Study Protocol

LANDMARK STUDY

LONGITUDINAL ASSESSMENT OF NEUROPATHY IN DIABETES USING NOVEL OPHTHALMIC MARKERS

also known as
OPHTHALMIC MARKERS OF DIABETIC NEUROPATHY

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Synopsis

Advanced diabetic neuropathy is a major cause of morbidity and mortality worldwide. The late sequelae of diabetic neuropathy include foot ulceration and lower extremity amputation. Therefore, the accurate early detection, characterization and quantification of diabetic neuropathy are important to define at-risk patients, anticipate and monitor deterioration, and assess new therapies. Nerve electrophysiology testing (NET) and quantitative sensory testing (QST) have only a limited capacity to assess small fibre pathology. Nerve and skin biopsy methods allow detailed pathological assessment of small nerves but are invasive and cannot be performed in routine clinical practice. Corneal confocal microscopy (CCM) is a relatively new technique that can be used to examine small nerve fibres; it is a real-time, non-invasive, time-efficient, cost-effective and reiterative means of imaging the sub-basal nerve plexus of the in vivo human cornea at 700X magnification, and has been shown to be capable of accurate diagnosis and stratification of neuropathic severity in diabetic patients. In addition, corneal sensitivity can be monitored using a novel, non-invasive and new form of QST, namely, non-contact corneal aesthesiometry (NCCA). The aim of this work is to employ these two novel non-invasive ophthalmic markers of peripheral nerve dysfunction – CCM and NCCA – to investigate longitudinal changes in peripheral nerve morphology and function in individuals with Type 1 diabetes and Latent Autoimmune Diabetes in Adults (LADA) with and without neuropathy. Diabetic participants will be recruited from those attending the Department of Diabetes and Endocrinology at the Princess Alexandra Hospital in Brisbane, Australia and the Manchester Diabetes Centre, Manchester Royal Infirmary, United Kingdom. Four cohorts will be recruited, each with 96 participants:

- Type 1 diabetic individuals without neuropathy,
- Type 1 diabetic individuals with neuropathy,
- LADA individuals with neuropathy,
- Type 2 diabetic individuals with varying degrees of neuropathy (Brisbane only)

One control cohort of 116 participants will be recruited as follows:

- Non-diabetic participants without neuropathy.

All participants will be examined annually for five years. At each visit, the following will be assessed: quantitative assessment of pain (using McGill pain questionnaire); neuropathic deficits (Neuropathy Disability Score); QST of vibration and thermal perception; NET; corneal sensitivity (using NCCA); corneal nerve fibre morphology (using CCM), risk factors (e.g. BP, smoking) and metabolic information (e.g. HbA1c). On a small subset of participants, a montage of the corneal nerve plexus will be generated. Skin punch biopsies will be performed periodically on a subset of participants in Manchester. As well, all study participants will be tested for antibodies to glutamic acid decarboxylase (GADAb) to help differentially diagnose individuals with LADA from those with Type 2 diabetes.
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<td>AVCC</td>
<td>Australian Vice Chancellors' Committee</td>
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<td>CCM</td>
<td>Corneal confocal microscopy</td>
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<td>CNFBD</td>
<td>Corneal nerve branch density</td>
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<td>CNFD</td>
<td>Corneal nerve fibre density</td>
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<td>CNFL</td>
<td>Corneal nerve fibre length</td>
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<td>CNFT</td>
<td>Corneal nerve fibre tortuosity</td>
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<td>CRF</td>
<td>Case Report Form</td>
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<td>CS</td>
<td>Corneal sensitivity</td>
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<td>DSMC</td>
<td>Data and Safety Monitoring Committee</td>
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<td>ETDRS</td>
<td>Early Treatment of Diabetic Retinopathy Study</td>
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<td>JDRFI</td>
<td>Juvenile Diabetes Research Foundation International</td>
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<tr>
<td>GADAb</td>
<td>Glutamic acid decarboxylase antibodies</td>
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<td>GCP</td>
<td>Good Clinical Practice</td>
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<td>HREC</td>
<td>Human Research Ethics Committee</td>
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<tr>
<td>HRV</td>
<td>Heart rate variability</td>
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<td>IGT</td>
<td>Impaired glucose tolerance</td>
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<tr>
<td>IHIBI</td>
<td>Institute of Health and Biomedical Innovation</td>
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<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
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<tr>
<td>LADA</td>
<td>Latent Autoimmune Diabetes in Adults</td>
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<tr>
<td>LMM</td>
<td>Linear mixed model</td>
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<tr>
<td>MRI</td>
<td>Manchester Royal Infirmary</td>
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<td>NCCA</td>
<td>Non-contact corneal aesthesiometry</td>
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<td>NDS</td>
<td>Neuropathy Disability Score</td>
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<tr>
<td>NET</td>
<td>Nerve electrophysiology testing</td>
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<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
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<td>NHS</td>
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<tr>
<td>OGTT</td>
<td>Oral Glucose Tolerance Test</td>
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<td>PAH</td>
<td>Princess Alexandra Hospital</td>
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<tr>
<td>PI</td>
<td>Principal Investigator</td>
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<td>QST</td>
<td>Quantitative sensory testing</td>
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<td>QUT</td>
<td>Queensland University of Technology</td>
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<tr>
<td>SOP</td>
<td>Standard operating procedure</td>
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<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
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<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
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<tr>
<td>UM</td>
<td>University of Manchester</td>
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1 Background

Diabetic neuropathy

Diabetic neuropathy is a significant clinical problem that currently has no effective therapy, and in advanced cases, it is a major cause of morbidity and mortality worldwide. The accurate detection, characterization and quantification of this condition are important to define at risk patients, anticipate deterioration, monitor progression and assess new therapies. The late sequelae of diabetic neuropathy include foot ulceration and ultimately, in some cases, Charcot’s neuroarthropathy and lower extremity amputation. Foot ulceration is much more common in neuropathic patients with the annual incidence rising from <1% in those without neuropathy to >7% in those with established neuropathic deficits.\(^7\) As 80% of amputations are preceded by foot ulceration, an effective treatment to prevent or slow the progress of peripheral neuropathy would therefore have a major medical, social and economic impact. Indeed, Ollendorf et al.,\(^8\) using a model based upon the incidence and cost of lower extremity amputations in diabetes, predicted potential savings of US $2 to $3 million over three years if this problem could be solved. Ramsey et al\(^9\) estimated the attributable costs for a middle-aged diabetic male patient, two years after a new foot ulcer, to be US $28,000. Such costs potentially could be minimized by the strategic application of CCM and NCCA should the efficacy of these approaches for detecting, characterizing and monitoring the progression of diabetic neuropathy be validated in this work.

Current methods of assessing nerve morphology

Conventional techniques of electrophysiology and quantitative sensory testing along with an assessment of neurological disability offer a relatively robust means of defining neuropathic severity.\(^10\) However, they have major shortcomings when they are employed to define therapeutic efficacy in clinical intervention trials.\(^11\) These are primarily those of an inability to target the fibre types demonstrating benefit but also limited ability to demonstrate regeneration and repair. The only techniques which allow a direct examination of thinly myelinated and unmyelinated nerve fibre damage and repair, are those of sural nerve biopsy with electron microscopy,\(^12,13\) and the newly refined skin punch biopsy.\(^14-17\) However, both are invasive procedures and particularly in the case of assessing therapeutic efficacy, demand repeat assessment which has to be at a different site from the original biopsy. Magnetic resonance imaging reveals a significant reduction in cross-sectional area of the cervical spine in early diabetic neuropathy,\(^18\) but again, this technique is not amenable to routine clinical use. Also, the functional relevance of the morphological changes observed using the above methodologies is not clearly defined.

Effective intervention must be aimed at a stage when there is a capacity for the nerve to repair i.e. in the sub-clinical or early phase of nerve damage. Several groups have demonstrated that up to 40% of patients with idiopathic neuropathy have impaired glucose tolerance (IGT)\(^14-16\) compared to less than 15% in the age-matched general population.\(^19\) This observation supports the hypothesis that IGT-related neuropathy may represent the earliest stage of diabetic neuropathy which is characterized by damage to the small fibres: indeed small fibre abnormalities as assessed by intra-epidermal nerve fibre (IENF) density was the only test which improved after lifestyle intervention.\(^20\) We have recently also
shown that diabetic patients with minimal neuropathy (normal electrophysiology and quantitative sensory tests) show significant unmyelinated fibre degeneration.\textsuperscript{13}

In an interventional study, whilst the peroneal motor nerve conduction velocity improved significantly, myelinated fibre density did not, and to complicate matters further, regenerative activity was confined almost exclusively to the small myelinated fibres, which are not assessed employing conventional electrophysiology.\textsuperscript{21} Furthermore, our studies show no relationship between quantitative sensory tests and unmyelinated fibre pathology.\textsuperscript{13,22} Thus, whilst nerve conduction studies and quantitative sensory testing are useful and well-validated measures of progression for established diabetic neuropathy, their utility in early neuropathy where small fibres are damaged is limited, because they primarily measure large myelinated nerve fibre function.

Whilst a number of candidate mechanisms for nerve fibre damage and repair in diabetes may be exploited to therapeutic advantage, a major barrier has been the availability of a relevant, non-invasive, sensitive but representative measure of nerve fibre damage and repair in human diabetic neuropathy. Although a range of tests have been used to assess therapeutic efficacy in diabetic neuropathy, regulatory authorities favour clinically relevant surrogate endpoints.\textsuperscript{10} Unfortunately, there are no unequivocal clinical markers for the early stage of nerve damage, nor are there any clear prognostic markers indicating progression of nerve damage. This goal is vital to the success of new therapies for human diabetic neuropathy.

**Corneal confocal microscopy**

The relatively new technique of CCM offers researchers and clinicians the opportunity to directly and non-invasively examine small nerve fibres in the living human cornea at a magnification of about 750X.\textsuperscript{23} In broad terms, the optical principle of CCM is that field of view is sacrificed for resolution but is regained by scanning. This allows essentially transparent tissue to be viewed with good contrast and high lateral resolution. A key advantage of CCM is that it is a simple and rapid clinical procedure that could be undertaken by a trained medical assistant. Although for statistical and analytical purposes the proposed research involves obtaining multiple scans and detailed image analysis, the clinical application would involve a 3-minute examination and we are currently developing paradigms that use automated image analysis software to provide an instant diagnosis of neuropathy without the need for specialist interpretation. At present this instrumentation is moderately expensive (around $60K), but the cost is likely to decrease as the instrument gains wider use in the ophthalmic community, at which time CCM could be readily translated into routine use in ophthalmic and/or diabetes clinics. The added attraction of this technique (as well as NCCA; see below) is that, as an ophthalmic procedure, it could be conducted as part of a slightly extended routine ophthalmic screening examination of diabetic patients who typically undergo annual fundus examination/photography for the detection of potentially sight-threatening diabetic retinopathy.

**Non-contact corneal aesthesiometry**

Murphy et al\textsuperscript{24-26} have described how corneal sensitivity can be measured using the NCCA. This instrument uses controlled pulses of air of different pressure to stimulate the cornea and measures the corneal nerve threshold to a composite stimulus consisting of air pressure, tear film evaporation and disruption. The advantage of NCCA over the traditional
and more conventional von Frey hair or Cochet-Bonnet aesthesiometer (using a fine nylon filament) is that a large, continuous range of stimulus intensity can be produced, the stimulus is more precise and sensory-specific, testing is less variable than when using a filament, and there is minimal patient apprehension. The NCCA can assess the corneal sensation threshold in an accurate and repeatable manner. Murphy et al have also determined that NCCA allows measurement of lower stimulus thresholds than Cochet-Bonnet aesthesiometry.

Significance of this work to Type 1 diabetes
This work is unique for two reasons. First, we will longitudinally monitor changes in small nerve fibre morphology non-invasively in humans in vivo; this has never been achieved before in any field of medical science. Second, we are the only group in the world investigating the application of both CCM and NCCA as markers of diabetic neuropathy in individuals with Type 1 diabetes and LADA.

The proposed longitudinal study will focus on the progression of diabetic neuropathy in individuals with Type 1 diabetes and LADA. The reasons for recruiting these categories of individuals (and not those with Type 2 diabetes) are that these individuals are likely to develop neuropathic changes sooner than individuals with Type 2 diabetes, and the neuropathic progression might be expected to occur more rapidly in these groups, both factors serving to increase the likelihood of observing the onset and some progression of neuropathy in a reasonably sized cohort within the limited time span of this study. Furthermore, evidence exists for differences in small fibre neuropathy between Types 1 and 2 diabetes, which may manifest as a difference in the rate of progression of diabetic neuropathy and probably differences in response to medical management e.g. improving glycaemic control and perhaps other risk factors (especially vascular risk factors in Type 2 diabetes) between Types 1 and 2 diabetic patients. Pooling Types 1 and 2 diabetic patients may mask or confound findings of specific relevance to either group.

Despite the considerable efforts into characterizing LADA, which some have termed ‘slowly-progressing Type 1 diabetes’, the literature is unclear as to prevalence of diabetic neuropathy in this group compared to classically-defined Types 1 and 2 diabetes. For example, Isomma et al suggest that patients with LADA have the same prevalence of neuropathy (29%) as patients with Type 2 diabetes (27%), but a higher prevalence of neuropathy compared with patients with Type 1 diabetes (13%). This finding contrasts with that of Baum et al, who report that patients with LADA have fewer features of diabetic neuropathy than patients with Type 2 diabetes, and are more similar to patients with Type 1 diabetes in this regard. Certainly, very little is known about the pathogenesis of small nerve fibres or the time-course of degenerative changes in small nerve fibres in humans suffering from either of these conditions; such issues will be addressed directly in this study.

Of secondary interest in this project – but of no less importance – are the clinical implications of corneal nerve degeneration on corneal health in patients with Type 1 diabetes and LADA. That patients with diabetes have reduced corneal sensitivity is well known. Creuzot-Garcher et al and Nuho et al have demonstrated that the loss of corneal sensitivity in diabetic patients is more significant in those with neuropathy. Patients exhibiting such losses in corneal sensitivity will be more at risk because the
protective function of corneal nerves is compromised; thus, such patients might be less aware of corneal trauma and infection, allowing such conditions to significantly worsen before therapeutic intervention. Corneal innervation also provides trophic functions in corneal repair in relation to disease, trauma or surgery. This could be relevant as a higher risk of developing post-operative epithelial complications and poorer refractive results have been demonstrated in diabetic patients undergoing laser refractive surgery for myopia.\textsuperscript{33}

1.1 Preliminary Studies

Cross-sectional studies of diabetic neuropathy employing corneal confocal microscopy

After documenting the appearance of the normal cornea as seen with CCM,\textsuperscript{39,40} we were the first to publish a detailed analysis of the corneal sub-basal nerve plexus viewed with this instrument.\textsuperscript{41} That work established the experimental paradigm for classifying various features of nerve morphology upon which subsequent studies of corneal nerve dysfunction were based.\textsuperscript{42-46} Of particular interest was the application of CCM for the assessment of corneal nerves in patients with various degrees of diabetic neuropathy (Figure 1).\textsuperscript{1}

![Figure 1. Representative CCM images of the sub-basal nerve plexus of a healthy non-diabetic subject (left) and a diabetic patient with severe neuropathy (right).](image)

Following on from the preliminary work of Rosenberg et al,\textsuperscript{36} we validated this approach by documenting a significant reduction in CNFD, CNFL and CNFBD,\textsuperscript{2} and an increase in CNFT,\textsuperscript{6} in diabetic patients compared to control subjects. Furthermore, we demonstrated a significant correlation between two markers of degeneration – CNFD and CNFL – and neuropathic severity in diabetic patients.\textsuperscript{2} These findings, especially in relation to CNFD, have been confirmed by subsequent workers.\textsuperscript{47-49} Our more recent observations of significant nerve pathology\textsuperscript{3} and reduced corneal sensitivity\textsuperscript{5} in non-neuropathic diabetic patients suggest a role for CCM and NCCA in the early detection of this debilitating condition.

Patel and McGhee\textsuperscript{50} have recently described a novel approach to the investigation of sub-basal corneal nerves, whereby CCM is used to capture a large number of images across the cornea, which are then mapped together to form a confluent montage. This approach allows the overall pattern of the sub-basal nerve plexus to be appreciated. Patel and McGhee\textsuperscript{50} demonstrated that the sub-basal nerve plexus radiates towards a whorl-like complex centred 1 to 2 mm below the corneal apex. We intend to adopt this technique to provide new insights into the overall pattern of nerve degeneration over time in the sub-
basal nerve plexus of the cornea in a subset of individuals with Type 1 diabetes and LADA suffering from diabetic neuropathy. Figure 2 is a montage, generated in our laboratory, of the nerve plexus of a healthy, non-diabetic participant.

Figure 2. Montage of images obtained using CCM, showing the overall distribution of nerves in the sub-basal nerve plexus of the cornea of a healthy, non-diabetic participant.

Cross-sectional studies of diabetic neuropathy employing non-contact corneal aesthesiometry

Using NCCA, Murphy et al\textsuperscript{26} demonstrated a loss of corneal sensitivity in diabetic patients. We have extended this work by subjecting 147 diabetic patients and 18 age-matched control subjects to assessment of corneal sensitivity using the contact Cochet-Bonnet aesthesiometer (CBE) and NCCA.\textsuperscript{5} Diabetic patients were stratified into differing severities of neuropathy using the Neuropathy Disability Score (NDS). Corneal sensitivity was significantly reduced using both CBE (P=0.0001) and NCCA (P<0.0001) in diabetic patients compared to control subjects. Using NCCA, loss of corneal sensation correlated significantly with increasing duration of diabetes (P = 0.002) and the severity of somatic neuropathy (P<0.0001).\textsuperscript{5}

Longitudinal studies of diabetic neuropathy

Only a small number of longitudinal studies of diabetic neuropathy have been undertaken, and most have used QST as an index of the severity of neuropathic deficit. These studies have revealed important associations and risk factors related to the development of neuropathy in diabetes. Dyck et al\textsuperscript{51} found that neuropathy in diabetic patients worsened over a 7-year period and van de Poll-Franse et al\textsuperscript{52} and Adler et al\textsuperscript{53} found that 21% and 50% of Type 2 diabetic patients, respectively, progressed toward developing significant neuropathy over 4 years. Forrest et al\textsuperscript{54} reported that 15% of children with Type 1 diabetes developed neuropathy over a 6 year period. Tesfaye et al\textsuperscript{55} observed that, after a mean of 7.3 years of follow-up, neuropathy had developed in 24% of patients with Type 1 diabetes without neuropathy at baseline. Partanen et al\textsuperscript{56} reported that the number of
patients with Type 2 diabetes who had nerve conduction abnormalities in the legs and feet increased from 8% at baseline to 16% after 5 years and to 42% after 10 years.

In the studies described above, risk factors positively correlated to the development of neuropathy in diabetes included age, height, body mass index, ankle-arm index, duration of diabetes, hypertension, smoking, poor glycemic control, lipid profile and albumin level. While providing valuable information relating to factors associated with diabetic neuropathy, these studies shed no light on the pathological changes taking place in small nerve fibres, or the degree to which changes in symptoms and signs correlate with the rate of nerve degeneration.

We have also demonstrated, in various contexts aside from diabetes, that CCM and NCCA can be used to monitor long term changes in corneal nerve morphology and sensitivity. In patients undergoing Laser In-Situ Keratomileusis (LASIK) and Laser-Assisted Sub-Epithelial Keratectomy (LASEK) – refractive surgery procedures in which corneal nerves are severed and/or ablated – we demonstrated the absence of nerves in the sub-basal layer and loss of corneal sensitivity immediately following these procedures followed by a significant recovery in nerve fibres and corneal sensitivity over the following 6 months. In a separate pilot study, we examined 24 diabetic patients (who maintained good health and were compliant with their therapeutic regimen) and 18 control subjects with CCM at baseline and after 25.2 ± 1.4 months. An increase in CNFD (19.1 ± 10.8 to 25.0 ± 9.2 nerves/mm²; p = 0.009) was observed during follow up, indicating reduced neuropathic severity. There was no significant change in this parameter in the control subjects. The increase in CNFD was related to a statistically significant improvement, albeit of low magnitude, in HbA1c (8.22% ± 1.48 to 7.59% ± 1.16; p = 0.02). We have also been able to demonstrate small fibre repair within 6 months of successful pancreas transplantation in patients with Type 1 diabetes. These findings suggest that CCM and NCCA can be used to assess longitudinal changes in nerve morphology, and it stands to reason that such techniques ought to be able to monitor the progress of neuropathic severity in patients with Type 1 diabetes and LADA.

Length of longitudinal study

No data currently exists on long-term changes in human nerve morphology in diabetes. A longitudinal study designed to generate this information necessarily needs to be of sufficient length to allow clinically and pathologically significant changes to be observed. Dyck et al has suggested that controlled clinical trials of patients with diabetic neuropathy need to be conducted for a period of at least 3 years to achieve clinically meaningful and statistically significant changes in QST results. The shortest longitudinal studies of diabetic neuropathy are those of van de Poll-Franse et al and Adler et al, both of these studies demonstrated significant sensory deficits in neuropathic severity over this period. In light of this information, and our previous demonstration of the link between NDS and deficits in both corneal nerve morphology and corneal sensitivity, it seems reasonable to suppose that significant changes in these parameters will be observed over a 4-year period. Given a one-year 'lead in' period at the commencement of the proposed study to allow for refinement of the experimental protocol, commissioning equipment, initiating recruitment etc., 4 years of longitudinal data will be collected on the planned cohort of 408 participants.
2 Trial Objectives

The objective of this clinical study is to employ two novel, non-invasive and reiterative ophthalmic markers of peripheral nerve dysfunction – corneal confocal microscopy (CCM)\(^1\)\(^-\)\(^4\) and non-contact corneal aesthesiometry (NCCA)\(^5\) – to investigate longitudinal changes in peripheral nerve morphology and function in individuals with Type 1 and Type 2 diabetes and Latent Autoimmune Diabetes in Adults (LADA) with and without neuropathy.

2.1 Specific Aims

We aim to investigate the following:

- Changes in nerve pathology using CCM and corneal sensitivity using NCCA over time.
- The progression of neuropathy, as measured with CCM and NCCA, compared with the results of neurological deficits (NDS); nerve electrophysiology, quantitative sensory testing (QST) and skin biopsy.
- The ability of CCM and NCCA to detect neuropathy earlier than established measures of diabetic neuropathy (electrophysiology, QST, skin biopsy).
- The rate of progression of pathology of corneal nerves, as determined using CCM and NCCA, in individuals with Type 1 diabetes, Type 2 diabetes and LADA.
- The sensitivity, specificity and discrimination capacity of CCM and NCCA.
- The identification of risk factors associated with development and progression of corneal nerve damage, observed using CCM, and nerve dysfunction, observed using NCCA.
- The development of a qualitative index of morphological changes to the extensive neural branching networks utilizing montages of the sub-basal nerve plexus.

3 Study Design

3.1 General Approach

This will be an investigator-masked prospective longitudinal controlled observational study conducted at two sites, one in Australia and one in the UK. The Anterior Eye Laboratory in Brisbane, Australia will coordinate the study. A cohort of 500 participants will be enrolled (298 in Brisbane and 202 in Manchester) according the inclusion/exclusion criteria, and followed annually for four subsequent years.

3.2 Research Plan

Individuals with Type 1 and Type 2 diabetes and LADA will be recruited from those attending the Centre for Diabetes and Endocrine Research at Princess Alexandra Hospital and Mater Hospitals in Brisbane, Australia, and the Manchester Diabetes Centre,
Manchester Royal Infirmary in Manchester, United Kingdom, the community and other clinics. Four cohorts will be recruited, each of 96 participants:

Group 1: Individuals with Type 1 diabetes and without neuropathy
Group 2: Individuals with Type 1 diabetes with neuropathy
Group 3: Individuals with LADA with neuropathy
Group 4: Individuals with Type 2 diabetes with varying degrees of neuropathy (Brisbane only).

One control cohort of 116 participants will be recruited:
Group 5: Non-diabetic subjects without neuropathy.

All participants will be examined at baseline and at 12-month intervals for a total of 5 visits over 4 years. Relevant demographic measures (age, height, body mass index, waist circumference, duration of diabetes, blood pressure, smoking) and metabolic information (e.g. HbA1c, lipid profile, creatinine, albumin levels) will be obtained from diabetic and control participants at baseline and throughout the study at each assessment.

All participants will have a test for glutamic acid decarboxylase antibodies (GADAb) to differentiate LADA and Type 1 from Type 2 diabetic individuals.

### 3.3 Outcome Markers

The two primary corneal outcome markers are (1) morphometric measures obtained from CCM of the corneal sub-basal nerve plexus, and (2) the corneal sensitivity threshold.

Specifically, the following morphometric measures obtained from CCM, which we have previously validated as indicators of corneal nerve fibre damage in diabetic neuropathy,\(^1\)\(^-\)\(^4\)\(^,\)\(^6\) will serve as markers of nerve fibre pathology:

- Corneal nerve fibre density (CNFD) – number of major nerves/mm\(^2\) of corneal tissue;
- Corneal nerve fibre length (CNFL) – length of nerves/mm\(^2\) of corneal tissue;
- Corneal nerve branch density (CNFBD) – number of nerve branches/mm\(^2\) of corneal tissue;
- Corneal nerve fibre tortuosity (CNFT) – degree of non-linearity of the nerve fibres.

Corneal sensitivity will be measured using a NCCA according to a procedure which we have described previously.\(^5\) In essence, the corneal sensitivity threshold is determined using the forced choice, double staircase psychophysical technique, and is expressed as air pressure in millibars. Corneal sensitivity results will serve as a marker of nerve fibre function.
3.4 Study Recruitment

The participants in the study will be recruited primarily from patients attending the Centre for Diabetes and Endocrine Research at Princess Alexandra Hospital and Mater Hospitals in Brisbane, Australia, and the Manchester Diabetes Centre, Manchester Royal Infirmary in Manchester, United Kingdom, the community and other clinics by the research team members. The general conduct of the study will be explained and the patients will be given an information sheet describing the conduct of the study and the commitment involved, shown in Appendix 1. Patients who indicate an interest in participating will be asked to sign the informed consent form prior to participation and undergo screening tests to determine eligibility, and if eligible, will be enrolled into the study.

Sample text for recruitment purposes is shown in Appendix 4.

Control participants will be recruited from staff, students and associates of Princess Alexandra Hospital, Manchester Royal Infirmary, Queensland University of Technology and University of Manchester. Newspaper and web advertisements may also be used.

3.5 Study Population

A total of 500 participants will be enrolled, 298 in Brisbane and 202 in Manchester. Participants at each site will be enrolled into each of the following five groups according to the inclusion/exclusion criteria:

Group 1: Individuals with Type 1 diabetes and without neuropathy (48 participants/site)
Group 2: Individuals with Type 1 diabetes with neuropathy (48 participants/site)
Group 3: Individuals with LADA with neuropathy (48 participants/site)
Group 4: Individuals with Type 2 diabetes (96 participants, Brisbane only), in the following subgroups:
   i. No neuropathy, NDS ≤ 2 (n=24)
   ii. Mild neuropathy, NDS 3-5 (n=24)
   iii. Moderate neuropathy, NDS 6-8 (n=24)
   iv. Severe neuropathy, NDS 9-10 (n=24)
Group 5: Non-diabetic participants without neuropathy (n=58 participants/site).

3.5.1 Inclusion Criteria

Participants must satisfy the following conditions prior to inclusion in the study:

a) Aged 14 to 75 years
b) Signed written informed consent (Appendix 1 Error! Reference source not found.)
c) Type 1 diabetes, Type 2 diabetes or LADA (or absence of diabetes for the control group)
d) Be willing to participate and comply with the experimental protocol.

3.5.2 Exclusion Criteria

Any of the following will render a participant ineligible for inclusion:

a) History of corneal trauma or surgery (NB cataract surgery does not preclude enrolment unless surgery occurred in the 12 months prior to enrolment date)
b) History of ocular disease or systemic disease which may affect the cornea   
c) Concurrent ocular disease, infection or inflammation 
d) History of systemic disease (e.g. malignant disease, congestive heart failure NYHA Grade III or IV, major psychosis (i.e. schizophrenia or bipolar), certain autoimmune diseases – hypothyroidism, coeliac disease) 
e) History of neuropathy due to non-diabetic cause e.g. alcoholism, amyloidosis, autoimmune disorders, chronic kidney failure, connective tissue disease, infectious disease (e.g., Lyme disease, HIV/AIDS, hepatitis B, leprosy), liver failure, radiculopathy, vitamin deficiencies (e.g. pernicious anaemia, B12 deficiency)
f) Presence of severe diabetic neuropathy indicated by NDS > 8 (NB. NDS > 8 is acceptable in the Type 2 diabetic group; Group 4).
g) Current or active diabetic foot ulcer or infection 
h) Participating in any other interventional (e.g. drug) research trial.

The following exclusion criteria apply to Group 5 (non-diabetic participants without neuropathy):

a) Diabetes, although impaired glucose tolerance (IGT) is acceptable
b) GADAb positive.

3.5.3 Group Assignment

Definition of Neuropathy

An individual is considered to have neuropathy if they meet the following criteria (Tesfaye et al, 2010), hereafter referred to as Toronto criteria:

1. Abnormal nerve conduction, based on age-matched control individuals at the site;

AND

2. A symptom or sign of neuropathy, defined as one or more of the following:
a) Diabetic neuropathy symptom (DNS) score of 1 or more out of 4 (Meijer et al, 2002)

b) Neuropathy Disability Score (NDS) of 3 or more out of 10 (Young et al, 1993).

The above definition will be used for classification of participants at baseline. San Antonio criteria may also be used for subsequent classification of participants.

**San Antonio Criteria**

The definition of neuropathy has been derived from the San Antonio criteria (American Diabetes Association, 1988), as used in the Rochester Diabetes study (after Dyck et al, 1993).

For the purpose of this protocol the above will be referred to as ‘San Antonio criteria’.

An individual is considered to have neuropathy if they have ≥2 abnormalities among criteria 1-5 (below) with at least one being #3 (electrodiagnostic studies) or #5 (autonomic testing);

1. **Clinical Symptoms**
   - Diabetic Neuropathy Symptom (DNS) score; a score of 1 or more out of 4. (Meijer et al, 2002) – see LANDMark MOP, SOP 10.

2. **Clinical Examination**
   - A Neuropathy Disability Score (NDS) of 3 or more out of 10. (Young et al, 1993) – see LANDMark MOP, SOP 12.

3. **Electrodiagnostic Studies (EDX)**
   - A peroneal nerve conduction velocity of <42 m/s. (Malik et al, 1990) – see LANDMark MOP, SOP 12.

4. **Quantitative Sensory Testing (QST)**
   - A vibration perception that is abnormal for the participant participant’s age (compared to normative values in Medoc; LANDMark MOP, Table 1), or;
• A thermal (hot or cold) perception threshold that is abnormal for the participant’s age (compared to normative values in Medoc; LANDMark MOP, Table 1) - see LANDMark MOP, SOP 4.

5. Autonomic Function Testing

• Postural hypotension with a fall in blood pressure of 20mmHg systolic and/or 10mmHg diastolic or more after 1 minute and/or 3 minutes of standing upright (Lahrmann et al 2006) – see LANDMark MOP, SOP 5, or;

• Loss of heart rate variability with an EI ratio that is reduced for the participant’s age and gender, as compared to published normal values shown in LANDMark MOP Table 2 (Agelink MW et al, 2001) – see LANDMark MOP, SOP 7.

Definition of LADA

The following criteria will be used to identify the LADA group:

1. Diabetes with no insulin prescribed in the first 6 months following diagnosis, and

2. GADAb positive.

Criteria for Groups

Once initial eligibility has been determined, the following additional criteria apply to the respective groups.

Group 1: Individuals with Type 1 diabetes and without neuropathy

a) Type 1 diabetes
b) No neuropathy by Toronto criteria

Group 2: Individuals with Type 1 diabetes with neuropathy

a) Type 1 diabetes
b) Neuropathy by Toronto criteria

Group 3: Individuals with LADA

a) Diabetes with no insulin prescribed in the first 6 months following diagnosis
b) GADAb positive
Group 4: Individuals with Type 2 diabetes (Brisbane only)

a) Type 2 diabetes
   i. No neuropathy, NDS ≤ 2
   ii. Mild neuropathy, NDS 3-5
   iii. Moderate neuropathy, NDS 6-8
   iv. Severe neuropathy, NDS 9-10
b) None to mild retinopathy, ETDRS ≤ 35
c) Visual acuity 6/9 (0.2 logMAR) or better

Group 5: Non-diabetic participants without neuropathy.

a) No diabetes (although IGT is acceptable)
b) GADAb negative
c) No neuropathy by Toronto criteria

Screening procedures and instrumentation are as follows:

- inclusion/exclusion criteria discussed - history / eligibility checklist
- intraocular pressure (mmHg) - Tiolat i-care handheld tonometer or equivalent (Brisbane only)
- anterior ocular health - Topcon slitlamp biomicroscope or equivalent
- visual acuity with current spectacle prescription - Bailey-Lovie Visual Acuity chart
- current spectacle prescription - Tomey TL-100 Autolensmeter or equivalent
- retinal fundus photograph - Zeiss Visucam Pro Non-Mydriatic Fundus Camera or equivalent
- Neuropathy Disability Score - Neurotips, tuning fork, metal rods, reflex hammer.

3.6 Enrolment Procedures

The process of informed consent will involve information exchange between the research team member and the potential participants in accordance with local university and hospital guidelines.

The local study coordinator will ensure enrolled participants are registered immediately with the coordinating centre (Anterior Eye Laboratory, IHBI) via entry into Distiller web-based, encrypted LANDMark Study database.

3.7 Study Procedures

The tests, procedures, instrumentation and outcome variables for the study are shown in Table 1. Each test will be conducted at each visit, typically on the hand-dominant side (unless otherwise indicated) and recorded on the case report form (CRF, Appendix 1). The
CRF referred to in this protocol will be the electronic online data entry form/CRF, or paper CRF where required. The following procedures will be conducted according to the Landmark Manual of Operations (Table of Contents, Appendix 7) and hospital protocol where applicable.

Table 1. Procedures and outcome variables.

<table>
<thead>
<tr>
<th>Procedure/Instrumentation</th>
<th>Outcome Variables</th>
</tr>
</thead>
</table>
| Corneal confocal microscopy | • corneal nerve fibre density (CNFD) – number of major nerves/mm² of corneal tissue  
| Heidelberg HRT III with Rostock | • corneal nerve fibre length (CNFL) – length of nerves/mm² of corneal tissue  
| Cornea Module (Heidelberg Engineering, Germany) | • corneal nerve branch density (CNFBD) – number of nerve branches/mm² of corneal tissue;  
| | • corneal nerve fibre tortuosity (CNFT) – degree of non-linearity of the nerve fibres.  
| Non-contact corneal aesthesiometry | • non-contact corneal sensitivity threshold  
| History, scales, blood pressure monitor, heart rate monitor, retinal fundus camera | • age, duration of diabetes, smoking, alcohol consumption  
| | • height, weight, BMI, waist circumference  
| | • blood pressure  
| | • heart rate variability  
| | • diabetic retinopathy  
| Nihon Kohden Neuropack S1; MS92a EMG (Medelec Limited, Old Woking Surrey UK) or equivalent | • peroneal motor nerve conduction velocity  
| | • peroneal max M-wave amp  
| | • peroneal F-wave latency  
| | • tibial motor nerve conduction velocity  
| | • tibial max M-wave amp  
| | • tibial F-wave latency  
| | • sural sensory nerve amplitude of action potential  
| | • sural sensory nerve latency to onset  
| | • sural sensory nerve latency to peak  
| | **NB BNE may include all visits, MAN baseline, 2-year and 4-year visits**  
| Neurotips, tuning fork, reflex hammer, metal rods | • Neuropathy Disability Score  
| Monofilament (10g nylon filament) | • quick test of foot ulceration risk  
| Medoc TSA-II NeuroSensory Analyzer and the VSA-3000 Vibratory Sensory Analyzer (Medoc Advanced Medical Systems, Ramat-Yishai, Israel) | • warm sensation threshold  
| | • cold sensation threshold  
| | • heat-induced pain threshold  
| | • cold-induced pain threshold  
| | • vibration threshold  
| Blood/Urine samples | • HbA1c  
| | • Lipids (total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, triglycerides)  
| | • Serum B12  
| | • Creatinine  
| | • Estimated Glomerular Filtration Rate (calculated from creatinine, age and gender)  
| | • Antibodies to glutamic acid decarboxylase (GADAb)  
| | • Spot urine Microalbumin / Creatinine Ratio (mg/g)
A more detailed description of the procedures is provided below.

**Blood/Urine Testing**

Each participant will undergo annual fasting blood and urine tests. Fasting for 12 hours prior to testing will be required. Any additional blood and urine parameters (Table 1) performed in the routine diabetic clinics will be recorded from patient records for diabetic participants (i.e. Groups 1-4). Blood and urine samples will be destroyed following testing, typically within 7 days of collection, according to local laboratory protocols. Separate consent will be sought for storage of blood (see Tissue Bank).

If impaired fasting glucose is observed in a control participant, they will be asked to undergo an oral glucose tolerance test (OGTT). A participant without diabetes but with impaired fasting glucose (IFG) is eligible for the study (and IGT recorded as a covariate), however a participant with impaired OGTT is not eligible for the study and will be discontinued.

**Heart Rate Variability**

The deep breathing heart rate variability (HRV) test will be performed on all participants according to the Landmark Manual of Operations (Appendix 7) as an indication of autonomic neuropathy. The tests will be performed with the participant in the supine position after training of the breathing (6 respiratory cycles per minute; the investigator will provide a verbal cue). High resolution ECGs (approx. 8 minutes, 2-lead) will be recorded at rest (Brisbane only) and with deep breathing. The heart rate variability results will be expressed as the expiration: inspiration (E:I) ratio.

**Tissue Bank**

It is possible that future developments in biomarkers for diabetes will occur in the next few years. Therefore approximately 30 ml of blood and, in Manchester only, 3 mm of skin biopsy tissue will be stored for subsequent investigation according to the local regulations and ethical requirements.
Separate consent (Appendix 2) will be obtained for tissue banking of blood and/or skin biopsy samples. Blood and tissues will be collected according to the LANDMark BioBank Manual of Operations (Table of Contents, Appendix 8). Secure storage of samples will occur in a -80°C locked freezer at IHBI and Manchester. The stored blood and tissue samples will be stored without participant name or identifying features to protect the privacy of the donor, however, will be re-identifiable for the purposes of linking the tissue to clinical data (identifiable) and in the instance any participant wishes to withdraw their donation. Tissue samples will be stored for up to 7 years. Disposal will occur according to the LANDMark BioBank Manual of Operations. HREC approvals will be sought for the use of tissue.

Retinal Fundus Photography

Annual retinal fundus photographs will be taken with (typically) a dilated pupil using the Zeiss Visucam Pro NM (non-mydriatic) fundus camera, or equivalent. Diabetic retinopathy will be graded according to the Early Treatment of Diabetic Retinopathy Study (ETDRS) scale, shown in Table 2. Images will be uploaded to the Distiller database.

Table 2. ETDRS scale.

<table>
<thead>
<tr>
<th>Level</th>
<th>Severity</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>No retinopathy</td>
<td>Diabetic retinopathy absent</td>
</tr>
<tr>
<td>20</td>
<td>Very mild NPDR</td>
<td>Microaneurysms only</td>
</tr>
<tr>
<td>35</td>
<td>Mild NPDR</td>
<td>Hard exudates, cotton-wool spots, and/or mild retinal haemorrhages</td>
</tr>
</tbody>
</table>
| 43    | Moderate NPDR    | 43A Retinal haemorrhages moderate (>photograph 1F) in four quadrants or severe (≥photograph 2A) in one quadrant  
        |                         | 43B Mild IRMA (<photograph 8A) in one to three quadrants                      |
| 47 A-D| Moderate NPDR    | 47A Both level 43 characteristics                                            |
|       |               | 47B Mild IRMA in four quadrants                                              |
|       |               | 47C Severe retinal haemorrhages in two to three quadrants                    |
|       |               | 47D Venous beading in one quadrant                                           |
| 53 A-D| Severe NPDR     | 53A ≥2 level 47 characteristics                                              |
|       |               | 53B Severe retinal haemorrhages in four quadrants                           |
|       |               | 53C Moderate to severe IRMA (≥photograph 8A) in at least one quadrant        |
|       |               | 53D Venous beading in at least two quadrants                                 |
| 53E   | Very Severe NPDR | ≥2 level 53A-D characteristics                                               |
| 61    | Mild PDR        | NVE < 0.5 disc area in one or more quadrants                                |
| 65    | Moderate PDR     | NVE ≥ 0.5 disc area in one or more quadrants                                 |
|       |               | 65B NVD <photograph 10A (<0.25-0.33 disc area)                              |
| 71, 75| High-Risk PDR   | NVD ≥photograph 10A, or NVD <photograph 10A or NVE < 0.5 disc area plus VH or PRH obscuring ≥ 1 disc area |
| 81, 85| Advanced PDR     | Fundus partially obscured by VH and either new vessels upgradable or retina detached at the centre of the macula |
NPDR: non-proliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy; IRMA: intra-retinal microvascular abnormalities; NVE: new vessels elsewhere; NVD: new vessels on or within 1-disc diameter of the optic disc; PRH: pre-retinal haemorrhage; VH: vitreous haemorrhage.

From Davis et al. IOVS, February 1998, Vol. 39, p233-252

**Corneal Confocal Microscopy**

Images of the sub-basal nerve plexus of the cornea will be captured from one eye (the non-dominant eye) of each participant using a commercially available laser scanning in vivo CCM – the Heidelberg HRT III with Rostock Cornea Module (Heidelberg Engineering, Germany) – after anaesthetising the cornea. This particular CCM has been demonstrated to be superior to other available instruments for corneal nerve assessment because of the higher contrast that is maintained to the edge of the field of view in all directions.

For each participant, five of the clearest images containing the maximum number of nerves at the level of the sub-basal nerve plexus of the central cornea will be uploaded to the Distiller database, and the metrics relating to each of the parameters described below averaged, giving a single mean metric for each parameter for each participant at each assessment.

The following parameters shall be assessed by the coordinating centre by automated methods using algorithms written in MATLAB® (TheMathWorks Inc, Natick, MA, USA): CNFD, CNFL, CNFBD and CNFT. Previously established manual methods using third party image analysis software (e.g. Scion Image for Windows, Scion Corporation, Frederick, MA, USA) may be used until automated methods have been validated.

**Neuropathic Severity and Questionnaire**

All participants will undergo a detailed clinical history and examination in an attempt to rule out any other causes of neuropathy.

The Diabetic Neuropathy Symptom (DNS) questionnaire will be administered and symptoms of pain will be quantified using the short form of the McGill pain questionnaire: both questionnaires are shown in the CRF (Appendix 5).

The Neuropathy Disability Score (NDS) will be based on a clinical scoring system obtained from a neurological examination which defines abnormalities of vibration perception using a tuning fork, pin prick perception and temperature perception as well as the presence or absence of ankle reflexes, producing a score ranging from 0–10 whereby: NDS = 0-2 'no neuropathy'; NDS = 3-5 'mild neuropathy'; NDS = 6-8 'moderate neuropathy'; NDS = 9-10 'severe neuropathy' according to the Landmark Manual of Operations (Appendix 7).

Quantitative vibration and heat pain assessment will be carried out with the Medoc TSA-II NeuroSensory Analyzer and the VSA-3000 Vibratory Sensory Analyzer (Medoc Advanced Medical Systems, Ramat-Yishai, Israel) according to the Manual of Operations (Appendix
7). Assessments will be made on the hand-dominant side of the laterodorsal aspect of the foot.

The risk of foot ulceration will be measured using a 10-gram nylon filament. The monofilament will be tested at 3 pre-determined points on the sole of the foot on the hand-dominant side. This test is a rapid, reproducible and cheap method for testing diabetic neuropathy and is a good predictor of ulceration risk.

Neuropad

The Neuropad® (miro Verbandstoffe GmbH, Wiehl, Germany) is an early detection test to ascertain the hydrosis status (moisture content) of the sole of the foot (early detection of diabetic foot syndrome). The indicator pad is 100% viscose, binder-reinforced, impregnated with defined cobalt (II) chloride solution. Its use is contraindicated for participants with chrome/nickel/cobalt allergy, or local inflammation, lesions or other skin disorders at the site being tested. The neuropad is stuck onto the skin of the ball of the big toe, or if there is hard skin there, on the skin on the ball of the small toe. After 10 minutes, the percentage blue area is estimated and a photograph is taken according to the Landmark Manual of Operations (Appendix 7). A 100% pink pad indicates a normal, non-neuropathic response and an abnormal response is indicated when the pad remains blue or is patchy.

Skin Punch Biopsy (Manchester only)

On a subset of 50 diabetic and 20 control participants at the Manchester site, two skin punch biopsies (3mm in diameter) will be performed on the dorsum of the foot on the hand-dominant side under local anaesthesia (1% lignocaine) at baseline, and then at the 2-year and 4-year visits.

The biopsy is cryoprotected, fixed and processed in accordance with a protocol which we have published previously 3 and described below.

Intraepidermal nerve fibre morphology will be assessed on frozen tissue thick floating sections. In brief, 50μm sections will be cut on a cryostat and the floating sections will be rinsed in TBS. 0.25% KMnO4 will be applied for 15 minutes followed by 5% oxalate for 3 minutes to bleach the melanin and a mixture of TBS with 0.5% powdered milk, 1% Triton X-100, 5% normal serum will be applied to protein block the tissue. It is then placed on the oscillator for 4 hrs and the primary Ab (dilution-1:1200 (Biogenesis rabbit anti-human PGP9.5) is applied at 125 RPM followed by the secondary Ab. Endogenous peroxidase is blocked with 1% H2O2 in 30% MeOH-PBS for 30 min. HRP-Streptavidin is applied for 1 hr followed by chromogen (DAB) and finally counterstained with 0.05% Eosin. The colour images are captured with the image analysis camera (Sony 2CCD, CCD-IRIS) and a computer program (Leica QWin Standard V2.4, Leica Microsystems Imaging Ltd, Clifton Road, Cambridge, CB1 3QH, England) is used to quantify intraepidermal nerve fibre morphology using a set of criteria we have developed6. Ideally, at least three sections per case are assessed and the average calculated. The following parameter will be recorded:
• Intraepidermal nerve fibre linear density (IENFD), i.e., the number of fibres per millimetre of basement membrane, will be expressed as number per millimetre,

Skin biopsy samples will be destroyed following testing, typically within 7 days of collection, according to local laboratory protocols. Separate consent will be sought for storage of tissue samples (see Tissue Bank).

Nerve Conduction Studies

Nerve conduction studies will be performed using a Nihon Kohden Neuropack S1 in Brisbane and a MS92a EMG machine (Medelec Limited, Old Woking Surrey UK) in Manchester using surface stimulation and recording electrodes (maintaining skin temperature above 31°C) at baseline, and then at least at the 2-year and 4-year visits.

The assessments listed in □ will be made in accordance with our previously-published protocols,²⁴ the Landmark Manual of Operations (Appendix 7) and hospital protocols. Abnormalities of parameters of nerve conduction velocity of peroneal and tibial motor, and sural sensory nerves will be values outside 3 standard deviations.

Non-Contact Corneal Aesthesiometry

Corneal sensitivity will be measured using a NCCA, as described previously.⁵ Both sites will have identical NCCAs specifically for this project. The corneal sensitivity threshold is determined using the forced choice, staircase psychophysical technique, and is expressed as air pressure in millibars. The test will be performed on the hand-dominant side.

Mapping the Sub-Basal Nerve Plexus

The technique of Patel and McGhee⁵⁰ will be used to map the overall architecture of the sub-basal nerve plexus. We have developed this capability in our laboratory and have published a nerve map of a non-diabetic participant.²³ Corneal nerve maps will be generated at the beginning of the longitudinal study on 12 control and 24 diabetic participants with no neuropathy or a mild level neuropathy (NDS score < 6). At the end of the longitudinal study, corneal nerve mapping will again be performed, taking attrition into account, on the 6 control and 12 diabetic participants of the original subgroup. This aim of this experiment is to generate a set of images to qualitatively demonstrate changes in the overall sub-basal nerve plexus of the cornea due to progressive diabetic neuropathy, with control images for comparison. This will be conducted by the Anterior Eye Laboratory.

Conjunctival flora and tear proteomics

Conjunctival flora is different in people with diabetes⁸³ which is believed to increase the risk of ocular infection. Conjunctival swabs will be taken from study participants to investigate the normal ocular microbiota in the study cohort. This relatively small and non-invasive procedure can give in return much information about the type of bacteria they are
harbouring as well as the likelihood of infection for these individuals. Bacterial strains will be identified and virulence factors of the bacterial strains will be characterized.

Nerve growth factor was recently reported to be elevated in patients with proliferative diabetic retinopathy and correlated with HbA1c and diabetic nephropathy. The accessibility of patient tears makes it an attractive source for discovery of diagnostic biomarkers, and novel factors that could shed light on the molecular mechanism of diabetic neuropathy. To compare the tear proteome and glycoproteome for identification of potential diabetic neuropathy biomarkers in the tears, a 50 µl sample of tears will be analysed.

Unilateral vs. Bilateral Assessments

All tests, with the exception of NDS, will be performed on the hand-dominant side, unless otherwise indicated. The hand dominant side will be determined by participant self-report. The ‘side’ i.e. left or right, will be recorded for each test. If the hand-dominant side is not used, a note can be included in the comment section.

3.8 Visit Schedule

There will be five scheduled visits. The following table summarises the acceptable visit ranges.

Table 3. Acceptable visit ranges are within 28 days of the target date.

<table>
<thead>
<tr>
<th>Visit</th>
<th>Target</th>
<th>Acceptable Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (Enrolment)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Year 1</td>
<td>336 days</td>
<td>308 to 364 days</td>
</tr>
<tr>
<td>Year 2</td>
<td>672 days</td>
<td>644 to 700 days</td>
</tr>
<tr>
<td>Year 3</td>
<td>1008 days</td>
<td>980 to 1036 days</td>
</tr>
<tr>
<td>Year 4 (and Study Exit)</td>
<td>1344 days</td>
<td>1316 to 1372 days</td>
</tr>
</tbody>
</table>

For example, Table 4 shows the visit dates that would apply if a participant is enrolled on 12\textsuperscript{th} January 2008.

Table 4. Sample enrolment and visit dates

<table>
<thead>
<tr>
<th>Visit</th>
<th>Target Date</th>
<th>Earliest Date</th>
<th>Latest Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (Enrolment)</td>
<td>12\textsuperscript{th} Jan 2008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year 1</td>
<td>14\textsuperscript{th} Dec 2009</td>
<td>16\textsuperscript{th} Nov 2009</td>
<td>11\textsuperscript{th} Jan 2010</td>
</tr>
<tr>
<td>Year 2</td>
<td>15\textsuperscript{th} Nov 2010</td>
<td>18\textsuperscript{th} Oct 2010</td>
<td>13\textsuperscript{th} Dec 2010</td>
</tr>
<tr>
<td>Year 3</td>
<td>17\textsuperscript{th} Oct 2011</td>
<td>19\textsuperscript{th} Sep 2011</td>
<td>14\textsuperscript{th} Nov 2011</td>
</tr>
<tr>
<td>Year 4 (Study Exit)</td>
<td>17\textsuperscript{th} Sep 2012</td>
<td>20\textsuperscript{th} Aug 2012</td>
<td>15\textsuperscript{th} Oct 2012</td>
</tr>
</tbody>
</table>
3.9 Masking
As this is an observational study, no randomization is necessary. Investigators however will be masked as to the group assignment of participants. All data analyses will be performed in a masked fashion. (Further details are given in Section 5.2).

3.10 Discontinuation
Participants will be discontinued from study when any of the criteria below applies. The reason for study discontinuation and the date the participant was discontinued must be documented in the online form/CRF, and the clinical record where appropriate. The criteria for discontinuation include:

- Illness that prevents further participation,
- Unacceptable adverse event(s),
- Participant decides to withdraw from the study*,
- General or specific changes in the participant’s condition render the participant unacceptable for further participation in the judgment of the investigator, or
- Two consecutive missed scheduled visits.

*Participants are free to withdraw from the project at any stage for any reason (stated or unstated) without comment or penalty.

In the event of discontinuation, the study online forms/CRF must be completed and the study exit data recorded on source document record. The Investigator will indicate the primary reason for discontinuation. Further details can be provided in the ‘comments’ section if necessary.

If a participant misses two consecutive visits they will be discontinued from the study and will be asked to return to fulfil discontinuation requirements if possible.

The reasons for discontinuation will be indicated by one of the following, with additional detail where possible: lost to follow-up; personal reasons; adverse event; other. If the participant is enrolled in the study then subsequently found to be not eligible, then “inclusion criteria not met” will be indicated in the record as the reason for non-inclusion.

Data will be utilised for individuals who complete some of the study as eligible participants.

3.11 Data Identification
Participants will be identified by a unique number code automatically generated by the Distiller database. This ID code will be recorded on all the CRFs, questionnaires and visit documents, where appropriate. The database will provide multilayered access to maintain security of participants’ information. The site coordinators will be able to access identified study information for their site.
3.12 Participant Retention

The research team will adopt strategies of flexibility, accessibility, personalized attention, and feedback to maximise participant retention.68

Flexibility

Study appointments will be offered during and after business hours and, if necessary, participants can attend two shorter visits rather than one longer visit (this may be necessary for NET). The research team shall provide pre-appointment reminder telephone calls and follow-up telephone calls for missed appointments. A research team member will also contact each participant within 48 hours of their participation visit as a goodwill gesture and to potentially identify any possible adverse effects of study participation.

Accessibility

Transport and parking information and reimbursement of travel costs will be provided. Cab vouchers, parking, and/or vouchers will be provided to participants related to their travel to and from their appointments.

Personalised Attention

Well-organized hospitality such as coffee/tea and refreshments will be provided at all visits. Inexpensive study trinkets (e.g. pens, mugs, shopping bags) will also be provided. If they wish, participants will be given digital photographs of their ocular fundus and/or nerve plexus – a strategy that may serve to enhance their interest in the project.

Feedback

A biannual newsletter will keep participants informed of the progress of the study as well as individual feedback via eye images will serve as an extra motivational influence to enhance retention rates. Publications arising from the study will also be provided to the participants at the completion of the study.

Retention strategies will be reviewed annually by the site coordinators, and altered where necessary.

3.13 Unscheduled Visits

Although extremely unlikely in the present study, the following guidelines apply to unscheduled visits:

An unscheduled visit is defined as any follow-up that occurs outside the visit window of the scheduled visit. A follow-up visit is also classified as unscheduled if the subject is seen a second time within the scheduled visit window.
Investigators should try as far as possible to schedule follow-up visits within the window. Exceptionally, if this is not possible, and the visit falls outside the window, the visit ID will be completed as unscheduled. Unscheduled visits will be made available anytime at the participant’s request.

Unscheduled visits will be recorded on the data entry screen/CRF with the visit ID completed as ‘Unscheduled’. All variables listed on the data entry screen/CRF must be completed unless the participant exhibits a condition that prohibits the completion of a full visit. If this is the case, a written explanation is required in the comments section (e.g. ocular discomfort prevented conduction of corneal confocal microscopy).

Presenting visual acuity must always be completed and the reason for the visit and any actions taken must be indicated in the ‘comments’ section.

### 3.14 Study Exit

The study exit procedures will be completed when a participant exits the study. This will occur either at study completion or if the participant is discontinued from the study at another time. Study exit details will be reported for all participants i.e. if they have been assigned a study ID number. The exit date will also be recorded on the source document record (clinical record, database and/or CRF as indicated). Post-study follow-up visits will be scheduled if the Investigator judges this is in the best interests of the participant.

The CRF will be completed at the final study visit and the best corrected distance high contrast visual acuity (logMAR). If the participant is being exited due to discontinuation, further details will be recorded on the CRF.

At the end of the study, or anytime during the study, specific test results will be reported to the participant’s general or other health care practitioner with participant consent, as stated in the information and consent form.

### 3.15 Post-Study Follow-up

A post-study follow-up visit will be scheduled if the participant exhibits any ocular findings, which are significantly different from baseline upon discontinuation or completion of the study, unless the Investigator judges that no follow-up is warranted. The date and reason of the post-study follow-up visit shall be recorded on the exit form. In addition, a CRF will be completed with the visit ID indicated as ‘Unscheduled’. In the comments section, the Investigator shall indicate that this is a post-study follow-up visit.

### 4 Assessment of Safety

#### 4.1 Data Safety Monitoring

The Data and Safety Monitoring Committee (DSMC) will meet once per year (or more if needed). The DSMC will monitor, on a regular basis, adverse effects, review outcome data and positive and negative feedback from participants about their participation. They will monitor the study data for evidence to support cessation of the study because of adverse safety or efficacy outcomes. All adverse effects and protocol changes will be reported to the ethics committees, the DSMC and the JDRFI.
In Brisbane, the DSMC may comprise of the following individuals: QUT Human Research Ethics Committee (HREC) Chair or representative, Nathan Efron, Anthony Russell, Andrew Cotterill and Nicola Pritchard as members, and one independent lay member (typically appointed by the HREC Chair).

In Manchester, the DSMC may comprise of the following individuals: HREC Chair or designate, Andrew Boulton, Rayaz Malik, Ann Knowles, RGN MS, Senior Diabetes Nurse Educator, Manchester Royal Infirmary, UK and one independent lay member (typically appointed by the HREC Chair).

This committee will receive an annual report to review all safety data relating to all other aspects of the study. In the unlikely event of any serious adverse events, an urgent meeting would be arranged. The Manual of Operations includes an SOP for LANDMark committees (Appendix 7).

### 4.2 Serious Adverse Events

It is a requirement of the regulatory and granting bodies that all serious and unexpected adverse events (SAEs) be reported by investigators to the HRECs.

According to NHMRC National Statement on Ethical Conduct in Human Research 2007, the definition of a Serious Adverse Event (SAE) is any untoward medical occurrence that:

- results in death;
- is life-threatening;
- requires in-patient hospitalisation or prolongation of existing hospitalisation;
- results in persistent or significant disability/incapacity;
- is a congenital anomaly/birth defect; or
- is a medically important event or reaction.

SAEs include all of the above events if they occur during the research project. The Principal Investigator (or most appropriate investigator) at each site will sign the form before submission to the HREC to indicate they have viewed the SAE and considered, in his/her expert opinion, whether the event is related or not related to the study. SAEs shall be reported to the Ethics Secretariats within 24 hours using the SAE form.

In the NHS hospital system in the UK, any Serious Adverse Events (SAE) occurring to a research participant in a study must be promptly notified to the local HREC that approved the study on the Serious Adverse Events form within 15 days of the Chief Investigator becoming aware of the event, where it is considered possible that the event resulted from participation in the research. Investigators are also required to complete a trust incident form.

### 4.3 Adverse Event Reporting

In the rare instance that an adverse event should occur as a result of participating in this study, the Investigator will complete the HREC-applicable adverse event form to document the condition. The Investigator will report the adverse event to the local HREC and to the study coordinator typically within 15 days of discovery of the event.
SAE’s are reported, as described above, typically within 24 hours.

4.4 Adverse Event Follow-up

The Investigator will use his/her clinical judgment as to whether or not the participant reporting with an adverse event should continue in the study.

5 Statistical Plan

5.1 Hypotheses

The following specific hypotheses will be tested:

H₁₀: change in corneal nerve fibre pathology, measured by CCM, will be different in T1DM, T2DM, LADA and controls over time

H₁₁: change in corneal sensitivity (CS), measured by NCCA, will be different in T1DM, T2DM, LADA and controls over time, specifically:

H₁₂: changes in corneal nerve fibre pathology (anatomic measure) and CS (functional measure) will be different in the group with T1DM and neuropathy compared to the group with T1DM without neuropathy over time.

H₁₃: progression of corneal nerve fibre pathology and decrease in function is greater in T1DM than LADA and T2DM groups over time.

H₁₄: changes in corneal nerve fibre pathology and function correlate with, and are early markers of, changes in neuropathic severity (electrophysiology, QST and skin biopsy) in T1DM, T2DM and LADA groups over time.

H₁₅: risk factors can be identified for a change in corneal nerve fibre pathology and function in individuals with T1DM, T2DM and LADA.

H₁₆: CCM and NCCA are sensitive and specific markers of diabetic neuropathy and both exhibit good discrimination capacity.

5.2 Statistical Methods

Hypotheses H₁₀ to H₁₆ relate to changes in outcome variables (CNFD and CS) over time. As there are a number of potential time-varying covariates included in this study (e.g. blood glucose), a linear mixed model (LMM) approach will be adopted for the repeated measures analysis. The LMM approach will allow the modelling of time-varying covariates and offers more flexibility regarding issues such as heterogenous within-subject and between-group variability and (sporadic) missing values. Where significant differences within the clinical groups are established, post-hoc analysis will be conducted using the Tukey-Kramer test.
Hypothesis H₁e relates to the degree of association between outcome variables (CNFD and CS) and traditional measures of neuropathy (nerve electrophysiology and QST). Measures of association (correlation) for variable combinations will be made using Pearson’s correlation coefficient if both continuously scaled variables are normally distributed. If one or more of the two continuously-scaled variables is not normally distributed, Spearman’s correlation coefficient will be applied.

Hypothesis H₁f relates to identification of risk factors. Hierarchical linear regression analysis will be undertaken to reveal possible risk factors associated with degree of neuropathy.

Hypothesis H₁g will be tested by calculating the specificity and sensitivity of CCM and NCCA against the ‘gold standard’ Neuropathy Disability Score, and a receiver-operating characteristic curve will be generated to establish the discrimination capacity of each technique.

Age distribution will inherently be different between groups; therefore age will be treated as a covariate. Rather than sex-matching, we will also treat gender as a covariate. Antibody status will also be treated as a covariate in the diabetic cohorts, as will IGT status and degree of retinopathy.

The experimental design will incorporate appropriate masking and randomization to avoid inadvertent experimenter bias. All CCM and NCCA examinations will be conducted by a single investigator at each site who will be masked with respect to the medical condition of the participants. A masked investigator based in Brisbane will analyse CCM images from both sites in large batches, with the images only identified by random number codes; thus, these measurements will be made without reference to NCCA results, participant history or study site, which otherwise could compromise masking. An unmasked investigator at each site will coordinate recruitment, gather all other relevant information about the study participants, administer the questionnaires, and undertake neuropathic examinations. At each site, NET will be conducted by a specialist electrophysiologist who will also be masked as to the participant group/history and all other study findings.

To ensure type I error rates are not inflated Bonferroni corrections will be applied where appropriate.

5.3 Sample Size Determination

The principle outcome measure for Hypotheses H₁a through H₁d relate to change in CNFD over a four-year period. In the absence of pre-existing longitudinal data, we anticipate from previous research showing differences between groups of varying degrees of severity of neuropathy² that the maximum change in this parameter is 25 nerves/mm². We expect this could occur in either direction i.e. an improvement or regression of neuropathy; therefore, the maximum range of possible change divided by six (i.e. 6 SD in a normal distribution) provides a conservative estimate of one standard deviation of 8 nerves/mm². An assumption of independent groups was used to calculate the sample size using within-subject (range of CNFD response estimated to be 25 nerves/mm²) and between-group estimates (clinically meaningful difference between groups of 5 nerves/mm²). The range
between groups is likely to be less than 25 nerves/mm², hence reducing the sample required. Therefore, recruiting a minimum of 27 participants for each diabetic group will provide 90% chance to detect a clinically meaningful change in CNFD of 5 nerves/mm² at the 0.05 significance level. This value, inflated by 20% to take into account the proposed multivariate modelling and a further 20% to account for variance attributable to the use of two clinical sites increases the required sample to 39.

Similar to the estimate for CNFD changes, previous research demonstrates differences in NCCA between groups of varying degrees of severity of neuropathy such that the maximum change anticipated in corneal sensitivity is 1.5 millibars. This change could occur in either direction i.e. an improvement or regression of neuropathy, giving a conservative estimate of one standard deviation of 0.5 millibars. A minimum of 29 participants for each diabetic group will provide 90% chance to detect a clinically meaningful change in air puff aesthesiometry of 0.3 millibars at the 0.05 significance level. With the aforementioned inflation factors applied, a sample of 42 is therefore required.

Previous longitudinal studies of diabetic neuropathy of 7-10 years duration suffered from non-completion rates of 31-41%. To compensate for a 40% non-completion rate in our study, and allowing a small 'safety margin', at least 78 participants need to be recruited into each of the three test groups and one control group. The power of the group*time interaction calculations is most at risk if attrition is high; therefore, we propose increasing the anticipated attrition factor to 60% to an overall sample of 96 per group. An additional 20 participants (20%) will be enrolled into Group 4 (controls) to account for the 16% impaired glucose tolerance (IGT) in the Australian population and IGT will be recorded as a covariate.

The NDS has been established as a simplified and valid equivalent to more elaborate QST. A correlation of approximately 0.3 to 0.4 has been recently observed between CNFD and NDS. To address Hypothesis H1e, we estimate 47 participants are required to demonstrate that a postulated correlation of 0.4 is significantly different from zero (power 0.8 and 0.05 significance level). Accounting for attrition, multivariate modelling and the use of two sites, a total sample of 134 participants is required. We expect a lower correlation between CNFD and nerve electrophysiology parameters and therefore are confident this sample will reveal a relationship, where one exists.

With respect to Hypothesis H1f, we have used the same assumptions as above to estimate the number of participants required to identify which risk factors predict significant changes in CNFD over a 4-year period. Using an alpha probability of 0.05, a power of 0.8 and a postulated correlation of 0.4, 47 participants should reveal statistical association between changes in CNFD and risk factors where they exist. Appropriately inflated for attrition and multivariate modelling and the use of two sites, a total sample of 134 participants is therefore required.

A comparison of two proportions was the statistical method used to calculate the sample size required to determine the sensitivity and specificity of CCM and NCCA (Hypothesis H1g). At a level of significance of 0.05 and power of 90%, an acceptable sensitivity of 80% was compared with an ideal of sensitivity of 95%. A sample of 75 participants with diabetic neuropathy (determined by the reference standard, NET/QST) is required to determine sensitivity of CCM and NCCA. Specificity will be determined by recruiting an equal number
of subjects without neuropathy (i.e. the control group). When an acceptable specificity of 75% is compared to the ideal value of 95% (significance of 0.05 and power of 90%), a sample of 49 is required. Therefore, to test the primary hypothesis a total of 150 participants is required.

At the Manchester site skin punch biopsy will be performed on a sub-cohort of 50 diabetic and 20 control participants. Power analysis demonstrates that a group of 22 participants will provide an 80% power to detect a difference of 6 nerves/mm² assuming an alpha of 0.05 and standard deviation of 10. Attrition of 50% has been assumed such that 50 diabetic participants should be enrolled. Due to reduced within-group variability of the control participants a sample of 20 will be used for comparison purposes. Diabetic participants will be recruited in approximately equal numbers from Groups 1, 2 and 3.

In consideration of all of the above estimates of sample size, the minimum number of participants enrolled will therefore be 96 in Groups 1, 2 and 3, and 116 in Group 4 i.e. a total of 404 participants, which will be equally split between Brisbane and Manchester. This sample will therefore be adequate to provide acceptable power to attend to the demands of the four key hypotheses of this work, and by default should also allow numerous secondary hypotheses to be tested.

5.4 Deviation Reporting

Any significant changes or deviations in the protocol will be the subject of a protocol amendment and must be pre-approved by the respective Research Ethics Committees. If an unexpected deviation from the protocol occurs the Investigators must notify the study coordinator immediately and the deviation from the protocol will be documented.

6 Access to Source Data and Documentation

The database ‘forms’ will be regarded as a source document. In addition the following minimum information will be recorded in the participant’s normal patient record, or as directed by the Director of the clinic at PAH or MRI:

- Enrolment and exit dates from the study
- Details of adverse events

The JDRF or representatives will be authorised to gain access to the source documentation for the purposes of monitoring and auditing the study.

The investigator will permit study-related monitoring, audits, HREC review and regulatory inspection by providing direct access to source data and documentation.

7 Quality Control and Quality Assurance

The study coordinator will endeavour to ensure that the trial is performed and data are generated, documented (recorded), and reported in compliance with good clinical practice (GCP) and the applicable regulatory requirements. Regular monitoring of data integrity and completeness will be conducted.
The JDRF, NHMRC, HREC or designates may conduct an audit as part of implementing quality assurance to ensure that the rights and welfare of the participants are being adequately protected. The study coordinator will assist in facilitating this process.

A Data Surveillance Committee (DSC) comprising Prof Efron, Prof Malik and the project statistician will meet biannually to assess the extent of variance in data generated from the two sites and will introduce strategies, where necessary, to minimise such variance. The Project Manager will forward a report for review every 6 months.

8 Ethical Considerations

This study will be conducted according to the National Statement of Ethical Conduct in Human Research. Applicable government regulations and Princess Alexandra Hospital and Queensland University of Technology research policies and procedures will also be followed.

This protocol and any amendments will be submitted to the Princess Alexandra Hospital and Queensland University of Technology Human Research Ethics Committees (HRECs) for formal approval to conduct the study. The decision of the HREC concerning the conduct of the study will be made in writing to the investigator.

All participants for this study will be provided a consent form describing this study and providing sufficient information for participants to make an informed decision about their participation in this study. This consent form will be submitted with the protocol for review and approval by the HRECs. The formal consent of a participant, using the HREC-approved consent form, will be obtained before that participant is submitted to any study procedure. This consent form must be signed by the participant or legally acceptable surrogate, and the investigator-designated research professional obtaining the consent.

This study will include children i.e. all individuals under 18 years of age. Taking into consideration the nature and seriousness of diabetes for all ages, and the prevalence of neuropathy in children with diabetes (up to 30%), the investigators believe the study warrants participation of children. Children will be included, unless a criterion for exclusion for involvement is met. Andrew Cotterill, MBBS MRCP MD FRACP, Associate Professor of Paediatrics and Child Health at the University of Queensland and Director of Paediatric Endocrinology at the Mater Children’s Hospital, South Brisbane has agreed to act as a consultant for the study in this regard. We will require concurrent assent from both guardian and child for those aged between 14-18 years. Participants who have their 18th birthday during their enrolment in the study will be asked to re-assent to participation as an adult i.e. without parental consent. In this instance the participant will be asked to sign and date the current consent document(s) for the study. Therefore, both adults and children will contribute to the data allowing results to be generalized across ages.

9 General Study Management

9.1 Coordination of Two Study Sites

Equal numbers of participants taking part in the longitudinal trial will be recruited and followed in Brisbane and Manchester, and study data will be pooled for analysis. A
number of measures will be adopted to minimize any possible data variance attributable to a multi-site study. The makes and models of study instrumentation as described in this protocol will be identical at both sites, and both centres will adopt the same procedures for instrument calibration, data acquisition and recording. These protocols and procedures will be documented in a Manual of Operations, and multiple copies will be available at both sites. All data will be analysed at a single site (Brisbane) by observers who will be masked with respect to participant history and study site. After unmasking, the distribution of the analysed data sets from the two sites will be scrutinized for systematic differences, and appropriate statistical adjustments will be made to align the data distributions.

10 Data Handling and Record Keeping

In accordance with the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), information will be stored for a minimum period of 15 years, or 5 years after the final publication.

Information will be gathered on a case report form (CRF)/data entry form and this information will be stored in a secure limited-access computer database, password-protected backup media and locked filing cabinets at PAH, QUT and UM. At the completion of the study, identifiers will be removed.

Data will remain the property of Queensland University of Technology, University of Manchester and the Manchester Royal Infirmary.

11 Finance and Insurance

All costs associated with conduct of the study will be met by QUT and UM in accordance with the predetermined budget. Hospitals and universities maintain appropriate public liability and professional indemnity insurance for their staff should injuries arise from the study.

12 Collaborative Arrangements

PI Efron at the Queensland University of Technology (QUT) will be responsible for the oversight and coordination of project management. His specific roles will include the following:

- further development and refinement of the experimental protocol
- overseeing preparation of the ethics approval process in Brisbane
- recruiting and appointing scientific staff in Brisbane
- supervising scientific staff in Brisbane
- academic collaboration with scientific staff in all aspects of the study, including the establishment of recruitment protocols, data analysis, scientific writing for publication, and reporting of results at international conferences
- monitoring overall progress of the study
• monitoring the budget assigned to the study
• writing progress reports

Co-PIs Boulton and Malik roles are as follows:
• further development and refinement of aspects of the experimental protocol
• overseeing preparation of the ethics approval process in Manchester
• recruiting and appointing scientific staff in Manchester
• supervising scientific staff in Manchester
• academic collaboration with respect to clinical staff in all aspects of the study, including the establishment of recruitment protocols, data analysis, scientific writing for publication, and reporting of results at international conferences

Co-PI Prins will be responsible for overseeing clinical aspects of the study at the Department of Diabetes and Endocrinology at the Princess Alexandra Hospital. His specific roles will include the following:
• further development and refinement of aspects of the experimental protocol relating to ongoing care and management of the diabetic patients, including clinical evaluation of neuropathy of all subjects
• ensuring the availability of clinical space, resources, support and equipment necessary for the conduct of the project
• collaboration with respect to preparation of the ethics approval process
• assisting PI in recruiting and appointing scientific staff
• monitoring the activities of clinical staff in Brisbane
• offering advice and support to clinical staff in Brisbane
• academic collaboration with respect to clinical staff in all aspects of the study, including the establishment of recruitment protocols, data analysis, scientific writing for publication, and reporting of results at international conferences
• assisting PI Efron in monitoring overall progress of the study, monitoring the budget assigned to the study and in writing progress reports

The PIs will communicate frequently by e-mail to discuss experimental design, data analysis, and all administrative responsibilities. PI Efron will share the research results with the other PIs, key personnel, and consultants on a regular basis. They will work together to discuss any changes in the direction of the research projects and the reprogramming of funds, if necessary. Video-conferencing is available to all PIs. In addition, PI Efron anticipates travelling to Europe at least once per year over the course of this project and will schedule meetings with Co-PIs Boulton and Malik, who are based in the UK, during these visits.

A publication policy will be established based on the relative scientific contributions of the PIs and key personnel. PI Efron will serve as contact PI and be responsible for submission of progress reports to JDRF and all communication, including HREC/IRB approvals, and other annual progress reports.
There are no pre-existing patents relating to this project, and it is not envisaged that this project will generate any intellectual property that will require protection. Rights in any pre-existing intellectual property will remain the property of the party that created and/or controls it.

In the unlikely event a conflict should develop, the University and Hospital administrators representing the PIs shall meet and attempt in good faith to settle any dispute, claim or controversy arising out of or relating to the interpretation, performance or breach of this disagreement. However, if the aforementioned administrators fail to resolve the disagreement within thirty business days, then such disagreement shall be referred for resolution to a designated senior executive of the parties who has the authority to settle the disagreement but who is not directly involved in the disagreement.

If one of the PIs moves to a new institution, it is not anticipated that this will in any way interrupt the progress of the study, as no transfers of the grant to the new institution will be necessary. In the event that a PI cannot carry out his duties, a new PI may be recruited as a replacement, subject to the approval of the PIs, the Institutions and the JDRFI.

The copyright of publications in journals is owned by the journal/publisher. Reports will be owned by the authors and institutions where they are employed.

13 Data and Sample Sharing

13.1 Overview

The LANDMark Study investigators are committed to sharing data and samples from the LANDMark studies shortly after their publication. It is envisaged data from completed LANDMark studies with published research findings will be made freely available via a formal collaboration between LANDMark investigators, JDRFI and investigators outside of LANDMark. Data, as defined by the NIH Data Sharing Policy and Implementation Guidance, are "recorded factual material commonly accepted in the scientific community as necessary to document, support, and validate research findings". The Manual of Operations for the LANDMark BioBank details sample sharing procedures. The data to be shared and the manner of sharing will be established by mutual consent of, and agreement between, the LANDMark investigators and the JDRFI. It is envisaged that data will be available for sharing approximately two years after the final publication and appropriate acknowledgments will be requested. To ensure confidentiality, data will be stripped of all personal identifiers and any variables that permit deductive identification of individual subjects.

13.2 Methods for Data Sharing

Methods for data sharing include, but are not limited to, the following:

- Sharing data under the auspices of the Principal Investigator (PI)
- Submitting data to an archive, or
- Submitting a documented, electronic version of the final dataset to JDRFI.
Sharing under the auspices of the PI may be accomplished by posting the data on an institutional website or by sending the dataset to the requester (e.g. on CD), and by specific collaborations. As stated above, the LANDMark team in consultation with the JDRFI, will develop a data-sharing agreement to impose appropriate limitations on users, such as requirements to protect participants' privacy, data confidentiality, and to ensure that the user will not further distribute the data. The NIDDK Data Repository (www.niddkrepository.org) is an example of a data archive which gathers, stores, and distributes incremental or finished datasets. If this repository is to be used, the PI will consult with the JDRFI program officer for assistance in interfacing with the NIH. The NIDDK Repository may also assist the LANDMark team in preparing the databases for sharing. Regardless of the mechanisms used to share data, each dataset requires proper documentation to prevent misinterpretation and ensure that others can appropriately use the dataset.

13.3 Methods for Sample Sharing

The LANDMark BioBank Manual of Operations outlines procedures for application to and distribution of blood and tissue samples archived during the LANDMark Study (Landmark Study BioBank MOP; Standard Operating Procedures 12 and 13). Biomaterials may be shared under the auspices of the PI or through a sample repository, or other suitable means. For sharing under the auspices of the PI, a material transfer agreement will be developed to impose necessary limitations on users. Appropriate sample repositories include the NIDDK Biosample Repository and Genetics Repository (see www.niddkrepository.org). If this repository is to be used, the PI will consult with the JDRFI program officer for assistance in interfacing with the NIH.

14 References


15 Appendices

Appendix 1   Participant Information and Consent Form (BNE PICF V5)
Appendix 2   Participant Information and Consent Form – Tissue Bank (BNE PICF V1)
Appendix 3   LANDMark BioBank Withdrawal Consent Form – Tissue Bank (BNE PIWC V1)
Appendix 4   Recruitment Text
Appendix 5   Case Report Form
Appendix 6   Participant Feedback Questionnaire
Appendix 7   LANDMark Manual of Operations - Table of Contents
Appendix 8   LANDMark BioBank Manual of Operations - Table of Contents
## 16 Revision History

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<thead>
<tr>
<th>Revision</th>
<th>Date</th>
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<td>V1</td>
<td>December 2008</td>
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<tr>
<td>V2</td>
<td>Updated 03/06/09</td>
<td>Definition neuropathy updated, coeliac added as exclusion criteria, blood relatives of landmark participant removed from control exclusion criteria</td>
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<td>V3</td>
<td>Updated July 17, 2009</td>
<td>Add Vit D to blood panel, frequency doubling thresholds, update references.</td>
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<tr>
<td>V4.1-3</td>
<td>Updated Dec 1, 2009</td>
<td>Added recruitment from Mater Hospitals, section on conjunctiva and tears, removed Vit D, update references.</td>
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<td>V4.4</td>
<td>Updated 14 Jan 2011</td>
<td>Modified definition of neuropathy, minor corrections.</td>
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<td>V5</td>
<td>2011 protocol unchanged, minor edit made on 6 August 2013</td>
<td>Coverpage “Sponsors” changed to “Financial Sponsors”</td>
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<td>V6</td>
<td>Updated May 2014</td>
<td>Added additional detail for procedure for discontinued participants</td>
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