilst it is accepted that statins could have potentially influenced both the lipid profiles and core 2 transferase co-effects, it should be emphasised that core 2 transferase activity remained significant despite such chemical intervention. Moreover, given the potentially different concentrations and brands of anti-lipid agent in use, direct comparisons could not have been readily made, even if this factor had been fully considered.

7.12.4 **Cholesterol / HDL Ratio and Core 2 Transferase**

The selection of the cholesterol / HDL ratio was based upon the apparent association between dyslipidaemia and diabetes (Nangle et al., 2003). However, no significant correlation was found to exist within this study ($p>0.05$). In furtherance to Section 7.12.3, anti-lipid agents have also been shown to improve HDL levels (Maron et al., 2000). Consequently, if total cholesterol levels remain static and HDL levels marginally improved, clearly, no significant correlation would be possible.

The data also revealed a positive correlation between the cholesterol / HDL ratio and HbA1c's ($p<0.05$). Hence, it could be proposed that this is indicative of HDL oxidation at high glucose levels and the subsequent removal of cardiovascular protection. Indeed, this supposition would correlate with those studies, which have established an increased risk of macrovascular disease in

diabetes generally (Kojda and Harrison, 1999, Honing et al., 1998, 1999, Ritter and Chowienczyk, 2001).

7.12.5 **HDL's and Core 2 Transferase**

The results demonstrated no significant correlation between HDL's and core 2 transferase ($p>0.05$), although a significant negative correlation was found with triglycerides ($p<0.01$). However, it should be noted that the inclusion criteria for this study required participants to have had no history of PVD (as determined by an ABPI ≤ 0.8 or previous vascular reconstruction). Consequently, those individuals who may have displayed the greatest alteration for HDL and LDL levels due to macrovascular disease (Dickinson et al., 2002), were not incorporated within this study.

7.12.6 **LDL's and Core 2 Transferase**

LDL oxidation has been implicated in the pathogenesis of macrovascular disease (Casino et al., 1993, Huang et al., 1993, El-Swefy et al., 2000). However, this study was unable to identify any significant correlation between core 2 transferase and LDL's ($p>0.05$). As previously mentioned, this may be attributed to the fact that all participants were free from PVD. Hence, the study would need to be extended to include individuals with macrovascular disorders, prior to definitive conclusions being drawn.

7.12.7 **Triglycerides and Core 2 Transferase**

Hypertriglyceridaemia has been reported in Type II diabetes (Riemens et al., 2000). Consequently, given the integral relationship between triglycerides and NEFA's (Mick et al., 2002), this particular biochemical marker was considered of relevance to this study. Notably, NEFA's provide vital substrates for hepatic triglyceride synthesis and their increased availability is actually thought to influence the pathogenesis of Hypertriglyceridaemia (Riemens et al., 2000). Indeed, this study revealed that triglycerides positively correlated with core 2 transferase ($p<0.05$).

Hence, it could be argued that elevated triglyceride levels are indicative of a source for raised NEFA's. The latter is known to stimulate the PKC / core 2 transferase cascade (Idris et al., 2001, Way et al., 2001), potentially explaining the observed positive correlation. This supposition can be further developed, as insulin is known to lower plasma NEFA's via its anti-lipolytic action (Bolinder et al., 1982, Lohnroth et al., 1993). However, this function appears to be impaired in Type II diabetes, leading not only to elevated NEFA levels, but also insulin resistance generally (Groop et al., 1989, 1991). Moreover, in diabetic obese individuals, NEFA expression appears to be directly related to expanded adipose tissue (Groop et al., 1989, 1992, DelPrato et al., 1990), which has been further implicated in the stimulation of VEGF and PKC (Mick et al., 2002).

The data also revealed a significant negative correlation between triglycerides and HDL's ($p<0.01$). These findings correspond with that of Riemens et al.,

(2000), who found that HDL's were lowest in obese Type II diabetic individuals, against a background of non-obese diabetic controls.

7.12.8 **Creatinine and Core 2 Transferase**

Plasma creatinine levels are used as a biochemical marker for glomerular filtration rate [GFR] (Haslett et al., 1999). Whilst all participants presented a normal creatinine reading [60 -130 µmol/l] (Porth, 1998) and displayed no history of albuminuria over the last 18 months, a significant positive correlation between creatinine and core 2 transferase ($p<0.01$) was recorded.

One potential theory for this outcome is based upon the premise that creatinine reflects muscle metabolism and as such, reduced muscle mass could adversely influence overall values. Notably, elevated core 2 transferase levels were chiefly observed amongst diabetic neuropathic individuals and it is this particular population, which is associated with muscle atrophy (Landsman et al., 1995). Consequently, it is proposed that, whilst absolute creatinine levels may reside within the normal range, those in the upper band unduly provoke a false reading. It is supposed that the microcirculatory changes associated with nephropathy exist concurrently with those of neuropathy. However, due to an atrophic reduction in muscle mass, creatinine output continues to be maintained in proportion.

Muscle atrophy to the lower limb leads to foot slap, increased velocity (Boulton et al., 1994) and thereby, aggravated plantar pressure. Hence, if tissue viability

is compromised due to core 2 transferase activity, the potential to cause both injury and ulceration is dramatically increased.

7.13 Linear Regression Modelling

Analysis of the data demonstrated several key factors, which significantly correlated with core 2 transferase. Given that the core 2 transferase assay is highly specialised and often outside the scope of standard laboratory facilities, the establishment of "rules of engagement" for future research and treatment protocols was viewed as a extremely fortuitous.

The Linear Regression Model for core 2 transferase was achieved by processing each significant variable through the method outlined in Section 2.17. This resulted in three significant predictive models: VPT's (both feet) and core 2 transferase [83.60%], 10g monofilament (both feet) and core 2 transferase [80.01%] and creatinine levels and core 2 transferase [77.86%].

7.13.1 VPT (both feet) and Core 2 Transferase - Linear Regression

Linear regression for this variable was significant, producing ≈ 84% predictability for core 2 transferase. This finding was considered to be particularly important, as it represents the first time that a standard clinical tool has been demonstrated to accurately predict core 2 transferase. Furthermore, in light of the continued positive correlation at a revised VPT threshold ≥ 15 V ($p<0.01$), the apparent role of core 2 transferase in the pathogenesis of diabetic peripheral neuropathy and thereby, foot ulceration, seems highly suggestive.

Indeed, by identifying individuals with a mean VPT of 15 - 25 V, intervention therapy could be introduced prophylactically to potentially help reduce the risk of ischaemic disorders. Consequently, it is intended that future investigation into core 2 transferase activity should expand upon the findings of this study to determine the use of VPT as an initial screening method for this enzyme.

Furthermore, new initiatives by pharmaceutical companies looking for biochemical markers of PKCβII inhibition may also benefit from this approach. As demonstrated by Chibber et al., (2003), core 2 transferase is a downstream event of PKCβII translocation. Hence, further employment of this model, using non-invasive and inexpensive clinical equipment, may prove pivotal to future PKC research.

7.13.2 **Monofilaments and Core 2 Transferase - Linear Regression**

The model produced an Rsq of 0.8001, indicating ≈ 80% predictability for core 2 transferase. As the 10g monofilament is used throughout both primary and secondary care, it is anticipated that individuals undergoing regular check-ups can be inexpensively screened for core 2 transferase up regulation and thereby, potential microcirculatory dysfunction.

Of note, VPT and 10g monofilament detection rates were also combined as a potential model for core 2 transferase. However, whilst the initial data appeared to be quite robust (82.7%), the model was considered too unstable. This instability was attributed to the apparent similarity between the two co-variates

and thus, their susceptibility to lurking variables. Accordingly, this particular example was abandoned.

7.13.3 **Creatinine and Core 2 Transferase - Linear Regression**

Creatinine was able to produce a significant model for core 2 transferase (Rsq 0.7786), indicating ≈ 78% predictability for this co-variate.

7.13.4 **VPT and Creatinine as Co-variates for Core 2 Transferase**

VPT was demonstrated to be the most effective co-variate for predicting core 2 transferase levels. Whilst further remedial statistical analysis had rendered a model utilising VPT's and 10g monofilaments unstable, a decision was made to statistically manipulate both the VPT and creatinine variables. In effect, this adjusted model would determine the efficacy of the two independently combined co-variates in predicting core 2 transferase. The result of this analysis provided an Rsq of 0.911, indicating ≈ 91% predictability for core 2 transferase.

The clinical application of this final model is profound, as it allows routine testing to be undertaken within a secondary care environment, yet with the potential capability to identify metabolic alteration. The timely multi-disciplinary approach by physician, podiatrist and dietician can therefore implement strategies to reduce the risk of further diabetic complications. This may take the form of strict glycaemic control via alteration and/or modulation of diabetic agents, foot care treatment and pressure displacement techniques, coupled with dietary advice on antioxidant intake and/or vitamin supplements.

7.13.5 **10g Monofilament and Creatinine as Co-variates for Core 2 Transferase**

This combined model provided a very robust 89.8% predictability for core 2 transferase. Indeed, this outcome supports the correlations observed during the initial statistical handling and furthermore, substantiates the use of the 10g monofilament as a general screening method. However, it was decided that this particular model should not be employed as a predictive tool, due to the inherent potential for human bias.

This supposition is founded upon basic mechanical law (Figure 1.4.2.i). Whilst the buckle load of the 10g monofilament is accepted as linear at room temperature and humidity, the impact velocity of the fibre onto the patient's plantar surface is not. As there is no prescribed rate of application (mm/sec) for the monofilament, there is scope for mechanical overload, i.e. stabbing. Additionally, the 1 cm deflection criterion remains subjective and in real terms, cannot be properly controlled for without formal "industrialisation" of the process.

Clearly, the above requirements are impractical in the care setting. Moreover, the skill level of an experienced clinician should be sufficient to negate these concerns. However, the 10g monofilament remains a non-linear method, due entirely to its hand operation. If it is to function, a predictive model must employ a uniform metrology system, free from repeatability error. Unlike the neurothesiometer, 10g monofilament detection cannot offer the required level of hysteresis and hence, must be removed from the final model.

7.14 Methodological Findings

Notably, the analysis of creatinine was not initially considered as part of the blood chemistry phase. However, Standard Operating Procedure (SOP) within the Diabetes Department of Poole Hospital NHS Trust dictated that these values, together with VPT data should be collected. Hence, whilst entirely serendipitous, this edict helped to form an important part of the final 91% predictive model for core 2 transferase. Therefore, it is imperative that health care professionals do not readily dismiss methods and advice, which initially appear superfluous.

An integral part of screening for peripheral neuropathy was based upon the use of the standard 10g monofilament. However, it should be noted that an inability to detect a 10g monofilament is actually consistent with an increased risk of ulceration, not neuropathy (Birke and Rolfson, 1997, Litzelman et al., 1997). Hence, it would appear that the indiscriminate use of the 10g monofilament has far exceeded its original remit, as it is difficult to see how nerve demyelination and loss of axonal fibres can be accurately detected without NCV measurement (Wang et al., 1999). Whilst there remains an undisputed correlation between peripheral neuropathy and the increased risk of foot ulceration (Kumar et al., 1991, Cavanagh, et al., 1993), the singular employment of the 10g monofilament as a "trawler" for these events is extremely suspect.

The rationale for this supposition is based upon the fact that individuals with poor glycaemic control are more likely to form AGE products (Wautier et al., 2001). These easily attach to collagen and can lead to tissue stiffening (Melling

et al., 2000), which may act as a protective shield against the 10g monofilament. However, it does not follow that the underlying nerve function is clinically neuropathic. Consequently, it is proposed that future protocols using monofilaments, irrespective of buckle load, should be modified to include a "prayer sign" test, to verify the absence of poor tissue malleability (Sauseng et al., 2002).

Finally, current medication should be recorded as part of the selection and recruitment process. However, resultant ethical and analytical complications, involving the modification of drug therapies, would have to be controlled for. Clearly, any aspect, which would lead to unnecessary patient discomfort or health risk, should be avoided entirely.

7.15 Limitations to the Study

In practical terms, the main limitation to this study was the inherent lack of geopolitical and socio-economic diversity within the participant banding. The relatively small sample population, whilst of sufficient statistical power, regrettably cannot be translated to the national level. In view of the restrictions on both time-frame and budget, which naturally accompanied this research programme, a decision was made to succumb to a "best fit" scenario, using the available assets. Accordingly, the control group did not accurately mirror the two diabetic study groups, although every effort was made to harmonise the latter. Consequently, the resultant data cannot be extrapolated to the UK diabetic population as a whole and thus, must be regarded contextually.

Given the known inhibitory effects of antioxidants on PKC expression (Chow, 1991, Cotter et al., 1995, Keegan et al., 1995, Baynes, 1991, Laight, et al., 2000, Cameron et al., 2001), it therefore remains probable that the nutritional condition of the sample population was preferentially affected by household income. Yet, despite this potential risk, core 2 transferase was still found to be significantly elevated between the groups ($p<0.001$).

As a consequence, the use of "food diaries" would have been extremely beneficial, as it would have allowed results from both the core 2 transferase assay and biochemical phase to be viewed in context. Moreover, this variable could have been further controlled through the establishment of dietary regimes and/or advocation of specific vitamin supplements. This would have had the effect of "normalising" the sample groups, thereby further isolating the association between diabetic neuropathic ulceration and core 2 transferase.

Lastly, this study deliberately barred participants with macrovascular complications, due to established associations with the biochemical markers under investigation (Mantey et al., 2000, Murphie, 2001, Jeffcoate and Harding, 2003, Wrobel et al., 2003). Whilst methodologically sound, subsequent analysis of the data was unable to provide any correlation between the HDL / LDL profiles and core 2 transferase. Hence, if only perfunctory, the use of a "macrovascular control" group would have permitted definitive conclusions to be drawn.

Despite the above concessions, this study offers considerable potential, in terms of the early detection of neuropathic plantar ulceration, according appropriate

intervention therapies and thereby, an improved quality of life for diabetic individuals. Therefore, for this objective to be realised, the supposed mechanisms require additional resources to conclusively determine their use as a clinical tool.

7.16 Recommendations for Future Investigation

During the course of this study, it became apparent that there is no formal datum for either VPT or the 10g monofilament, within the context of diabetic peripheral neuropathy and the gold standard of nerve conduction velocity. The only previous work in this area provided logarithms for VPT's against an age related mean reduction in nerve function (Coppini et al., 1998). However, these findings neither establish VPT thresholds for neuropathy, nor employ "standard issue" tools, i.e. the neurothesiometer. Similarly, current VPT boundaries tend to be used anecdotally by clinicians and are, in strict terms, only representative of the increased risk of ulceration (Young et al., 1994). Therefore, it is entirely probable that early neuropathic development is allowed to flourish due to systematic error. Hence, it is essential that VPT's are benchmarked against NCV, if the accurate prediction of neuropathic foot ulceration is to be achieved.

Accordingly, this study provides a natural springboard for future research to include:

1. Analysis of VPT's against NCV to develop a model for the early detection of core 2 transferase up regulation and diabetic peripheral neuropathy

2. PKCβII inhibition and its effect upon core 2 transferase activity and nerve conduction velocity over a 12 month period

3. NEFA saturations in diabetic individuals and their potential role in the up regulation of core 2 transferase. This would further include the modulation of core 2 transferase via the dietary intake of antioxidants and use of weight reduction programmes

4. Establishment of a finite model for creatinine versus core 2 transferase activity. By extending the scope of the present study, it is anticipated that a predictive threshold for creatinine can be determined. This will facilitate the early detection of microcirculatory changes using a low cost, routine protocol

8.0 CONCLUSION

Core 2 transferase is significantly increased within the Type II diabetic population, most particularly, amongst neuropathic individuals with a history of plantar ulceration ($p<0.001$). Consequently, this result not only establishes a potential pathogenesis for diabetic neuropathic ulceration, but plausibly, peripheral neuropathy itself. Given apparent consistency with the findings of Chibber et al., (2000, 2003) in relation to retinopathy, it remains highly probable that the two complications are intrinsically linked. Indeed, as this study has shown, the postulated mechanism is wholly indiscriminate, relying entirely upon the diabetic individual's antioxidant and glycaemic status, as both antagonist and determinate.

Notably, VPT's were found to positively correlate with core 2 transferase up regulation, even at a non-standard [15 V] benchmark ($p<0.01$), implying early neurological disturbance. Further expansion of this relationship using linear regression modelling, resulted in a 91 % predictability of elevated core 2 transferase, with VPT and creatinine as co-variates. Therefore, it is imperative that this model is further developed, in order to facilitate the timely identification of the "at risk" patient, in both the primary and secondary care setting.

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Appendix A

Non-Proliferative Diabetic Retinopathy Grading Sheet Adapted From The Early Treatment Diabetic Retinopathy Study

Level	Features
Mild	At least one microaneurysm
Moderate	Severe haemorrhages (> 4 blot haemorrhages) in one quadrant and/or cotton wool spots or venous beading or IRMA definitely present
Severe	Any one of the following: severe haemorrhages in four quadrants, venous beading in two quadrants, IRMA in one quadrant
Very Severe	Any of the two features in the "severe" categories

Appendix B

20 APR 2001

Poole Hospital NHS
NHS Trust

COPY

19 April 2001

Ms Michelle Spruce
74 Cereleton Park
Charleton Marshall
Blandford Forum
Dorset DT11 9PN

Dear Ms Spruce

INTRINSIC AND EXTRINSIC FACTORS IMPLICATED IN THE SEQUENCE OF DIABETIC FOOT ULCERATION
LREC NO 28/01/S

Thank you for your letter dated 5 April 2001 and for the revised patient information sheet etc.

Dr Coppini also gave me a final version of the study protocol.

I am happy to give you Chairmans Approval now to proceed with the study.

Conditions of Approval are set out in the attached sheet.
Protocol amendments should be precised onto one page and accompany any documentation.
Serious adverse events should be listed on the attached form and accompany any documentation.

Yours sincerely

G P CLEIN
CHAIRMAN, East Dorset Local Research Ethics Committee

Appendix C

Patient Information Sheet

Ethics Ref. No. 29/01/S

Date:

TAKING PART IN RESEARCH

WORKING TITLE: Factors which may lead to diabetic foot ulceration
(STUDY TITLE): *Intrinsic factors implicated in the sequence of diabetic foot ulceration*

You are being invited to take part in a research project. Here is some information to help you decide whether or not to take part. Before, you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you so wish. Ask us, if there is anything you do not understand or if you would like more information. Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London, N16 0BW. Thank you for reading this.

Background and aim of study

Diabetes is a common condition affecting about 2-4% of the population, where high blood sugar levels can cause you to feel unwell. In some patients, over a period of time, diabetes can cause other complications like eye disease, kidney disease, nerve disease and foot ulceration. A recent study in the UK has shown that good blood sugar control reduces the long term problems considerably. So, we believe that finding those who may have an increased risk at an early stage will help us provide them with better treatment.

Further research information has indicated that people with diabetes are more likely to produce Advanced Glycation End-Stage products. These products are formed within the body due to high glucose levels and have been linked to the appearance of vascular, kidney and eye complications. At present, we do not know if Advanced Glycation End-Stage products are an important marker for diabetic complications. The importance of these findings could affect how diabetes education material is delivered, so that it is given at a time which is best suited to the individual.

This study will form part of a Ph.D. research project which aims to find out if poor glucose control and Advanced Glycation End-Stage products can be linked to an increased risk of foot ulceration and nerve disease.

Who is suitable for the study?
The focus of the study will be on individuals aged between 30-75 years who have developed diabetes later in life. To make proper comparison, we will require three groups of patients with diabetes. The first diabetic group will represent those individuals with peripheral neuropathy (nerve disease to the foot or leg), whilst the

second diabetic group will include those individuals who have diabetes, but without any evident complications , i.e. nerve or eye disease and are currently free from foot or leg ulceration. We are asking all individuals with diabetes aged 30-75, who fall within the study criteria and have attended the Diabetic Centre at Poole Hospital within the last 2 years, to consider taking part.

If you do not wish to take part, it will not in any way affect the care/treatment you currently receive at the departments within or associated to Poole Hospital. If you fulfil the initial requirements for participation, we will arrange to see you at a mutually convenient time to explain the study in and answer any remaining questions you may have.

What does the study involve?
The study will require you to make one additional attendance to the Diabetes Centre, Poole Hospital. This appointment will last for approximately 30 minutes.

At this visit, your height and weight will be recorded along with some health related questions. You will have our feet examined by a State Registered Chiropodist to look for signs of foot problems. We will them check your ability to feel a vibrating device applied to your feet and measure your blood pressure at your arm and ankle. Thereafter, a blood sample will be taken. The purpose of this blood test is to determine the levels of Advance Glycation End Stage Products and the enzyme Core 2, both of which have been implication in the cause of foot ulceration and nerve disease. Due to the equipment required for the tests, it is necessary that the blood samples are transported to St. Thomas's Hospital, London. The transportation of all samples will be supervised by the researcher and all bloods taken will be coded to preserve your total confidentiality. Furthermore, on completion of the proposed tests, all blood samples will be destroyed, so that no further analysis can be performed.

Will this study benefit you directly?
You will not benefit directly from your involvement in the study. However, information arising from this research may help us improve the quality of care for patients with diabetes in the future. All your medical and personal information will remain strictly confidential and will be used only for the purpose of this Ph.D. research study. Any information, which leaves the hospital will be sanitised to protect your identity. All participants will have the right to view the results of the research prior to completion of the Ph.D. project. Notification of this point will take the form of a letter and a copy of the research results will be available from the Diabetes Centre, Poole Hospital.

Appendix C (cont)

The design of this study has been approved by the Local Research ethics Committee Ethics Ref. No. 29/01/S. Your decision to help with this study or not will be respected and will not affect your medical care whatsoever.

For further information, you are invited to contact:-

Mrs Michelle Spruce
Diabetic Research Podiatrist to Dr DV Coppini
Diabetes Centre
Poole Hospital, Dorset BH15 2JB
Tel: 07729136517

Thank you very much for your interest

Michelle Spruce BSc (Hons). S.R.Ch.
Diabetic Research Podiatrist

Appendix D

Data Collection Sheet

Date Hospital No.

Patient Name Date of birth

Address

Data Group:

Height Weight

Glucose level

VPT 1st 2nd 3rd
Right

Left

Bloods taken for: (3 tubes)

HbA1c Yes/No

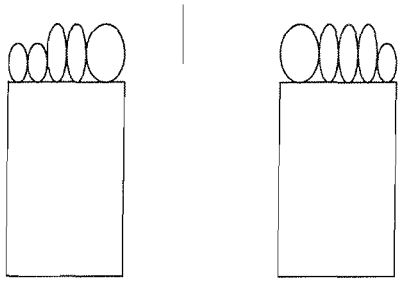
Core 2 (10g) Yes/No

Lipids Yes/No

Neuropathic Pain Present/Absent - Left/Right/Both

10g monofilament L R

Ankle
Tibial tuberosity



Appendix E

Table E-1

PATIENT	HISTORY OF ULCERATION	CORE 2 VALUE	HbA1c	BLOOD GLUCOSE	HEIGHT	WEIGHT	BMI	GENDER	VPT R	VPT (L)
Cont. 1	NO	103.75	5.7	4.6	164	48	17.85	F	5	6
Cont. 2	NO	127.39	5.4	4.2	163	49	18.4	F	7	5
Cont. 3	NO	160.2	5.1	4.8	165	60	22	F	3	3.5
Cont. 4	NO	146.61	6	4.7	185	77	22.5	M	5	3
Cont. 5	NO	184.7	5.1	5	175	80	26.1	M	6.5	6
DNC 1	NO	438.09	8.3	5.8	168	73.5	26	F	6	4
DNC 2	NO	369.95	8.9	7.4	182	117	35.3	M	18	15.5
DNC 3	NO	473.41	8.3	13.3	175	84.3	27.5	M	10.5	5.5
DNC 4	NO	443.71	8.9	15.6	161.5	54	20.7	F	3.5	4
DNC 5	NO	383.15	9.4	11.9	175.5	96	31.4	M	17	14
DNC 6	NO	256.19	5.9	6.8	160.2	57.8	22.5	F	4	4
DNC 7	NO	295.22	7.7	9	165	86.8	31.9	F	4	7
DNC 8	NO	481.25	12	26.1	171	103.5	35.4	M	10	6
DNC 9	NO	312.06	7	5.3	175.5	101.9	33.1	M	14	20
DNC 10	NO	390.11	7.4	6.1	171	120.5	41.2	M	10	10
DN 1	YES	1297.03	10	15.1	172	106.5	36	M	40	40
DN 2	YES	1027.43	9.4	18.1	182.5	120.2	36.1	M	36	40
DN 3	NO	954.48	10.3	14.6	177	115.6	36.9	M	29	30
DN 4	YES	1005.2	11.9	16.5	171.2	98.9	33.7	F	40	40
DN 5	NO	913.76	7.9	10.2	171	76.5	26.2	M	28	29
DN 6	NO	873.73	7.5	9.6	175.5	86.6	28.1	M	32	26
DN 7	NO	891.34	10	16.1	190	138	38.2	M	40	40
DN 8	NO	1067.92	9.6	16	174	101.5	33.5	M	40	33
DN 9	NO	926.1	9.2	8.8	175	95.1	31.1	F	27	38
DN 10	YES	1448.1	8.4	23.5	197	97.7	35	M	29	40

Table E-1 (cont)

PATIENT	10G MONO RIGHT	10G MONO LEFT	10g MONO TOTAL	ANKLE REFLEX	CHOLES.	CHOL/ HDL	CREAT.	TRIGS.	HDL	LDL	DOB	AGE
Cont. 1	6	6	12	present	3.9	2	77	0.6	1.99	1.6	14/9/1972	30
Cont. 2	5	6	11	present	4	3.3	87	1	1.21	2.3	25/9/1961	42
Cont. 3	6	6	12	present	5.5	2.3	87	1	2.41	2.6	18/7/1944	59
Cont. 4	6	6	12	present	4.7	3.7	84	1.7	1.26	2.2	3/7/1963	39
Cont. 5	6	6	12	present	3.5	3	82	0.8	1.16	1.1	24/8/1967	37
DNC 1	6	6	12	present	5.5	3	74	1.5	1.84	3	9/8/1944	58
DNC 2	4	5	9	present	5.9	3.6	80	1.3	1.62	3.7	23/6/1946	56
DNC 3	5	6	11	absent	4.4	4.1	94	1.9	1.07	2.5	17/12/1946	56
DNC 4	5	5	10	present	3.5	1.6	92	0.6	2.14	1.1	22/4/1962	41
DNC 5	4	5	9	absent	5.2	6.9	88	2.7	0.75	3.2	23/1/1942	61
DNC 6	6	5	11	present	4.7	3.9	76	1.9	1.2	2.6	19/5/1953	50
DNC 7	5	5	10	present	3.5	3.9	74	3	0.9	1.2	30/11/1953	49
DNC 8	4	5	9	absent	5.8	4.3	97	2	1.35	3.5	2/11/1949	53
DNC 9	5	4	9	present	3.5	3.8	77	2.8	0.93	1.3	23/1/1942	61
DNC 10	4	5	9	absent	5	3.9	92	2.6	1.28	2.5	6/10/1949	53
DN 1	0	0	0	absent	2.9	3.7	123	3.3	0.79	0.6	23/9/1947	55
DN 2	0	0	0	absent	5	6.6	97	3.9	0.76	2.5	16/6/1962	40
DN 3	3	2	5	absent	5	3.7	95	4.8	1.36	1.3	22/6/1951	51
DN 4	0	0	0	absent	5.8	5.9	105	3.6	0.99	3.2	10/3/1942	61
DN 5	2	1	3	absent	4	2.2	100	1.5	1.86	1.5	27/7/1948	54
DN 6	1	1	2	absent	4.3	4.1	104	1.7	1.06	2.5	29/12/1930	72
DN 7	0	0	0	absent	5	4.6	98	3.6	1.09	3.6	13/9/1938	64
DN 8	0	0	0	absent	4.1	3	105	2.1	1.38	2.2	15/12/1940	62
DN 9	2	0	2	absent	4.8	4.1	104	2.1	1.18	3.5	26/8/1933	69
DN 10	2	0	2	absent	5.9	2.9	127	1.6	1.99	4.5	21/2/1935	68

Table E-2

PATIENT	MEDICATIONS			
	ASPIRIN	**ACE-INHIBITOR**	**STATIN**	**ORAL GLYCAEMIC**
DNC 1	75 MG 1D	LISINOPRIL 20MG 1D	ATORVASTATIN 10MG 1D	GLICLAZIDE 160MG 2D
DNC 2	75 MG 1D	IMIDAPRIL 10MG 1 D	ATORVASTATIN 10MG 1D	METFORMIN 500MG 2D & GLICLAZIDE 160MG 1D
DNC 3	75 MG 1D	LISINOPRIL 20MG 1D	ATORVASTATIN 10MG 1D	METFORMIN 500MG 2D & ROSIGLITAZONE 4MG 2D
DNC 4	75 MG 1D	INNOZIDE 1D*	SIMVASTATIN 20MG 1D	GLICLAZIDE 80MG 2D & METFORMIN 500MG 2D
DNC 5	75 MG 1D	PERINDOPRIL 4MG 1D	ATORVASTATIN 10MG 1D	GLICLAZIDE 160MG 2D & METFORMIN 500MG 2D
DNC 6	75 MG 1D	ENALAPRIL 20MG 1D	SIMVASTATIN 10MG 1D	GLICLAZIDE 120MG 2D
DNC 7	75 MG 1D	RAMIPRIL 10MG 1D	ATORVASTATIN 10MG 1D	GLIMEPIRIDE 4MG 1D
DNC 8	75 MG 1D	PERINDOPRIL 8MG 1D	SIMVASTATIN 10MG 1D	GLICLAZIDE 80MG 2D
DNC 9	75 MG 1D	RAMIPRIL 10MG 1D	SIMVASTATIN 10MG 1D	METFORMIN 500MG 3D
DNC 10	75 MG 1D	LISINOPRIL 20MG 1D	SIMVASTATIN 20MG 1D	METFORMIN 500MG 2D & GLICLAZIDE 80MG 1D
DN 1	75 MG 1D	ENALAPRIL 20MG 1D	ATORVASTATIN 10MG 1D	METFORMIN 500MG 3D
DN 2	75 MG 1D	VALSARTAN 80MG 1D**	ATORVASTATIN 20MG 1D	METFORMIN 1G 2D & PIOGLITAZONE
DN 3	75 MG 1D	ENALAPRIL 20MG 1D	SIMVASTATIN 20MG 1D	METFORMIN 850MG 2D &V ROSIGLITAZONE 4MG 1D
DN 4	75 MG 1D	ENALAPRIL 20MG 1D	ATORVASTATIN 10MG 1D	GLICLAZIDE 80MG 1D & METFORMIN 500MG 2D
DN 5	75 MG 1D	RAMIPRIL 10MG 1D	ATORVASTATIN 10MG 1D	GLICLAZIDE 150MG 2D & PIOGLITAZONE 15MG 1D
DN 6	75 MG 1D	RAMIPRIL 10MG 1D	SIMVASTATIN 40MG 1D	METFORMIN 850MG & PIOGLITAZONE 15MG 1D
DN 7	75 MG 1D	LISINOPRIL 5MG 1D	SIMVASTATIN 20MG 1D	METFORMIN 500MG 2D GLICLAZIDE 160MG 2D
DN 8	75 MG 1D	ENALAPRIL 10MG 1D	FLUVASTATIN 20MG 1D	GLICLAZIDE 40MG 2D & METFORMIN 850MG 2D
DN 9	75 MG 1D	RAMIPRIL 10MG 1D	SIMVASTATIN 20MG 1D	METFORMIN 850MG 2D
DN 10	75 MG 1D	RAMIPRIL 10MG 1D	FLUVASTATIN 40MG 1D	METFORMIN 850MG 2D & ROSIGLITAZONE 8MG 2D

Table E-2 (cont)

PATIENT	OTHERS	OTHERS CONT.	OTHERS CONT.	CO-MORBIDITY
DNC 1	GAVISCON			Acid reflux
DNC 2	RANITIDINE 150MG 1D	FRUSEMIDE 40MG 1D		GI irritation, hypertension, oedema
DNC 3	COPROXAMOL	THYROXINE 100MG 1D		Hypothyroidism
DNC 4	SALBUTAMOL 10MG	TERMAZEPAN 20MG 1D		Asthma, insomnia
DNC 5	CO-CODAMOL 8/500	AMITRIPTYLINE 30MG 1D	AMLODIPINE 5MG 1D	Hypertension, angina, depression
DNC 6	ATENOLOL 50MG 1D			Hypertension
DNC 7	PREDNISOLONE 7.5 MG 1D	CO-AMILOFRUSE 5/40 1D***		Rheumatoid Arthritis, hypertension
DNC 8	BENDROFLUAZIDE 2.5MG 1D			Oedema
DNC 9	FRUSEMIDE 40MG 1D	BISOPROLOL 5MG 1D		Hypertension, oedema
DNC 10	FRUSEMIDE 80MG 1D			Oedema
DN 1	FRUSEMIDE 20MG 1D	AMLODIPINE 5MG 1D	ATENOLOL 25MG 1D	Hypertension, oedema
DN 2	BENDROFLUAZIDE 2.5MG 1D	ZOPICLONE 3.75MG 1D		Insomnia (pain related), hypertension
DN 3	FRUSEMIDE 40MG 1D	AMITRIPTLYINE 25MG 1D	ALLOPURINOL 300MG 1D	Gout, hypertension, neuropathic pain
DN 4	CARBEMAZEPINE 200MG 2D	BETAHISTINE 16MG 2D	PRIMIDONE 250MG 2D	Epilepsy, tinnitus
DN 5	FRUSEMIDE 80MG 1D	ATENOLOL 25MG 1D	NITROLINGUAL SPRAY	Angina, hypertension
DN 6	LANSOPRAZOLE 15MG 1D	IBUPROFEN 800mg 2D	AMITRIPTYLINE 25MG 1D DILTIAZEM 60MG 3D	
DN 7	FRUSEMIDE 40MG 1D	NEFEDIPINE 20MG 1D	BISOPROLOL 5MG 1D	
DN 8	BECLOMETHASONE 100MG	FRUSEMIDE 20MG 1D		Hypertension
DN 9	ATENOLOL 50MG 1D	BENDROFLUAZIDE 2.5MG 1D		Hypertension
DN 10	ATENOLOL 50MG 1D	SALAZOPYRIN 500MG 4D		Crohn's disease, hypertension

* 20mg Enalapril maleate & 12.5mg Hydrochlorothiazide
** Angiotensin-II receptor antagonists
*** Amiloride hydrochloride 5mg & furosemide 40mg

Appendix F

1.0 Statistical Methods

1.1 Unrelated *t* test (2-tailed)

This compares two separate or unmatched groups in different conditions. The test calculations are:

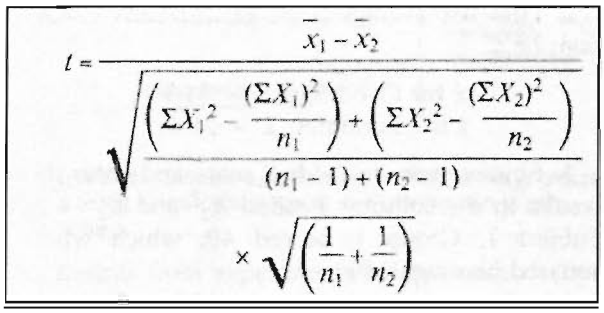
- Find the total (Σ) for each condition
- Find the average score for each condition
- Square every individual score and enter the result under columns X_1^2 and X_2^2
- Sum the squared scores for each condition separately, i.e. ΣX_1^2
- Square the total for each condition separately, i.e. $(\Sigma X_1)^2$

Table 1.1.1 **Example of *t* test table**

GROUP 1			**GROUP 2**		
Subject	**Scores (X_1)**	**X_1^2**	**Subject**	**Scores (X_2)**	**X_2^2**
1	40	1600	1	20	400
2	30	900	2	25	625
3	35	1225	3	30	900
4	25	625	4	25	625
5	30	900	5	15	225
6	40	1600	6	40	1600
7	45	2025	7	35	1225
8	35	1225	8	40	1600
9	25	625	9	25	625
10	40	1600	10	30	900
ΣX_1 =	345	ΣX_1^2 12325	ΣX_2 =	285	ΣX_1^2 8725
χ_1 =	34.5		χ_2 =	28.5	

The ***t*** value is calculated using the formula:

Figure 1.1.2 **Formula for *t* value**



$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{\left(\Sigma X_1^2 - \frac{(\Sigma X_1)^2}{n_1}\right) + \left(\Sigma X_2^2 - \frac{(\Sigma X_2)^2}{n_2}\right)}{(n_1 - 1) + (n_2 - 1)}} \times \sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

Reproduced from Hicks, C. M. (1995) Research for Physiotherapists: Project Design and Analysis, 2nd ed., Churchill Livingstone, London. pp170.

Figure 1.1.3 **Degrees of Freedom (df)**

df $=(n_1 - 1) + (n_2 - 1)$

The ***t*** value is ascertained from the probability tables using the **df**, thus indicating the chances of random error within the results.

1.2 One Way Analysis of Variance (ANOVA)

This parametric test is used to compare mean results from three or more conditions, with different, unmatched subject groups in each condition. The test reveals if the values from the groups are sufficient to be classified as significant. Inherent variation is termed "between groups variance". Such variation occurs due to individual differences amongst the individuals within each group, i.e. the result of random error or "within groups variance". The variance is calculated by the Sum of Squares (SS), Degrees of Freedom (df) and the Mean Sum of Squares (MS).

Figure 1.2.1 **ANOVA components**

SS = The sum of the squared total for each group/condition.

DF = df_{bet} = number of conditions -1

$df_{tot} = N - 1$

$df_{error} = df_{tot} - df_{bet}$

MS = SS divided by its own df value

$$MS_{bet} = \frac{SS_{bet}}{df_{bet}}$$

Comparison between the experimental conditions (between groups) and random error (within groups) results in an "F ratio".

F ratio = $\frac{MS_{bet}}{MS_{error}}$

Table 1.2.2 **Example of ANOVA table**

Source of variance	SS	df	MS	F ratio
Between Groups variation	195	2	97.5	1.915
Within Groups variation	1375	27	50.926	
Total	1570	29		

To find the F ratio in the probability tables, the df values for "between group" and "within group" variation are required. Consequently, the test displays if the differences are due to the experimental conditions ($p<0.05$) or alternatively, due to random error.

1.3 Tukey Honest Statistical Difference (HSD)

Although ANOVA analysis indicates a difference between two or more group means, it does not actually locate the area of significance. The Tukey Test is a post hoc test designed to perform pair wise comparisons of the means. The Tukey Test is calculated thus:

- Arrange the means in ascending order in a comparison table and calculate the difference between each pair of means

Table 1.3.1 **Comparison table**

		Means in Ascending Order				
		Grp 4	**Grp 5**	**Grp 3**	**Grp 2**	**Grp 1**
		17.8	32.5	33.4	46.6	70.2
Grp 4	17.8	----	14.7	15.6	28.8	52.4
Grp 5	32.5	----	----	0.9	14.1	37.7
Grp 3	33.4	----	----	----	13.2	36.8
Grp 2	46.6	----	----	----	----	23.6
Grp 1	70.2	----	----	----	-----	----

- Calculate the minimum pair wise difference needed:

$$\mathbf{D_{min} = Q_t\ (sq\ rt(MS_{within\ group}))/(sq\ rt(S))}$$

Where: $\mathbf{D_{min}}$ is the minimum pairwise difference needed for significance

$\mathbf{Q_t}$ is the t statistic for df within the number of means being tested

MS is the Mean Square Error (within group) from ANOVA

S is the group sample size (number of data points in each cell of the ANOVA)

Figure 1.3.2 **Example**

$D_{min} = Q_t$ (sq rt($MS_{within\ group}$))/(sq rt(S))

$D_{min} = 4.89$(sq rt(113.4))/(sq rt(36))

$D_{min} = 4.89(10.65)/(6)$

$D_{min} = 8.68$

- Finally, compare the difference between the means in the table to the minimum pair-wise difference. Those larger than the minimum are significantly different.

1.4 Pearson Product Moment Correlation Coefficient (2-tailed)

The Pearson Product Moment Correlation Coefficient test is used to compare two sets of data for the degree of association. This will result in a figure between -1 and + 1. The closer the figure is to -1, the stronger a negative correlation exists. A negative correlation can be defined, where small scores on one variable associate with large scores on the other. Alternatively, the closer the figure is to + 1, the stronger a positive correlation is present. A positive correlation is described, when large scores on one variable are associated with large scores on the other.

The calculations for the Pearson test are:

- Sum all the scores for variable A, i.e. $\sum A$
- Sum all the scores for variable B, i.e. $\sum B$
- Multiply each variable A score by the variable B score
- Enter the result under column "A x B"
- Add up all the scores in column "A x B", i.e. $\sum(A \times B)$
- Square each variable A score and enter the result under column "A^2"
- Square each variable B score and enter the result under column "B^2"
- Add up all the scores in column A^2, i.e. $\sum A^2$
- Add up all the scores in column B^2, i.e. $\sum B^2$
- Find the value of *r* with the formula:

Figure 1.4.1 **Formula for *r* value**

$$r = \frac{N\Sigma(A \times B) - \Sigma A \times \Sigma B}{\sqrt{[N\Sigma A^2 - (\Sigma A)^2][N\Sigma B^2 - (\Sigma B)^2]}}$$

Reproduced from Hicks, C. M. (1995) Research for Physiotherapists: Project Design and Analysis, 2nd ed., Churchill Livingstone, London. pp185.

- Once the *r* value has been determined, df must be calculated:

$$df = N - 2$$

Using the df, the *r* value is read against the critical values of *r* to ascertain its significance ($p<0.05$).

2.0 **Analyses**

Table F-1 **Group statistics – core 2 transferase**

Absolute values of core 2 transferase					95% Confidence for	Interval Mean
(pmmol/l/h/mg protein)	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	144.53	30.90	13.82	106.16	182.90
Diabetic no complications	10	384.31	77.15	24.40	329.12	439.50
Diabetic neuropathic	10	1040.51	188.78	59.70	905.47	1175.55
Total	25	598.84	399.01	79.80	434.13	763.54

Table F-2 **ANOVA – core 2 transferase**

	Sum of Squares	df	Mean Square	F	Sig
Absolute values for core 2 transferase:					
Between Grps	3442917	2	1721458.49	100.16	**0.000**
Within Grps	378115.10	22	17187.05		
Total	3821032	24			

Table F-3 **Group statistics – duration of diabetes**

Group	N	Mean (Years)	SD	Std. Error Mean
DNC	10	11.30	9.63	3.04
DN	10	11.60	9.54	3.02

Table F-4 **Independent samples test – duration of diabetes**

	F	Sig	T	df	Sig	Mean Δ	Std. Error Δ
Equal variances assumed	0.01	0.93	-0.07	18	0.95	-0.30	4.29
Equal variances Not assumed			-0.07	18	0.95	-0.30	4.29

Table F-5 **Group statistics - VPT**

VPT Readings (Volts)					95% Confidence Mean	Interval for
		Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
VPT Right Foot						
Controls	5	5.30	1.57	7.00	3.36	7.24
Diabetic no complications	10	9.70	5.36	1.70	5.87	13.53
Diabetic neuropathic	10	34.10	5.65	1.79	30.06	38.14
Total	25	18.58	13.90	2.78	12.84	24.32
VPT Left Foot						
Controls	5	4.70	1.40	0.62	2.97	6.43
Diabetic no complications	10	9.00	5.67	1.80	4.94	13.06
Diabetic neuropathic	10	35.60	5.54	1.75	31.64	39.56
Total	25	18.78	14.93	2.99	12.62	24.94
VPT Both Feet						
Controls	5	5.10	1.29	0.58	3.49	6.71
Diabetic no complications	10	9.45	5.26	1.66	5.69	13.21
Diabetic neuropathic	10	34.85	4.73	1.49	31.47	38.23
Total	25	18.74	14.21	2.84	12.88	24.60

Table F-6 **ANOVA – VPT**

	Sum of Squares	Df	Mean Square	F	Sig
VPT (Right):					
Between Grps	4079.04	2	2039.52	80.80	**0.000**
Within Grps	555.30	22	25.24		
Total	4634.34	24			
VPT (Left):					
Between Grps	4776.84	2	2388.42	91.59	**0.000**
Within Grps	573.70	22	26.08		
Total	5350.54	24			
VPT (Both):					
Between Grps	4388.61	2	2194.31	105.76	**0.000**
Within Grps	456.45	22	20.75		
Total	4845.06	24			

Table F-7 **Group statistics – 10g monofilament**

10g Monofilament					**95% Confidence Mean**	**Interval for**
(Sites Detected)		**Mean**	**Std. Deviation**	**Std Error**	**Lower Bound**	**Upper Bound**
10g Mono (R)						
Controls	5	5.80	0.45	0.20	5.00	6.00
Diabetic no complications	10	4.80	0.79	0.25	4.00	6.00
Diabetic neuropathic	10	1.00	1.16	0.37	0.00	3.00
Total	25	3.48	2.28	0.46	0.00	6.00
10g Mono (L)						
Controls	5	6.00	0.000	0.000	6.00	6.00
Diabetic no complications	10	5.10	0.57	0.18	4.00	6.00
Diabetic neuropathic	10	0.40	0.70	0.22	0.00	2.00
Total	25	3.40	2.58	0.52	0.00	6.00
10g Mono total						
Controls	5	11.80	0.45	0.20	11.00	12.00
Diabetic no complications	10	9.90	1.10	0.35	9.00	12.00
Diabetic neuropathic	10	1.40	1.71	0.54	0.00	5.00
Total	25	6.88	4.79	0.96	0.00	12.00

Table F-8 **ANOVA – 10g monofilament**

	Sum of Squares	df	Mean Square	F	Sig
10g Mono (R)					
Between Grps	105.84	2	52.92	63.27	**0.000**
Within Grps	18.40	22	0.84		
Total	124.24	24			
10g Mono (L)					
Between Grps	152.70	2	76.35	230.10	**0.000**
Within Grps	7.30	22	0.33		
Total	160.00	24			
10g Mono total					
Between Grps	512.54	2	256.27	147.98	**0.000**
Within Grps	38.10	22	1.73		
Total	550.64	24			

Table F-9 **Group statistics - height**

Height (cm)					95% Confidence Interval for Mean	
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	170.40	9.48	4.24	158.63	182.17
Diabetic no complications	10	170.47	6.89	2.18	165.55	175.39
Diabetic neuropathic	10	178.52	8.73	2.76	172.27	184.77
Total	25	173.68	8.81	1.76	170.04	177.31

Table F-10 **ANOVA - height**

	Sum of Squares	df	Mean Square	F	Sig
Height:					
Between Grps	391.09	2	195.54	2.92	**0.075**
Within Grps	1471.82	22	66.90		
Total	1862.91	24			

Table F-11 **Group statistics - weight**

Weight (Kg)					95% Confidence Mean	Interval for
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	62.80	15.12	6.76	44.02	81.58
Diabetic no complications	10	89.53	22.77	7.20	73.24	105.82
Diabetic neuropathic	10	103.66	17.54	5.55	91.11	116.21
Total	25	89.84	24.08	4.82	79.90	99.78

Table F-12 **ANOVA - weight**

	Sum of Squares	Df	Mean Square	F	Sig
Weight:					
Between Grps	5566.70	2	2783.35	7.34	**0.004**
Within Grps	8347.79	22	379.45		
Total	13914.48	24			

Table F-13 **Group statistics - BMI**

BMI (*x* : *y*)					95% Confidence Mean	Interval for
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	21.37	3.36	1.50	17.19	25.55
Diabetic no complications	10	30.50	6.33	2.00	25.97	35.03
Diabetic neuropathic	10	33.48	3.90	1.23	30.69	36.27
Total	25	29.87	6.58	1.32	27.15	32.58

Table F-14 **ANOVA – BMI**

	Sum of Squares	df	Mean Square	F	Sig
BMI:					
Between Grps	495.54	2	247.77	10.04	**0.001**
Within Grps	542.77	22	24.67		
Total	1038.31	24			

Table F-15 **Group statistics - age**

Age (Years)					95% Confidence Mean	Interval for
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	41.40	10.78	4.82	28.01	54.79
Diabetic no complications	10	53.80	6.09	1.93	49.44	58.16
Diabetic neuropathic	10	59.60	9.72	3.07	52.65	66.55
Total	25	53.64	10.71	2.14	49.22	58.06

Table F-16 **ANOVA - age**

	Sum of Squares	df	Mean Square	F	Sig
Age:					
Between Grps	1104.56	2	552.28	7.37	**0.004**
Within Grps	1649.20	22	74.96		
Total	2753.76	24			

Table F-17 **Group statistics – HbA1c's**

HbA1c's (%)					95% Confidence Mean	Interval for
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	5.46	0.39	0.18	4.97	5.95
Diabetic no complications	10	8.38	1.64	0.52	7.21	9.55
Diabetic neuropathic	10	9.42	1.28	0.40	8.51	10.33
Total	25	8.21	1.96	0.39	7.40	9.02

Table F-18 **ANOVA – HbA1c's**

	Sum of Squares	df	Mean Square	F	Sig
HbA1c's:					
Between Grps	52.74	2	26.37	14.69	**0.000**
Within Grps	39.50	22	1.80		
Total	92.25	24			

Table F-19 **Group statistics – glucose levels**

Glucose Levels (mmol / l)					95% Confidence Mean	Interval for
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	4.66	0.30	0.13	4.29	5.03
Diabetic no complications	10	10.73	6.44	2.03	6.13	15.33
Diabetic neuropathic	10	14.85	4.43	1.40	11.68	18.02
Total	25	11.16	6.12	1.22	8.64	13.69

Table F-20 **ANOVA – glucose levels**

	Sum of Squares	df	Mean Square	F	Sig
Glucose:					
Between Grps	349.26	2	174.63	6.99	**0.004**
Within Grps	549.94	22	25.00		
Total	899.20	24			

Table F-21 **Group statistics - cholesterol**

Cholesterol Levels (mmol / l)					95% Confidence Mean	Interval for
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	4.32	0.79	0.35	3.34	5.30
Diabetic no complications	10	4.70	0.95	0.30	4.02	5.38
Diabetic neuropathic	10	4.68	0.89	0.28	4.04	5.32
Total	25	4.62	0.87	0.17	4.26	4.98

Table F-22 **ANOVA - cholesterol**

	Sum of Squares	df	Mean Square	F	Sig
Cholesterol:					
Between Grps	0.55	2	0.28	0.34	0.714
Within Grps	17.70	22	0.81		
Total	18.25	24			

Table F-23 **Group statistics – cholesterol / HDL ratio**

Cholesterol / HDL (x : y)					95% Confidence Interval for Mean	
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	2.86	0.70	0.31	1.99	3.73
Diabetic no complications	10	3.90	1.31	0.41	2.96	4.84
Diabetic neuropathic	10	4.08	1.35	0.43	3.12	5.04
Total	25	3.76	1.27	0.26	3.24	4.29

Table F-24 **ANOVA – cholesterol / HDL ratio**

	Sum of Squares	df	Mean Square	F	Sig
Chol / HDL:					
Between Grps	5.27	2	2.64	1.72	0.202
Within Grps	33.69	22	1.53		
Total	38.96	24			

Table F-25 **Group statistics - creatinine**

Creatinine Levels (µmol / l)					95% Confidence Interval for Mean	
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	83.40	4.16	1.86	78.24	88.56
Diabetic no complications	10	84.40	9.07	2.87	77.91	90.89
Diabetic neuropathic	10	105.80	10.76	3.40	98.10	113.50
Total	25	92.76	13.98	2.80	86.99	98.53

Table F-26 **ANOVA - creatinine**

	Sum of Squares	df	Mean Square	F	Sig
Creatinine:					
Between Grps	2837.36	2	1418.68	16.86	**0.000**
Within Grps	1851.20	22	84.15		
Total	4688.56	24			

Table F-27 **Group statistics – triglyceride levels**

Triglyceride Levels (mmol / l)					**95% Confidence Interval for Mean**	
	N	**Mean**	**Std. Deviation**	**Std Error**	**Lower Bound**	**Upper Bound**
Controls	5	1.02	0.41	0.19	0.51	1.54
Diabetic no complications	10	2.03	0.76	0.24	1.49	2.57
Diabetic neuropathic	10	2.82	1.16	0.37	1.99	3.65
Total	25	2.14	1.10	0.22	1.69	2.60

Table F-28 **ANOVA – triglyceride levels**

	Sum of Squares	df	Mean Square	F	Sig
Triglycerides:					
Between Grps	11.02	2	5.51	6.75	**0.005**
Within Grps	17.95	22	0.82		
Total	28.97	24			

Table F-29 **Group statistics – HDL's**

HDL's (mmol / l)					95% Confidence Mean	Interval for
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	1.61	0.56	0.25	0.91	2.31
Diabetic no complications	10	1.31	0.44	0.14	0.99	1.62
Diabetic neuropathic	10	1.25	0.41	0.13	0.95	1.54
Total	25	1.34	0.46	0.09	1.15	1.53

Table F-30 **ANOVA – HDL's**

	Sum of Squares	df	Mean Square	F	Sig
HDL's:					
Between Grps	0.45	2	0.23	1.09	0.354
Within Grps	4.57	22	0.21		
Total	5.02	24			

Table F-31 **Group statistics – LDL's**

LDL's (mmol / l)					95% Confidence Mean	Interval for
	N	Mean	Std. Deviation	Std Error	Lower Bound	Upper Bound
Controls	5	1.96	0.60	0.27	1.21	2.71
Diabetic no complications	10	2.46	0.96	0.30	1.78	3.15
Diabetic neuropathic	10	2.54	1.19	0.38	1.69	3.39
Total	25	2.39	0.99	0.20	1.98	2.80

Table F-32 **ANOVA – LDL's**

	Sum of Squares	df	Mean Square	F	Sig
LDL's:					
Between Grps	1.20	2	0.60	0.59	0.566
Within Grps	22.54	22	1.03		
Total	23.74	24			

Table F-33 **Independent Samples Test – History of Ulceration and Core 2 transferase**

Core 2 transferase (pmoles/h/hg protein)	**t**	**Df**	**Sig. (2-tailed)**	**Mean Difference**	**Std. Error Difference**
Equal variances assumed	3.91	18	**0.01**	602.54	426.06

Appendix G

Table G-1 **Specification of Biomen 8160 analyser**

Samples	Whole Blood and/or Haemolysate
Measured Items:	Diabetes Monitoring Mode: HbA1c, HbA1, HbF, Main Variant Hbs Detection Diabetes Monitoring and Thalassaemia Screening Mode: HbA1c, HbA1, HbF, HbA2, Main Variant Hbs Quantification
Measurement Principle:	Reversed-phase cation exchange chromatography
Detection Method:	Dual-wavelength calorimetry (415-500 nm)
Resolution:	0.1%
Throughput:	Diabetes Monitoring Mode: 2.9 minutes per sample Diabetes Monitoring and Thalassaemia Screening Mode: 4.2 minutes per sample
Sample Volume:	Approximately 4 µl whole blood
Sample Tubes:	Blood Collection Tube 12,3 to 15 diameter x 75 to 100 mm length Sample Cup 500 µl
Sampling method:	Cap Piercing
Rack:	Sysmex compatible
Column Temperature:	Approximately 40° C
External Output:	RS-232C (2-way), 1 channel, 300 to 9600 bps
Operating Temperature:	10 to 30° C
Operating Humidity:	20 to 80% (no condensation)
Display:	2-row x 20-digit LCD
Built-in Printer:	58 mm wide thermal printer
Sample Loading Capacity:	A maximum of 100 samples. Continuous loading possibility
Min. Required Sample Volume:	Blood Collection Tube: 1 ml Sample Cup: 400 µl
Power Supply:	200 V AC, 50/60 Hz
Power Input:	320 VA
External Dimensions:	530W x 530D x 530H mm
Weight:	Approximately 43 Kg