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Genetic analysis of the microvascular complications of diabetes mellitus

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diabetes mellitus

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ABSTRACT

Genetic analysis of the microvascular complications of
diabetes mellitus
by
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There is increasing evidence to suggest that genetic factors are involved in the pathogenesis of microvascular complications in diabetes mellitus. Recent studies have suggested that genetic variations in the aldose reductase (ALR2) gene may contribute to the genetic susceptibility to microvascular complications. Aldose reductase is the first and rate-limiting enzyme of the polyol pathway and is implicated in the pathogenesis of diabetic microvascular disease (nephropathy, retinopathy and neuropathy). It has recently been shown that the three polymorphisms of the ALR2 gene are associated with susceptibility to microvascular complications in both T1DM and T2DM. The aim of this study was to investigate the CA dinucleotide repeat polymorphism (5'ALR2) that is located –2100bp and the C-106T substitution in the promoter region of the ALR2 gene, and also the A+11842C within intron 8 of the ALR2 gene itself. DNA from 285 Caucasoid patients with T1DM and well-defined microvascular disease and 120 normal healthy controls, as well as 60 Southern Indian patients with T2DM and 43 non diabetic controls were typed. The 5'ALR2 Z-2/X genotype was significantly increased in patients with nephropathy (n=92), retinopathy (n=160) and neuropathy (n=104) compared to those with no microvascular disease after 19 years duration of diabetes (uncomplicated, n=66) (46%, 41%, 42% vs. 24%, respectively). In contrast, the frequency of the Z+2/Y genotype (where Y is not Z-2) was significantly reduced in the patients with nephropathy, retinopathy and neuropathy compared to the uncomplicated (17%, 23%, 23% vs. 52%, respectively). Similar observations were made in the Southern Indian T2DM patients, however no significant differences were found. In the patients with T1DM the C-106 allele was associated with the Z-2 5'ALR2 allele. The C/Z-2 haplotype was present in 32% of the nephropaths, 32% of the retinopaths and 35% of the neuropaths compared to 11.5% of the uncomplicated. The A+11842 allele was also associated with the C-106 allele in T1DM patients with microvascular disease. The reported mitochondrial polymorphism (mt5178A/C) was not found in this T1DM population, possibly due to differences in the background frequencies between ethnic groups. Family studies investigating the transmission of the 5'ALR2 and C-106T alleles from parents to offspring with diabetic nephropathy found preferential transmission of the Z-2 allele although this was not statistically significant. Functional studies of the activity of the ORE in T1DM patients with and without microvascular disease showed differences in the mean OREBP binding activity. OREB and OREC were found to have increased activity in response to hyperglycaemia in the complicated patients compared to the uncomplicated and normal controls. In conclusion, these results confirm the role of the aldose reductase gene in the genetic susceptibility to diabetic microvascular complications, and a possible role of the D17S934 polymorphism in T2DM. These results also provide a novel insight into the role of the ORE of the ALR2 gene in the pathogenesis of diabetic microvascular complications. Further studies are now required to determine the molecular basis of these observations. Hopefully, in the future it will be possible to offer 'high risk' patients therapeutic intervention that will prevent the ravages of the long term complications of diabetes mellitus.

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and polyol pathway activation.

Authors declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other award.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes and several papers prepared for publication.

Presentations and Conferences Attended;

The 17th Annual Meeting of the Anglo-Danish-Dutch Diabetes Group (ADDDG)- The Netherlands, May 1999 **(Oral Presentation)**

The American Diabetes Association (ADA)- San-Diego, California, July 1999. **(Poster Presentation)**

Publications/ Abstracts:

Cross DF, Heesom AE, Millward BA, Demaine AG. Investigation of the Essential Hypertension Locus D17S934 and the 5'ALR2 locus in patients with Type 1 Diabetes Mellitus and Microvascular Complications. *Diabetes, May 1999, Vol. 48, Supplement 1.*

Demaine AG, Heesom AE, Cross DF, Millward BA; Investigation of polymorphisms of the aldose reductase promoter region in patients with type 1 diabetes and diabetic microvascular complications. *Diabetes, May 2000, Vol. 49, Supplement 1.*

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Cross DF, Millward BA, Demaine AG; Association of diabetic complications with the 5'ALR2 microsatellite polymorphisms and an intragenic polymorphism of aldose reductase gene. *Paper in preparation*

Cross DF, Millward BA, Demaine AG: Osmotic response elements in the onset of T1DM microvascular complications. *Paper in preparation.*

Zhao HX, Stenhouse E, Sanderson E, Soper C, Hughes P, Cross D, Demaine AG, Millward BA; Continued rising trend of childhood Type 1 diabetes mellitus in Devon and Cornwall, England. *Accepted for publication by Diabetic Medicine.*

About the Author-

I read for my B.Sc. Degree in Molecular and Cellular Biology at the University of Plymouth. Following this, the Plymouth Postgraduate Medical School offered me studentship for a Ph.D. to research into diabetic complications, which forms the basis of this thesis. Since completing my experimental work for the PhD I have also been involved in the recruitment of patients for the Diabetes UK Warren 3 Nephropathy Collection. I have also since been offered a place at Lucy Cavendish College, University of Cambridge to enter onto the graduate course in Medicine and Surgery, upon completion of my PhD.

List of Abbreviations

%	percent
μ	micro
μM	micromolar
β	beta
δ	delta
γ	gamma
γ32PdATP	gamma 32P ......
δn-1	standard deviation
χ2	chi square
/	per
+/-	plus or minus
3'	three prime
5'	five prime

A

AA	amino acids
ACE	angiotensin converting enzyme
ACE-I	angiotensin converting enzyme inhibitors
ACR	albumin:creatinine ratio
ADP	adenosine di-phosphate
ADA	american diabetic association
AER	albumin excretion rate
AGEs	advanced glycation end-products
AGEIs	advanced glycation end-product inhibitors
ALR2	aldose reductase
AR	aldose reductase
ARBs	angiotensin II receptor blockers
ARIs	aldose reductase inhibitors
ARMS	amplification refractory mutation system
ASA	allele specific amplimer
ASP	affected sib pair
ATP	adenosine triphosphate

B

BB	bio-breeding
BB/Wor	bio-breeding/Worcester
bp	base pair
BP	blood pressure
BDA	british diabetic association
bFGF	basic fibroblast growth factor
bn	billion
BSA	bovine serum albumin

C

Ca⁺⁺	calcium
CCK	cholecystokinin
CD	cluster of differentiation
CHD	coronary heart disease
cM	centimorgan
CML	carboxymethyl lysine
CO₂	carbon dioxide
CMP	cows milk protein
cpm	counts per minute

D

DAG	diacylglycerol
DC	uncomplicated diabetic controls
DM	diabetes mellitus
DMEM	dulbecco's modified medium
DMSO	dimethyl sulfoxide
dNTPs	2' deoxyribonucleotide 5'triphosphate
DN	diabetic nephropathy
DNA	deoxyribonucleic acid
DNu	diabetic neuropathy
DR	diabetic retinopathy
DCCT	diabetes control and complications trial

E

EASD	european association for the study of diabetes
ECACC	european collection of animal cell culture
ecNOS	enhance nitric oxide synthesis
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
EMC	encephalomyocarditis
EMSA	electrophoretic mobility shift assay
ESRD	end stage renal disease
et al	and others

F

FCS	foetal calf serum
FFA	free fatty acids
FHP	full house patient
FPG	fasting plasma glucose

G

GAD	glutamic acid decarboxylase
GBM	glomerular basement membrane
GDM	gestational diabetes mellitus

H

H₂O	water
HbA1c	glycated heamoglobin
HCl	hydrochloric acid
HDL	high density lipoprotein
HK	hexokinase
HLA	human leukocyte antigen
HMPS	hexose mono-phosphate shunt
HWE	hardy weinberg equilibrium

I

I-A2	autoantibodies to protein tyrosine phosphatases
IAA	insulin auto antibody
ICA's	islet cell antibodies
IDDM	insulin dependent diabetes mellitus
IFG	impaired fasting glycaemia
IGF	insulin-like growth factor
IGT	impaired glucose tolerance
IMS	industrial methylated spirit
INS	insulin gene
IRS	insulin receptor substrate
I.U.	international units

K

KRV   kilhams rat virus

L

L.t.d.   limited
LDL   low density lipoprotein
LDS   lipids in diabetes study

M

MAP   mitogen activated protein
MEM   minimum essential medium
MHC   major histocompatibility complex
MODY   maturity onset type diabetes of the young
MRDM   malnutrition related diabetes mellitus
mRNA   messenger ribose nucleic acid

N

n   number of subjects
NaCl   sodium chloride
NADPH   nicotinamide adenine dinucleotide phosphate
NAD   nicotinamide adenine dinucleotide
NAOH   sodium hydroxide
NC   normal controls
NDDG   national diabetes data group
NHS   national health service
NIDDM   non insulin dependent diabetes mellitus
no.   number
NOD   non obese diabetic
NSF-D   national service framework for diabetes
nt   nucleotides

O

ORE   osmotic response element
OD   optical density

P

PAGE   polyacrylamide gel electrophoresis
PCR   polymerase chain reaction
pg   page
PBMCs   peripheral blood mononuclear cells
PBS   phosphate buffered saline
PICs   polymorphism information contents
PKC   protein kinase C
PNK   polynucleotide kinase
PP   pancreatic polypeptide

R

RAS   renin angiotensin system
RFLP   restriction fragment length polymorphism
RNA   ribonucleic acid
RPA   ribonuclease protection assay
RPMI   roswell park memorial institute
RSP   restriction site polymorphism

S

SORD   sorbitol dehydrogenase
SD   standard deviation
SDH   sorbitol dehydrogenase
SDS   sodium dodecyl sulphate
SI   international system of units
SSRIs   serotonin reuptake inhibitors
STZ   streptozotocin

T

T1DM   type 1 diabetes mellitus
T2DM   type 2 diabetes mellitus
TBE   tris/borate electrophoresis buffer
TCR   T-cell receptor
TDT   transmission disequilibrium test
TEMED   N,N,N',N' tetramethylenediamine
TGF   transforming growth factor
TH   tyrosine hydroxylase
TNF   tumour necrosis factor
Tris   2 amino-2-(hydroxymethyl)-1,3-propanediol

U

U.K   united kingdom
UKPDS   united kingdom prospective diabetes study
USA   united states of america
U.V.   ultra violet

V

VNTR   variable number tandem repeat
Vs   versus

W

WESDR   wisconsin epidemiologic study of diabetic retinopathy
WHO DiaMond   world health organization diabetes mondiale
WHO   world health organization

Chapter 1

Diabetes Mellitus and its Complications

Preface

Diabetes and its associated complications, which include retinopathy, nephropathy and neuropathy, is one of the leading causes of morbidity and mortality in both the developed western world, and also world-wide. The Department of Health's (DOH) 'National Service Framework for Diabetes (NSF-D)' was published early this year (2002), and implementation in England and Wales is also scheduled to start in 2002. The NSF-D which includes diabetic complications, is in the top four priority program's for the National Health Service (NHS), preceded only by mental health, coronary heart disease and older people (Keen 2001). In April of 1999 Frank Dobson (Secretary for Health) announced a new £10 million research and treatment fund for diabetes and related diseases, and announced "guaranteed national and local standards of care and treatment" for people with diabetes (NSF press release 1999; Saving Lives: Our Healthier Nation 1999). This degree of recognition pinpoints the disease as a priority target, indicating a real need for research into the molecular biology and genetics involved in the underlying aetiology of diabetes mellitus and its associated complications. It is imperative in the interest of future diabetic medicine that we aim to fully understand this disease in order to improve healthcare and medicine of tomorrow. This perspective is encapsulated by the 61st American Diabetes Association (ADA) scientific session mission statement ' to prevent and cure diabetes and to improve the lives of all people affected by diabetes' (American Diabetes Association 2001).

The medical history of diabetes mellitus

Diabetes was once a universally fatal disease, which led to a slow consuming death involving a gradual wasting away. Diabetic children had a life expectancy of 2-3 years and 90% died from ketoacidosis. The symptoms of what we currently describe as 'diabetes' have been recorded since ancient times, but ideas about its causes have evolved over the centuries and still remain uncertain. The story of diabetes is an extensive one, and

importantly an indeterminate one, to which a degree of ambiguity still remains within its field.

Although clinical descriptions of diabetes were well recognised from as early as Egyptian times, and had been depicted in the Ebers papyrus, 1550 BC, it wasn't until later that the disease was named. Various sources of information attribute the name 'diabetes' to various people. The earliest record, however, is ascribed to Demetrios of Apamaia in the second century BC who derived the name from the Greek word "diabeinein", meaning to go to excess. Later in the second century AD, Arataeus of Cappadocia in Asia Minor, lays claim to the term 'diabetes' which is Ionian Greek, likening its symptoms to the passing of water through a siphon. Arabic texts from the 9th to 11th centuries detail accurate descriptions of the clinical features of diabetes, and in particular mentions two specific complications of the disease to be gangrene and the collapse of sexual function.

More recent advancements began to take place in the 17th century when Thomas Willis (1621-1675) an Oxford physician to King Charles II noticed the sweet taste of urine in many diabetic patients. Later into the 18th century Matthew Dobson reported that the sweet taste of diabetic urine was due to an excess of sugar in the blood, suggesting that diabetes may be a systemic disease rather than a problem of the kidneys. John Rollo, the Surgeon General to the royal artillery, conducted early attempts at treatment in 1798. He observed that careful dietary control with carbohydrate restriction improved glucosuria and greatly improved the patients' prognosis. At a similar time Thomas Cawley 1788, observed pancreatic tissue damage in diabetic cadavers, in particular he observed diabetes occurring alongside 'calcific pancreatitis'. Pioneering work however, really only began to take place in the nineteenth century by Claude Bernard's experimental work on dogs which lead to some important discoveries. He surmised that the liver stored glycogen and secreted a

sugary substance (glucose) into the blood. He also concluded that the secretory organs in particular the pancreas, were controlled by the sympathetic nervous system. Claude Bernard also demonstrated links between the central nervous system and diabetes where hyperglycaemia was caused by transfixing the medulla of conscious rabbits.

In 1867 Paul Langerhans used microscopic techniques to identify clusters of cells within sections of pancreas tissue, which were not connected to the ducts carrying digestive juices to the gut. These were later to be named the 'islets of Langerhans', and much later in the mid 20th century proved to become the anatomical site of insulin production. Joseph Von Mering and Oscar Minkowski quite unexpectedly made the link between diabetes and the pancreas in 1889 (Von Mering and Minkowski 1889). They succeeded in performing the first total pancreatectomy in dogs and in doing so produced severe and fatal diabetes, concluding that the anti-diabetic substance was to be found in the pancreas. These findings sparked 30 years of frantic research right across Europe and North America to isolate a pancreatic extract that could reverse the symptoms of diabetes. Georg Zuelzer working in Berlin and Nicholas Paulesco in Rumania separately isolated a crude pancreatic extract that improved patient's symptoms dramatically, but both showed toxic effects due to hypoglycaemia.

In the summer of 1921 the breakthrough was finally made in Toronto, Canada and led to the award of a Nobel Prize. The discovery of insulin or 'iletin' as it was first called, by Frederick Banting and Charles Best was a significant milestone in the chronological history of the disease. By tying off the pancreatic duct and allowing the glandular part of the pancreas to atrophy, they were able to make an extract of the remaining gland (Banting and Best 1922 (a)). With assistance from a visiting biochemist named J.B. Collip they applied standard biochemical extraction techniques and produced an extract that could be tried on patients. The effects of administering this insulin extract to patients were dramatic.

In a paper published later that same year they describe a young male patient who had been slowly dying of diabetes, who, upon admission showed all signs of severe juvenile diabetes with ketosis. His condition became dramatically worse and the pancreatic extract was administered by daily injection. This resulted in immediate improvement, a loss of consuming thirst and he recovered his strength (Banting and Best 1922 (b)). Insulin provided a lifesaving remedy for many diabetic patients and led to a new era for diabetes. This once fatal disease could now be controlled, providing patients took their daily insulin injections normal life could be restored.

It was however, soon recognised that not all diabetes sufferers responded to the insulin treatment in the same way. In 1936 Himsworth suggested that different forms of diabetes mellitus might exist. The first type involving a lack of insulin and an acute onset of symptoms, and a second type involving a lack of a sensitising factor where patients show little or no clinical symptoms. Their experiments involved injecting insulin intravenously followed by an oral glucose drink. It was apparent that the diabetic patients under investigation could be divided into two types, those in whom the injected insulin produced an immediate suppression of hyperglycaemia and those in whom the insulin had little or no effect in suppressing the hyperglycaemia. They named the two forms 'insulin sensitive', and 'insulin insensitive' diabetes mellitus (Himsworth 1936).

The advent of insulin has enabled diabetic patients to control their diabetes through stringent insulin administration. However, this has unmasked the devastating consequences of persistent hyperglycaemia, namely the diabetic microvascular and macrovascular complications. Dr Elliot Joslin, one of the first clinicians to specialise in the treatment of diabetes mellitus, wrote in 1931, 'with the advent of insulin, we moved from the era of diabetic coma to the era of diabetic complications' (Lee *et al* 2000). Unfortunately these words remain true today as despite significant development in antihypertensive therapy

and glycaemic control we still do not understand the underlying causes of diabetic complications well enough to allow preventative treatment to be fully effective. The history of diabetes was comprehensively reviewed in a paper published by Allan 1972.

Diabetes today and its clinical implications

Since the discovery of insulin a great deal of interest has established itself world-wide for the clinical management of diabetes. Dr Elliot Joslin founded the first diabetic clinic in Boston, USA in 1910, which has today become one of the worlds leading clinical and research establishments for diabetes. Many other research centres have since been established within hospitals and universities throughout the world, all striving for the improved management and treatment of diabetes.

In 1938 the British Diabetic Association (BDA) was founded (now called 'Diabetes United Kingdom, DUK) primarily for patient education and a medical and scientific section was formed in 1960. The 'American Diabetes Association' (ADA) was founded in 1942 for doctors and scientists, publishing the monthly journal 'Diabetes', and similarly the 'European Association for the Study of Diabetes' (EASD) in 1965, which publishes 'Diabetologia'. These organisations all hold annual research meetings, which attract both scientists and medical doctors from around the world.

Today diabetes afflicts large numbers of people of all social conditions, ethnic and economic groups throughout the world and is one of the most common chronic childhood diseases in developed countries. According to demographic and epidemiology studies, the prevalence of diabetes is rapidly increasing on a world-wide scale, and in the absence of effective intervention will continue to proliferate extensively. A study carried out by the World Health Organisation (WHO) in 1985 (WHO 1985) estimated approximately 30 million diabetics world-wide, and more recently in 1994 estimates for the year 2000 were

that more than 100 million people would be affected by diabetes (WHO 1994). In 1997 an estimated 124 million people world-wide had diabetes, and it is thought through projection analysis that this figure will potentially reach 221 million world-wide by the year 2010 with 3 million in the UK alone (Amos *et al* 1997; Zimmet 1999; Dobson; 2000).

Diabetes clearly imposes a vast public health problem in terms of the clinical implications of the disease. The proposals for a 'National Service Framework for Diabetes' (NSF-D) published in 2001, state that diabetes is now one of the UK's governments top health priorities. On 12th January 1999 Frank Dobson (Secretary for Health) announced a new £10 million research and treatment fund for diabetes and related diseases (NSF press release 1999). He also stated that 'The new standards will be set out in a new 'National Service Framework' for diabetes, which will ensure that top quality standards of care and treatment for diabetes are available in all primary care, local hospitals, and specialist centres'.

It is therefore apparent that it remains important to investigate the underlying environmental, molecular and genetic factors influencing the development of diabetes and its associated complications. This will pave the way for advancement in medical knowledge towards treatment and prevention of disease, improve medical care, and improve patient prognosis and quality of life. Considerable advances have been made in our understanding of diabetes and diabetic complications yet we do not sufficiently understand the underlying pathogenesis of these complications to allow for fully effective preventative measures. The fundamental biochemical mechanisms underlying the pathogenic processes of diabetic neuropathy, nephropathy and retinopathy remains unsolved. A wider approach is therefore now required, as we are still somewhat unable to explain satisfactorily the role of obesity, insulin resistance and genetic factors influencing diabetes and its complications.

A major scientific advancement in the study of diabetes has been the mapping of the human genome, enabling research into identifying people who are genetically susceptible to developing diabetes and diabetic complications. With further research, it should be possible to prevent the onset of complications or to control the damaging processes of the disease before they become irreversible. There is still a long way to go in terms of research in diabetic medicine and how new information might be used to prevent the disease.

Definition and classification of diabetes mellitus

In any clinical research the first requirement is to have a clear uniform definition of the parameters of the disease. This is especially true for the study of diabetes where various forms and stages of the disease exist and need to be clearly classified. In the late 1970's the International Working Group for the National Diabetes Data Group (N.D.D.G) of the National Institute of Health, USA, and the World Health Organisation (W.H.O) Expert Committee on Diabetes published their criteria for classification and diagnosis of diabetes mellitus (NDDG 1979; WHO 1980). These criteria for a classification system were based largely on the pharmacological treatment of the disease, and recognised a distinction between insulin dependent (IDDM) and non-insulin dependent (NIDDM) types of diabetes mellitus. The World Health Organisation 1980, endorsed the NDDG proposed diagnostic criteria for diabetes mellitus, and revised and updated in 1985 (WHO Study Group on Diabetes Mellitus 1985).

There has since been an explosive growth in the scientific and medical knowledge into the aetiology and pathogenesis of diabetes mellitus. The earlier classification system based largely upon the treatment of the disease, rather than its underlying pathogenic mechanisms has therefore led to a requirement for a revised classification criteria. In 1997 and 1998 the American Diabetic Association (ADA) Expert Committee on the Diagnosis and Classification of Diabetes Mellitus published updated recommendations on the classification of diabetes (The Expert Committee on Diagnosis and Classification of Diabetes Mellitus 1997, 1998). The report came as a response to the need for revised nomenclature, diagnostic criteria and classification of the disease. The W.H.O. group also presented their conclusions for newer recommendations in 1998 in a report by Alberti and Zimmet 1998. The most recently updated classification is a review by the ADA 'Clinical Practice Recommendations 1999' (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1999; Unwin *et al* 1998). A report by Ravi Shankar

presents an up-to-date review on the current diagnostic criteria for diabetes (Ravi-Shankar *et al* 2001).

The general consensus of the key groups concerned is that diabetes mellitus is a group of metabolic diseases characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action or both. The reports recognise that there are disturbances of carbohydrate, fat and protein metabolism, which can lead to chronic hyperglycaemia as well as microvascular, macrovascular, and neurological complications of this disorder. To date these main organisations have recognised that the vast majority of cases of diabetes mellitus fall into two main aetiopathogenic categories, both of which exist in the UK. The first group which is currently termed 'type 1' or 'T1DM' is insulin dependent diabetes mellitus or juvenile onset diabetes, which includes the immune mediated beta cell dysfunction resulting in absolute deficiency of insulin secretion. There is also a subgroup of T1DM where there is no evidence of autoimmunity and this is termed 'type 1 idiopathic diabetes'. Approximately one-quarter of all diabetics suffer from the T1DM form and it will be this disorder that will be the main focus of interest during this thesis. Secondly, the more prevalent form of diabetes, which accounts for approximately three-quarters of patients, is termed 'type 2' or 'T2DM' and is 'non-insulin-dependent diabetes mellitus', which is characteristically onset during adulthood. Several subgroups of T2DM are also known to exist, an obese form and a non-obese form, as well as rare Maturity Onset Diabetes of the Young (MODY). The cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response.

Various lines of evidence also exist to classify further sub-groups of diabetes, which are numerically small in comparison to the T1DM and T2DM conditions, and are etiologically very heterogeneous. These include diabetes secondary to some other disorder such as pancreatic or endocrine diseases, gestational diabetes where the onset occurs in women

during pregnancy where glucose tolerance often returns to normal postpartum, and thirdly, all other types of diabetes mellitus associated with fibrocalcific pancreatitis and malnutrition related diabetes mellitus (MRDM). The up-to-date classification of diabetes subgroups proposed by the A.D.A. and adopted by the W.H.O is summarised in table 1 (Alberti and Zimmet 1998).

During the development of this thesis a collection of British Caucasoid T1DM subjects and Southern Indian T2DM subjects were studied. Therefore, both subgroups require a brief summation here. The classification criteria used throughout this thesis are based upon the recommendations of the 'aforementioned 'American Diabetes Association' clinical practice recommendations 1998/1999, along with World Health Organisation criteria 1998.

Type	Sub-groups
T1DM (Type 1)	(beta cell destruction, usually leading to absolute insulin deficiency) • Autoimmune • Idiopathic
T2DM (Type 2)	• Predominantly insulin resistance with relative insulin deficiency. • Predominantly secretory defect with insulin resistance.
Other specific types	• Genetic defects of β-cell function. • Genetic defects in insulin action. • Diseases of the exocrine pancreas • Endocrinopathies • Drug or chemical induced • Infections • Uncommon forms of immune-mediated diabetes • Other genetic syndromes associated with diabetes
Gestational diabetes (GDM)	• Gestational impaired glucose tolerance • Gestational diabetes

Table 1. Classification of diabetes mellitus as outlined by WHO 1998. Adapted with slight modification from 'World Health Organisation' recommendations 1998 (Alberti and Zimmet 1998).

Diabetes mellitus

In investigating any disease it is important to understand the fundamental underlying factors leading to the patients disease symptoms. This is particularly important in the case of diabetes, especially as two different forms of the disease are being studied within this thesis. This section will aim to define the two forms of diabetes under investigation 'T1DM' and 'T2DM', which differ in their aetiological, pathogenic and epidemiological characteristics.

Type 1 diabetes mellitus (T1DM)

During the development of this thesis a collection of T1DM subjects of British Caucasoid origin were investigated as well as a family trio collection where the proband had T1DM. The T1DM subclass of diabetes is generally characterised by abrupt onset of severe symptoms, which indicate the presence of hyperglycaemia. Evidence suggests that there is a long pre-diabetic period where the initiation of pathogenesis may begin up to several years prior to the onset of diabetes (Gorsuch 1981). In some patients diabetes is brought to light by the onset of distinct physical symptoms which include polyuria, polydipsia, unexplained weight loss in spite of polyphagia and glucosuria. In extreme cases patients may also present with ketosis, acidosis and even coma. Other symptoms can include genital itching, impairment of visual acuity, repeated skin sepsis and unaccountable pain and paraesthesiae in the limbs. All patients are dependent upon exogenous insulin in order to sustain life and its omission results in life-threatening diabetic ketoacidosis caused by absolute insulin deficiency (insulinopenia).

There are widespread biochemical abnormalities, but the fundamental defects to which most of the abnormalities can be traced are (1)-reduced entry of glucose into various peripheral tissues, and (2) increased liberation of glucose into the circulation from the liver. Only the T1DM form has an autoimmune aetiology with a genetic predisposition

linked to certain class 2 major histocompatibility (MHC) genes. There is also an idiopathic form of T1DM where the aetiology is unknown, there is no evidence of autoimmunity however patients are prone to insulinopenia and ketoacidosis.

Diagnostic criteria for autoimmune T1DM

The detection of glucose in the urine (glucosuria) is usually the first diagnostic indication of the diabetic state, and can be easily performed using the non-invasive 'Diastix' method. Although glucosuria is not a definitive marker of diabetes, it indicates a requirement for a blood-screening test. T1DM subjects also have an extra-cellular glucose excess and, in many cells, an intracellular glucose deficiency, a situation that has been described as starvation in the midst of plenty. The definitive clinical diagnosis of T1DM is therefore relatively straightforward and involves testing the patient's fasting blood glucose levels. In non-diabetic people under normal conditions of life, blood glucose concentration is regulated at values between about 3 mmol/l (54 mg/dl) and 6 mmol/l (108 mg/dl). In people with diabetes, both glycaemic setting levels and homeostatic corrective mechanisms are deranged in varying sometimes gross, degrees. Blood glucose values in untreated diabetes are often found to be in the range of 15-25 mmol/l (270-450 mg/dl).

In accordance to the new and updated 'ADA-expert committee' criteria guidelines for diagnosis, diabetes can be confirmed by one of three methods. These diagnostic figures vary slightly from earlier definitions reflecting the increase in knowledge with regard to the development of complications. The first indication of diabetes is the presentation of classic symptoms of diabetes such as polydipsia, polyuria and unexplained weight loss with a casual/random plasma glucose concentration greater than or equal to 11.1 mmol/l (200mg/dl) (where casual is defined as anytime of the day irrespective of time since last meal). Secondly, diagnosis can be achieved by taking plasma glucose tests following 8 hours of fasting (no calorific intake). This test requires repeat testing on a different day as

confirmation, and is set at 7.0 mmol/L (126 mg/dl) using the fasting plasma glucose (FPG) test. The third technique, which is not recommended for routine clinical use, involves using a glucose load containing 75-g anhydrous glucose dissolved in water and testing glucose levels 2 hours post load, where positive levels are greater than or equal to 11.1mmol/l (200mg/dl). The ADA diagnostic criteria as proposed by 'The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus' in 1997 are shown in table 2.

Interest has also been shown in the use of glycated haemoglobin A_{1c} (HbA_{1c}) in conjunction with the FPG value as a tool for screening and identification of impaired glucose tolerance and diabetes (Peters *et al* 1996; Rohlfing *et al* 2000). The mean blood glucose level as a means of estimating glucose control can be measured by the HbA_{1c} value, which is a component of HbA_1. It is known that glucose can attach to many proteins via a nonenzymatic, post-translational process through the formation of a Schiff base followed by an Amadori rearrangement to form an irreversible ketoamine. Haemoglobin within newly formed red blood cells enters the circulation free from any glucose attachment. Red blood cells are freely permeable to glucose, and glucose becomes attached to haemoglobin at a rate that is dependent upon blood glucose concentration. Importantly, the turn over of red blood cells is relatively rapid and as a consequence of this, the average amount of glycated haemoglobin change reflects the mean blood glucose blood concentration over a 6-8 week period (Nathan *et al* 1984; Goldstein *et al* 1984). Suggestions have been made that diabetes should not be diagnosed in subjects with a FPG less than 7.8 mmol/l (140mg/dl) unless accompanied with a high HbA_{1c} value (Davidson *et al* 1999). However, due to problems in the standardisation of HbA_{1c} assays this is not a technique routinely used in the diagnosis of diabetes mellitus.

	Glucose concentration (mmol l^{-1} (mg dl^{-1})			
	Whole blood		^{a} Plasma	
	Venous	Capillary	Venous	Capillary
Diabetes Mellitus				
Fasting or	≥ 6.1 (≥ 110)	≥ 6.1 (≥ 110)	≥ 7.0 (≥ 126)	≥ 7.0 (≥ 126)
2-h post glucose load or both	≥ 10.0 (≥ 180)	≥ 11.1 (≥ 200)	≥ 11.1 (≥ 200)	≥ 12.2 (≥ 220)
Impaired Glucose Tolerance (IGT):				
Fasting concentration and	<6.1 (<110)	<6.1 (<110)	<7.0 (<126)	<7.0 (<126)
2-h post glucose load	≥ 6.7 (≥ 120) and < 10.0 (<180)	≥ 7.8 (≥ 140) and < 11.1 (<200)	≥ 7.8 (≥ 140) and < 11.1 (<200)	≥ 8.9 (≥ 160) and < 12.2 (<220)
Impaired Fasting Glycaemia (IFG):				
Fasting	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 6.1 (≥ 100) and < 7.0 (< 126)	≥ 6.1 (≥ 110) and < 7.0 (< 126)
2-h	< 6.7 (< 120)	< 7.8 (< 140)	< 7.8 (< 140)	< 8.9 (< 160)

Table 2. Values for diagnosis of T1DM as recommended by WHO 1998 criteria for diagnosis of diabetes mellitus (adapted from Alberti and Zimmet 1998).
^{a} Corresponding values for capillary plasma are: for Diabetes Mellitus, fasting ≥7.0 (≥126), 2-h ≥12.2 (≥220); for Impaired Glucose Tolerance, fasting < 7.0 (< 126) and 2-h ≥8.9 (≥160) and <12.2 (<220); and for Impaired Fasting Glycaemia ≥6.1 (≥110) and <7.0 (<126) and if measured, 2-h <8.9 (<160).

Glycaemic control in T1DM

As a consequence of the immune mediated genetically programmed destruction of the islet β-cells, T1DM patients have much reduced or complete loss in the production of insulin. In some rare patients where there are still some remaining β-cells adequate glycaemic control can be achieved by weight reduction, exercise and/or the use of oral agents to stimulate insulin production. These patients do not require insulin providing they follow a strict dietary control of carbohydrate and fat intake. Other diabetic subjects require insulin in order to maintain adequate glycaemic control, but its absence is not life threatening. Most T1DM cases however, fit into the category where survival of the patient is only made possible by the administration of insulin.

Insulin therapy

Administration of insulin has become the primary treatment for T1DM. Firstly in the form of animal insulin's such as bovine or porcine, and later with genetically engineered semi-synthetic human insulin (Fletcher 1990). Almost all insulin now used in the United States is synthetic recombinant human insulin. Today insulin still requires injecting subcutaneously and there is no form of administering orally, however pulmonary and rectal routes are currently under investigation (Laube *et al* 1998), as well as a surgically implanted programmable insulin pump (Dunn *et al* 1997). The primary aim of insulin injection therapy is to restore the plasma concentration of insulin to a normal level that would have been maintained by the islet β-cells, and consequently achieve long-term glycaemic normalisation. Several different insulin regimens can effectively control hyperglycaemia in subjects through multiple daily injection of regular, intermediate-acting and long acting insulin's or continuous subcutaneous insulin infusion with a pump. Therapeutic problems do however exist such that β-cell secretion of insulin is stringently controlled to a more accurate degree than can be achieved by injection regimes, and also

that there is a physiological difference in the ratio of portal to peripheral insulin concentration to normal.

Pancreas and islet transplantation

Pancreatic transplantation and immunosuppressive regimes using cyclosporin and anti-T-cell antibodies have been successful in the treatment of diabetic patients with renal failure where success results in independence from exogenous insulin therapy and a normalisation of blood glucose concentrations and HbA$_{1c}$ values. Recently a great deal of research has been employed to establish techniques for a far less invasive procedure of islet cell transplantation. This involves 500,000 or more islets from cadaveric pancreases injected into the portal vein of the recipient. Current success in diabetic patients is however very low and improvement in immunosuppressive drugs and islet harvesting techniques are required. This has begun to be seen in one study carried out in Edmonton, Canada, where seven T1DM subjects were transplanted with ~800,000 islet cells and maintained on sirolimus, tacrolimus and daclizumab. All subjects showed normal HbA1c values without exogenous insulin one year after transplantation (Shapiro *et al* 2000; Shapiro *et al* 2001). Improvements in experimental harvesting techniques have also been seen in studies that have so far only been carried out in animals to generate islet cells from islet-producing stem cells (Ramiya *et al* 2000; Halban *et al* 2001).

Epidemiology of T1DM

The onset of T1DM is rare in the first few months of life but rises gradually throughout early childhood and peaks during the pubertal years in both males and females. Following this, there is a sharp fall in incidence with approximately 30% of cases being diagnosed after the age of 20 (Lounamaa 1996). Although the incidence is highest in schoolchildren and adolescents it is also now clear that T1DM may develop at any age and there are indications that the age-at-onset pattern of the disease is changing (Tuomilehto 1992;

Melbak and Marner 1994). The presentation of diabetes is similar in both children and adults, and the same diagnostic criterion applies to both (Pinkey 1994). There is little difference in the incidence of T1DM between males and females, but the EURODIAB ACE study showed a slightly higher incidence in males compared to females but without statistical significance (Green 1992). A family history of diabetes has been shown to be a contributing factor to the onset of diabetes where approximately 5% of first degree relatives of a diabetic proband also have the disease. Twin studies have also shown a 50% concordance rate for the disease (Barnett 1981 [b]). The majority of diabetic patients however do not have a first degree relative with the disease. Other influences affecting the incidence of T1DM also appear to exist and are thought to include temporal trends where most North European countries show a steady increase in incidence, and also seasonal variations where a decline in incidence is often seen during the summer months (Karvonen 1993; Karvonen 1996; Karvonen 1998).

Incidence in UK population and worldwide

T1DM has an annual incidence of 15.6 cases per 100,000 of the population under 21 years in the U.K. There is a weak North-South gradient in incidence with Scotland and Northern Ireland having overall higher rates in incidence than England (Amos *et al* 1997; Zimmet 1999; Dobson. 2000). The incidence of T1DM is currently being monitored by the WHO Diabetes Mondiale (DiaMond) study project, consisting of diabetes monitoring registries throughout the world (WHO 1991). On a world-wide scale diabetes currently affects 135 million people of which 13 million have the T1DM subgroup. The incidence of T1DM accounts for 10-15% of all diabetic cases in European populations. The incidence is highest in Finland/Scandinavia, affecting more than 45 cases/year/100,000 inhabitants (Tuomilehto *et al* 1999), and Sardinia affecting 30/100,000 per year (Muntoni and Songini 1992). It is of medium incidence in Europe and the USA (approximately 10-15 cases/year/100,000). The incidence decreases dramatically towards the Mediterranean area

affecting 8/100,000 inhabitants per year, and is lowest in oriental groups (0.5 cases/year/100,000) and populations living in the tropics (Green *et al* 1992; Yang *et al* 1998).

Karvonen *et al* 1993 reported a steady temporal increase in the incidence of diabetes in North America, Northern Europe, Japan and New Zealand between 1960 and 1989. The same group also published a later report, which further substantiated this, as well as highlighting a significant global variation in the incidence of T1DM (Karvonen *et al* 2000). Gardner reported data from Finland and the UK showing that the main increase in the incidence of diabetes is occurring in children under 5 years (Gardner *et al* 1997). The world-wide prevalence of diabetes was predicted to more than double between 1994 and 2010, to 239 million people (McCarty and Zimmet 1994).

Pathogenesis of T1DM

The exact processes initiating the onset of T1DM are not yet completely understood, although several environmental and genetic influences have been found to be strongly associated to the disease. The pathogenesis of T1DM is known to be associated with an organ specific autoimmune reaction against the pancreatic islet β-cells for which the pathognomic sign is a nearly complete loss of the pancreatic β-cells at the time of clinical diagnosis.

Anatomy and physiology of the non-diabetic and T1DM pancreata

The pancreas is a composite and lobulated gland, which lies immediately behind the peritoneum of the posterior abdominal wall. It consists of a head, neck, body and tail and normally weighs between 60g and 160g with a length of about 15cm. The pancreas is composed of two types of secretory cells. Approximately 98% of the pancreatic cells are exocrine 'serous acini', which are under the influence of secretin and cholecystokinin

(CCK) produced by enteroendocrine cells of the small intestine. These cells discharge various digestive enzymatic secretions such as trypsin, lipase and bicarbonate into the duodenum to assist in digestion. The remaining 2-3% are pale staining metabolic endocrine cells, which cluster together in a cellular aggregate known as the islets of Langerhans, and are involved in hormone secretion into the portal vein. The normal adult human 'islets' are ovoid, with an average diameter of 76 x 175μm, and consist of a compact mass of epithelial cells permeated by a dense network of capillaries. These collections of cells are scattered throughout the pancreas, although they are more plentiful in the tail than in the body and head (Lazarus and Volk 1962). There is a wide variation in the number of 'islets' in the human pancreas, however there are approximately $10^5 - 10^6$ scattered throughout the exocrine parenchyma of the pancreas. Each aggregate contains approximately 1000 endocrine cells, of which four cell types can be distinguished on the basis of their staining properties, and morphology using electron microscopy. Approximately 80% are the insulin producing 'beta' (β) cells, and about 15% are glucagon secreting 'alpha' (α) cells. The remaining 5% are composed of 'delta' (δ) cells that produce somatostatin, and 'pancreatic polypeptide'-containing (PP) cells. The beta cells are generally located in the centre of each islet, and tend to be surrounded by the alpha cells (Goldstein *et al* 1968; Deconinck *et al* 1971).

Insulin is synthesised in the beta cells firstly as preproinsulin in the ribosomes of the rough endoplasmic reticulum (ER) which is then cleaved to form proinsulin and transported to the golgi apparatus where it is packed into secretory granules located close to the cell membrane. Insulin secretion is regulated primarily by extracellular glucose concentration whereby glucose is taken up into the beta cells via glucose transporters (GLUT2 and GLUT1) and is consequently metabolised to glucose-6-phosphate. This results in an intracellular increase in ATP concentration closing potassium dependent ATP (KATP) channels in the beta-cell membrane causing membrane depolarisation and an influx of

calcium. This increase in intracellular free calcium promotes margination of the secretory granules, their fusion with the cell membrane and the release of their contents into the extracellular space. The secretion of insulin in normal subjects is pulsatile with a periodicity of 9-14 minutes, and loss of this is an early sign of β-cell dysfunction.

Pancreata from patients with recent onset T1DM are normal in appearance, size and weight, however, patients with chronic T1DM display a considerable reduction in weight and volume of the pancreas. This volume reduction, results from severe, pathognomonic changes which mainly involves the reduction of β-cells by pancreatic endocrine cell atrophy (Gepts 1965; Gepts and Lecompte 1981). Quantitative evaluation using immunohistochemistry and immunoflourescence techniques on islet tissue from newly diagnosed diabetic pancreatic biopsy specimens has revealed a marked decrease in the total β-cell insulin containing cells to about one-third to one-seventh of that of non-diabetics. These studies have also shown that the diabetic pancreas exhibits preservation of glucagon containing cells, as well as evidence of some immunological changes (Stefan *et al* 1982; Rahier *et al* 1983; Hanafusa *et al* 1990). The β-cells do not always disappear completely, but are still present in most patients with diabetes of less than 10 years duration, and in 40% of patients with disease duration between 11 and 40 years. Severe acinar cell atrophy is seen in the surrounding cells of insulin deficient islets, and the peri-lobular spaces are enlarged and contain loosely arranged connective tissue, but acinar tissue around insulin-containing islets is normal (Foulis and Stewart 1984). The large arteries are often affected by arteriosclerosis, while the small arterial vessels show diabetic microangiopathy. The islet cell organisation is also distorted with many endocrine cells scattered as single cells in the exocrine tissue (Gepts and Lecompte 1981; Cossel *et al* 1983; Kawanishi *et al* 1966; Kloppel 1985).

Aetiology of β-cell destruction in T1DM

The precise aetiology of autoimmune β-cell destruction of T1DM is still not completely clear and strong evidence indicates a multifactoral basis, determined by both genetic and non-genetic (environmental) factors (Tisch and McDevitt 1996; Haverkos 1997). Several groups have written comprehensive reviews with respect to the pathogenesis of T1DM (Atkinson and MacLaren 1994; Mandrup-Poulsen 1998; Chowdhury *et al* 1999 [c]). Evidence suggests that T1DM results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas, determined by defects in immune regulatory genes, which is often initiated several years prior to the onset of diabetes. However, twin studies have shown that there is also considerable evidence for the involvement of environmental factors in the pathogenesis of β-cell destruction. It has been hypothesised that telomeres might play a role in the pathophysiology of T1DM, where altered cell turnover and different rates of attrition in the length of the telomeres leads to premature replicative senescence of the cell (Jeanclos *et al* 1998). It has also, recently been suggested (Pipeleers *et al* 2001) that the β-cell itself may actively participate in its own destruction, rather than being a passive victim of a cytotoxic process. They suggested that the surrounding non-β-cells also influence the process of β-cell death by making the islets internal milieu more protective or toxic. Evidence for the immunologically mediated destruction of insulin secreting β-cells comes from several lines which will be discussed here:

Autoimmune markers

One indication of diabetes is the presence of insulitis, where the 'islets of Langerhans' of recently diagnosed diabetic patients are infiltrated by mononuclear cells. This islet infiltrate consists primarily of T lymphocytes, from both of the two major subtypes 'cluster of differentiation'- 4 and 8 (CD4 and CD8) with a predominance of CD8+ cells together with macrophages (Foulis *et al* 1986). The presence of markers of the autoimmune

processes, which lead to the destruction of the β-cells of the pancreas, is also a factor. 'Islet cell auto-antibodies' (ICA's) are a feature of newly diagnosed diabetes and comprise autoantibodies to a number of antigens (Wilkin 1990; Bonifacio *et al* 1990; Genovese *et al* 1992). Such antibodies include autoantibodies to insulin (IAA's), autoantibodies to glutamic acid decarboxylase (anti-GAD_{65}), and autoantibodies to protein tyrosine phosphatases (I-A2) which can be detected in up to 90% of patients (Atkinson *et al* 1986; Christie *et al* 1992; Baekkeskov *et al* 1990; Leslie *et al* 1999 [a]; Lernmark 1987; Hawa *et al* 1997). Approximately 10% of new cases of T1DM occur in first degree relatives of a diabetic proband. Amongst these, positivity for ICA's and at least one other autoantibody gives an 88% risk of developing T1DM, and identifies 75% of those who are actually progressing to disease. It is important to note that none of the autoantigens described to date have been found to be the definitive diabetes autoantigen.

Metabolic markers also provide a prediction tool for the onset of T1DM where the acute insulin response to several secretagogues such as glucose, arginine, glucagon and isoproterenol decrease progressively during the preclinical period. The most widely performed test is the acute insulin response to glucose (AIR-g) test where the serum insulin increase after an intravenous glucose challenge is measured, the result of which correlates with functioning beta cell mass (McCulloch *et al* 1993).

Animal studies

Animal studies into immune-related β-cell destruction using BioBreeding (BB) rats and Non Obese Diabetic (NOD) mice have served as models for human T1DM. Studies carried out by Like *et al* 1982, using 'BB' rats have shown evidence for cell mediated autoimmune pathogenesis of diabetes by administering antiserum to rat lymphocytes which prevented the onset of diabetes. Further studies using the BioBreeding/Worcester (BB/Wor) rat, showed that pancreatic islet insulitis occurs in the presence of enhanced class I antigen

expression (Weringer and Like 1988). Studies using transplantation methods on the NOD mouse were carried out by Stein *et al* 1992, which showed that destructive insulitis can be transferred to non-diabetes prone mice.

Genetic susceptibility to T1DM

The genetics of T1DM have been comprehensively reviewed by Cavan *et al* 1992; Julier *et al* 1996; and Todd 1997. These articles have discussed the various lines of evidence for the involvement of several key genetic components in the aetiology of T1DM, and it is these points that will also be discussed here. The involvement of a genetic component in the underlying aetiology of T1DM has been confirmed, based upon findings from twin studies and also from family studies, suggesting that susceptibility to developing T1DM may be in part due to a genetic component. Monozygotic and dizygotic twin studies have argued a strong case for a genetic involvement in the genesis of T1DM. Twin study's where the 'index' twin has developed T1DM has been carried out on large patient collections from several populations. These include studies carried out by Olmos *et al* 1988; Barnett *et al* 1981 (b); Kaprio *et al* 1992 on a population of Finnish twins, by Kumar *et al* 1993 on North-American twins, Kyvik *et al* 1995 on young Danish twins, and recently by Redondo *et al* 1999 and 2001 on monozygotic twins in Great Britain and the United States. These studies have collectively shown a higher disease concordance rates for T1DM in identical monozygotic twin pairs (45% to 96%), compared to non-identical twins (3% to 37%). These concordance rates are however below unity, implying that there is also an environmental component involved in the aetiology. These studies have also concluded that the twins that progressed to heterogeneity of diabetes (25%), did so if the patients were diagnosed at a younger age, and also that the rates of developing diabetes in the co-twins declines sharply in the years after diagnosis of the index twin.

A positive family history is also a risk determinant, with familial clustering occurring among first-degree relatives. In siblings of diabetic probands the risk of developing the disease is about 5% (WHO 1994), and there is evidence of preferential transmission where the risk is higher if the child's father (5-6%) rather than the mother (2-3%) has T1DM. Between 80-90% of all newly diagnosed diabetics have no family history of the disease, however the remaining 20% of patients show familial aggregation (Warram 1984; The EURODIAB ACE Study Group 1998). Genome wide searches of families with two or more affected sib pairs have been carried out using fluorescence based technology and linkage analysis. These searches have identified more than 30 genomic susceptibility intervals and potential candidate genes for T1DM situated on different chromosomes, with varying degrees of evidence for linkage (Easton 1989; Davies *et al* 1994; Todd 1997; Friday *et al* 1999). In addition to the argument for a genetic involvement, epidemiological data suggests that disease incidence rate is increasing at a rate which is faster than can be explained by changes in the gene pool alone (Karvonen *et al* 1993).

Susceptibility genes for T1DM

Two main chromosomal influences have been found to be associated with T1DM. Firstly, and the most influential is the IDDM1 in the Major Histocompatibility Complex (MHC) on chromosome 6p21, and the second is the IDDM2 in the insulin gene region (INS) on chromosome 11p15 and these will be briefly discussed below.

Major Histocompatibility Complex (MHC) (IDDM1)

The Major Histocompatability Complex (MHC) is situated in the region associated with the genes for the immune system recognition molecules known as the Human Leucocyte Antigen (HLA). The MHC is situated on chromosome 6p21, consists of around 3500 Kb of DNA which code for more than 200 genes and has been shown to be a principle region associated with the genetic predisposition to T1DM. Most of these genes are known to be

involved in the antigen presentation and processing events of the immune response. The MHC is composed of three major regions, namely class I, class II and class III, which are described by Campbell and Trowsdale 1993 and Trowsdale and Campbell 1997. The class I region (covering HLA-A, -B, -C, -E, -F, -G and –H) encodes the single polypeptide beta-chain peptide which forms part of the trans-membrane molecules expressed on the surface of nucleated cells to activate CD8+ cytotoxic T-lymphocytes. The class II region contains genes, which code for the alpha and beta chains of the HLA-DR, DQ and DP molecules, which are involved in activating CD4+ T-helper (Th) cells. The class III region encodes a large number of genes related and unrelated to the immune response. Such genes include complement factors, tumour necrosis factors (TNF) α and β, heat shock proteins and many others.

The pathological processes that take place during β-cell death are thought to begin with the expression of interferon-alpha by the beta cell. Secretion of this cytokine is associated with hyperexpression of class I MHC by all endocrine cells within the islet and the presence of aberrant class II MHC molecule expression by B-cells (Foulis 1996). Early studies for genetic markers of T1DM in the MHC 'class I' region showed a positive association between HLA-B15, B8 and B18. The strongest associations came from B8 where positive associations were found in both Caucasoid and Negroid populations with a neutral association in Asians (Cudworth and Woodrow 1975, 1976; Nerup et al 1974; Singal and Blajchman 1973). A number of other studies have also shown evidence that the 'class I' region contains genes that play an important role in the susceptibility to T1DM (Demaine et al 1995; Fujisawa et al 1995). Also, studies carried out by Hodgkinson et al 1999 have found an association between the HLA-E locus and age at onset and susceptibility to T1DM in British Caucasoids. It is within the 'class II' and 'class III' regions of the MHC however, that several stronger associations have since been made for candidate genes for T1DM. The pathology involving autoimmune destruction of pancreatic islets in T1DM has

been found to have a strong association with possession of Human Leukocyte Antigen (HLA) -DR3 and/or –DR4 antigens (Platz *et al* 1981; Rotter *et al* 1983; Wolf *et al* 1983). In the British Caucasoid population, approximately 90-95% of patients with T1DM has the DR3 and/or DR4 HLA antigens, compared with 45-54% of the general population (Jenkins *et al* 1990; Cavan *et al* 1997). Approximately 50% of T1DM patients are heterozygous for HLA-DR3/DR4 whereas this is only seen in 5% of the normal population (Gottlieb and Eisenbarth 1996). It has been suggested that the HLA association with diabetes relates to the affinity of the MHC molecules for different diabetogenic peptides (Nepom and Robinson 1990).

In conclusion, the combination of these regions within 'class I, II and III' of the MHC represents the genetic susceptibility associated to the MHC (Lie *et al* 1999). However it is important to note that this does not completely confer susceptibility and that other genes within and outside of the MHC are also important in the susceptibility or protection from the onset of T1DM.

Insulin gene (INS) (IDDM2)

The insulin gene region located on chromosome 11p15 has also been found to show association with, and linkage to T1DM. This 20-kb chromosome region is known to contain genes encoding tyrosine hydroxylase (TH), insulin (INS) and insulin like growth factor II (IGF-II). The human insulin gene and its flanking regions are known to exhibit conserved and highly variable sequences, which have previously been sequenced, originally by Bell *et al* 1982, and showed DNA sequence polymorphisms. It is known that there is a highly polymorphic region flanking the 5' end of the insulin gene (Ullrich *et al* 1982; Owerbach and Aagaard 1984). A T1DM susceptibility region has been found to be present in the 5' region of chromosome 11p15.5, in a case control study (Bell *et al* 1984). Owerbach and Gabbay 1993 reported finding that suggest the localisation of the T1DM

susceptibility locus on chromosome 11p15.5 is in the 5' variable number tandem repeat (VNTR) region of the insulin gene. Also Lucassen *et al* 1993 reported susceptibility to T1DM to be mapped to a 4.1-kb region including the insulin gene and VNTR, but excluding TH and IGF2. By cross-match haplotype analysis and linkage disequilibrium mapping, Bennett *et al* 1995 and 1997 mapped the mutation to within the VNTR itself. They have shown that IDDM2 expression is influenced by parent-of-origin effects, and that allelic variation of the VNTR may correlate with the level of INS- ribonucleic acid (RNA).

A transracial study carried out by Undlien and colleagues looked at T1DM patients from three different ethnic groups, namely Tanzanian blacks, Norwegian Caucasoids and Japanese Orientals. They concluded that polymorphisms in the insulin gene only conferred significant susceptibility to T1DM in Caucasoids (Undlien *et al* 1994). Mijovic *et al* 1997 reported further studies on INS region genotypes in Japanese, Hong Kong Chinese, North Indian Asians and Afro-Caribbean's finding no INS polymorphism across all races.

It would therefore appear that the additional risk of T1DM conferred by the insulin gene is small in comparison to the contribution of particular HLA-DQ alleles. However, it is clear that it plays an important role as an additional marker and the contribution from other INS gene region polymorphisms cannot be excluded and warrants further investigation.

Other genes contributing to T1DM

Theoretical calculations suggest that the combined effects of IDDM1 and IDDM2 do not count entirely for the genetic component of susceptibility to the disease. The respective contributions of the genes to the susceptibility to T1DM is approximately 42% for IDDM1 and 10% for IDDM 2, indicating that other genes must also be involved (Davies *et al* 1994). Several other genes have been proposed to be associated with susceptibility to

T1DM. Such genes include a locus on chromosome 15q26 (IDDM3) (Field *et al* 1994),

11q13 (IDDM4) (Hashimoto *et al* 1994; Nakagawa *et al* 1998), 6q (IDDM5) (Davies *et al*

1996), and 2q (IDDM7) (Copeman *et al* 1995). Also several investigators have found an

association between the T-cell receptor (TCR) constant beta chain polymorphism located

on chromosome 7q35 and T1DM (Hibberd *et al* 1992; Robinson *et al* 1993; Zhao *et al*

1994). Analysis of a large collection of 356 United Kingdom affected sib pair (ASP)

families (Warren 1) also found the IDDM1/MHC to be the major locus associated to

T1DM, and only four other regions outside of the MHC were not excluded. Two of these

regions on chromosome 10p13-p11chromosome 16q22-16q24 showed evidence of linkage,

along with two possible susceptibility genes located at chromosome 14p12-q21 and

chromosome 19p13-q13 (Bain *et al* 1990; Mein *et al* 1998). It is therefore likely that the

genetic aspect of the autoimmune pathogenesis of T1DM outside of the established MHC

region, is an amalgamation of several different contributory genes (Friday *et al* 1999).

Non genetic risk factors of T1DM

There are strong arguments for the involvement of an environmental influence in the

pathogenesis of T1DM (Cooper *et al* 1999). These primarily include a low rate of

concordance in monozygotic twins, where more than 70% of monozygotic twin pairs have

been found to be discordant for the disease (Barnett *et al* 1981 [a and b]. The incidence of

diabetes also varies significantly according to geographical location. Large differences in

frequency between different countries has been shown (Diabetes Epidemiology Research

International Group 1988), and also migrants from areas of low risk to areas of high risk

have been seen to acquire an increased risk of disease (Bodansky *et al* 1992). The clinical

manifestation of the disease also shows a seasonal pattern with fewer cases diagnosed in

the summer months and a peak in the autumn months (LaPorte and Cruickshanks 1984).

Karvonen *et al* 1993 reported that in some countries of the world the rise in incidence of

T1DM is occurring at a rate that cannot be explained by changes in the gene pool. Whilst it

has been proven to be difficult to elucidate a direct putative environmental agent involved in the onset of T1DM, research has shown several candidate factors to have a modulating or disease-triggering effect, and these are briefly outlined below;

Viral Infection

A role for an infectious agent has been indicated by the occurrence of epidemics, non-familial clustering and seasonality in the incidence of disease (Leslie and Elliot 1994). A viral infection can initiate the autoimmune destruction of the islet cells of the pancreas through autoreactive lymphocytes by molecular mimicry mechanisms. This process occurs through an immune response against a viral protein that shares an amino acid sequence with a beta-cell protein resulting in the appearance of antiviral cytotoxic CD8 lymphocytes that react with self protein on the beta cells. Among the most strongly suspected environmental agents are certain viruses, enteroviruses and Coxsackie B viruses in particular and exposure early in life, possibly in utero may contribute to the aetiology of T1DM (Lounamaa 1996). Diabetes has been seen to occur in subjects with congenital rubella (Forrest *et al* 1971), Coxsackie B, cytomegalovirus, hepatitis C and other viruses which have been implicated in inducing the disease (King *et al* 1983; Karjalainen *et al* 1988; Pak *et al* 1988). Several studies in animals have demonstrated that several viruses trigger the onset of diabetes and these include encephalomyocarditis (EMC) virus, Coxsackie B4 virus, Kilhams rat virus (KRV) and rubella virus. These viruses trigger diabetes either by directly infecting and destroying the insulin producing pancreatic beta cells, or by initiating an autoimmune response against the beta cells.

Environmental Insult

A second process could be an environmental insult which may generate cytokines and other inflammatory mediators that induce the expression of adhesion molecules in the vascular endothelium of the pancreatic islets, resulting in extravasation of circulating

leukocytes and the presentation of beta cell antigens (Atkinson *et al* 1994). Environmental insult may be in the form of exposure to certain food proteins or certain toxins and drugs. Early studies into the effects of poor nutrition during infanthood and the incidence of T1DM came from research carried out on a Scandinavian population (Borch-Johnson *et al* 1984; Borch-Johnson *et al* 1984). The studies show some evidence to suggest that reduced breast-feeding during infancy is associated with the development of islet cell autoimmunity. The association is suggested to be due to early exposure to cows milk protein (CMP) triggering an immune destruction of the β-cells (Martin *et al* 1991; Virtanen *et al* 1994; Akerblom and Knip 1998). Amino acid homologies between the cows milk protein bovine serum albumin (BSA) and the islet cell autoantigen ICA69 may explain the onset in some patients, in particular the CMP variants β-casein A1 variant and β-casein (A1 + B). Many drugs are also known to impair insulin secretion or insulin action, such as nicotinic acid and glucocorticoids (Pandit *et al* 1993; O'Byrne *et al* 1990), and toxins such as Vacor (rat poison) and pentamidine can permanently destroy pancreatic beta cells (Gallanosa *et al* 1981; Assan *et al* 1995).

Prevention of T1DM

Effort has been made to find effective preventative therapy for subjects at high risk for T1DM that can be identified using a combination of immune, genetic and metabolic markers. Recent data from prospective studies in humans suggests that it will soon be possible to predict with reasonable certainty from genetic and autoantibody screening people who are likely to develop T1DM. Immunotherapy using a combination of drugs including azathioprine, cyclosporine, nicotinamide and insulin to reduce the immune mediated destruction of beta-cells has been investigated in pilot studies (Skyler and Marks 1993). Success in these uncontrolled pilot trials has led to larger controlled trials such as the European Nicotinamide Diabetes Intervention Trial (ENDIT) and the Diabetes Prevention Trial for type 1 diabetes (DPT-1), the outcome of which are still to be

determined. Other approaches are looking at the expression of intercellular adhesion molecules (ICAM) such as ICAM-1 that is involved in accelerating beta cell destruction by cytotoxic T cells (Yagi *et al* 1995). Animal studies have also indicated that vaccination with insulin or its peptides looks very promising as a prevention method. Results from pilot trials in humans are also encouraging for the prospect of using insulin for primary prevention (Graves and Eisenbarth 1999).

Type 2 diabetes mellitus (T2DM)

During the development of this thesis a collection of Dravidian T2DM subjects from the Southern Indian region of Madras were also investigated. It is therefore necessary to differentiate between the T2DM subjects and T1DM subjects that are also included in the study. T2DM is often asymptomatic for many years and frequently remains undiagnosed because the level of hyperglycaemia is often not severe enough to provoke noticeable symptoms of diabetes. Patients may present either as a result of complications of diabetes as such patients are at increased risk of developing macrovascular and microvascular complications, or incidentally with an abnormal blood or urine test (Harris *et al* 1992). Subjects have relative insulin deficiency and do not normally require insulin treatment to survive. T2DM is characterised by hyperglycaemia caused by impaired insulin secretion, impaired insulin resistance in muscle, and elevated hepatic glucose production. Due to its clinical heterogeneity T2DM can be divided into obese and non-obese forms and also into early and late onset forms. The early onset form includes the formerly named 'maturity onset diabetes of the young' (MODY), which is a genetically heterogeneous monogenic form of T2DM characterised by an early onset <25 years of age, and autosomal dominant inheritance. The majority of T2DM subjects are obese or have characteristically increased percentage of body fat distributed predominantly in the abdominal region (Kissebah 1982). It occurs more frequently in women with prior gestational diabetes, and individuals with hypertension or dyslipidaemia. Ketoacidosis is seen rarely in T2DM and when it does it is usually in association with illness or infection.

Diagnostic criteria for T2DM

The diagnostic criteria for T2DM are the same as for T1DM where the fasting plasma glucose test is used. Insulin resistance may improve with weight reduction and/or pharmacological treatment of hyperglycaemia. Patients with T2DM are not dependent on insulin for prevention of ketonuria and are not prone to ketosis. However they may require

insulin for correction of symptomatic or persistent fasting hyperglycaemia if unable to correct using diet controls or oral hypoglycaemic agents (sulphonylureas/biguanidines). T2DM is often also accompanied by hypertension, high serum low-density-lipoprotein (LDL) cholesterol concentrations and low serum high-density-lipoprotein (HDL) cholesterol concentrations that can increase cardiovascular risk.

Treatment of T2DM

As with T1DM it is necessary to control the blood glucose levels in T2DM subjects, which can be achieved through both non-pharmacological treatment and pharmacological therapy. The United Kingdom Prospective Diabetes Study (UKPDS) suggested that as with T1DM maintaining as near normal blood glucose concentration in T2DM subjects by oral hypoglycaemic drugs or insulin markedly reduces the risk of microvascular complications (UKPDS 33). Non-pharmacological intervention in T2DM can improve many aspects of the disease including hypertension, obesity and insulin release and effect. Weight reduction and calorific control are known to greatly improve glycaemic control and insulin sensitivity in T2DM subjects (Henry *et al* 1985). However, although diet, weight reduction and exercise are known to improve glucose metabolism compliance with these interventions is not sustained in many patients (Uusitupa *et al* 1993). As a consequence, drug or insulin therapeutic intervention is usually required for which there are currently four options, which can be applied independently or through polypharmacy. An increase in insulin release can be achieved with sulfonylureas (glipizide or glyburide) or meglitinides (Repaglinide). Increased insulin responsiveness can be achieved with a biguanide (metformin) or a thiazolidinedione (rosiglitazone- Avandia or pioglitazone- Actos). The intestinal absorption of carbohydrate can be modified with an alpha-glucosidase inhibitor (acarbose and miglitol), and a lipase inhibitor (Orlistat- Xenical) can be used to modify the absorption of fat. The administration of exogenous insulin can also be applied in subjects who have persistent hyperglycaemia despite other intervention.

Epidemiology of T2DM

T2DM is the most common form of diabetes, accounting for 80-90% of all cases in developed countries, and the majority of cases in developing countries. The prevalence of T2DM varies enormously from population to population and between countries throughout the world. The highest prevalence has been recorded in Pima Indians and the Micronesian population in the Central Pacific where up to 25% of each population is affected (Trevisan 1998). In most industrially advanced societies, the incidence of T2DM rises throughout adult life and is usually highest in old age. It has been noted that while the incidence of new cases of T2DM seems fairly stable, the prevalence in the general population may be rising. A study carried out in Canada showed that the prevalence of T2DM rose from 4.5 to 7 percent between 1986 and 1991 (Blanchard 1996). Several reviews have highlighted differences in the clinical profiles of diabetes between the developed countries of the temperate region and tropical countries (Mohan 1986). The T2DM subjects included in this study were collected from the Southern Indian region of Madras, a mainly Dravidian sub-population region where T2DM is the most common form of diabetes seen. Previous studies carried out in the Southern Indian town of Kudremukh have shown that the overall prevalence of diabetes was as high as 5%, and in those older than 40 years it rose to 21% (Ramachandran 1988 [a and b]). In the region of Madras studies have shown a higher prevalence of diabetes in the urban population (8.2%) compared to the rural areas (2.4%) (Ramachandran *et al* 1992). Ramachandran *et al* 1988 [a] also demonstrated that there is a very high risk of diabetes in Southern Indian families with one diabetic parent.

Pathogenesis of T2DM

In the pathogenesis of T2DM there are at least two pathological defects either of which may be the predominant feature and it is uncertain whether one causes the other or whether both are necessary to cause T2DM (Beck-Nielsen and Groop 1994; Kahn 1994). One is insulin resistance where there is a decreased ability of insulin to act on peripheral tissue

(muscle and liver) to stimulate glucose metabolism or inhibit hepatic glucose output. A second defect is due to insulin deficiency where the endocrine pancreas is unable to compensate for insulin resistance due to a defect in the glucose stimulated insulin secretion and glucose desensitisation of the pancreatic β-cell. This impairment has been associated to a beta cell dysfunction in the processing of proinsulin to insulin, where the proportion of immunoreactive proinsulin is greater in T2DM subjects (40%) compared to normal subjects (10%) (Kahn 1997).

Following the stimulation of insulin secretion, insulin action can be divided into three stages. The first stage involves the binding of the hormone insulin to the insulin receptor (tyrosine kinase enzyme) on the plasma membrane of the cell. This binding leads to a conformational change in the receptor and stimulation of kinase activity. The second stage is the cascade of serine phosphorylation and dephosphorylation by mitogen-activated protein kinase (MAP), and thirdly the biological effectors of the insulin cascade involving translocation of the glucose transport molecules to the plasma membrane (Kahn 1994). Several cell mechanism defects are thought to be associated with increased insulin resistance including decreased activation of enzymes (glucokinase, glycogen synthase), reduced levels of cell-membrane glucose transporters and increased levels of circulating fatty acids. The cause of B-cell failure is still undetermined, however β-cell mass is only modestly reduced by 20-40%. It is likely that the defect in insulin secretion is due to glucose toxicity in beta cells by which chronic sustained hyperglycaemia leads to impaired insulin secretion possibly by decreasing insulin gene expression (DeFronzo 1992; Moran 1997).

Aetiology of T2DM

It is recognised that defects of insulin secretion and insulin sensitivity in the pathogenesis of T2DM are multifactoral in nature influenced by both genetic and environmental factors.

Animal and human studies have indicated that insulin resistance alone is insufficient to cause diabetes in humans and that multiple abnormalities are required in the expression and interaction of genes controlling insulin secretion and action. Primary diabetogenes are involved in the initiation of diabetes, and secondary changes involve altered gene expression. In certain populations where there is evidence of a major genetic component, a 'thrifty genotype' has been implicated where a metabolic efficiency is advantageous when food is scarce, but becomes disadvantageous when food is plentiful (Dowse 1993; McCance 1994; Hales *et al* 1992). Also, environmental factors such as diet, activity and environmental toxins such as use of steroids and antihypertensive agents may act as initiating or progression factors.

Genetic susceptibility to T2DM

It is becoming more and more apparent that the aetiology of T2DM is genetically and clinically very heterogeneous in nature and that several genes may be involved in its aetiology (Rotter *et al* 1981). Studies in unaffected co-twins of T2DM parents, different prevalence between ethnic groups, familial clustering and the high concordance rate for T2DM monozygotic twins all suggest a predominantly inherited aetiology for T2DM (Newman *et al* 1987; Turner *et al* 1995; Carter 1996). Thirty nine percent of patients with T2DM have at least one parent with the disease (Klein *et al* 1996), and among monozygotic twin pairs with one affected twin, 60 to 90 percent of unaffected twins eventually develop the disease (Barnett *et al* 1981 [b]). The importance of genetic factors in T2DM is also highlighted by the observations that lean, normoglycaemic offspring of parents with T2DM have reduced non-oxidative glucose metabolism associated with reduced muscle glycogen synthesis (Eriksson *et al* 1989; Rothman 1995). Several attempts have been carried out to identify T2DM genes by linkage studies which has led to conflicting results, indicating that distinct genes are probably involved in different

populations. It is likely that different genes may be involved in different families within the same ethnic group.

Candidate genes for T2DM

The search for possible candidate genes has focused on genes coding for proteins that might be involved in insulin secretion or action. For early onset T2DM (MODY) linkage studies have identified genes that are mutated in different MODY pedigrees. The MODY1 locus is found on chromosome 20 (hepatocyte nuclear factor-4α gene), MODY2 locus is on chromosome 7 (glucokinase gene), the MODY3 locus is on chromosome 12 (hepatocyte nuclear factor-1α). A fourth form formerly referred to as MODY4 associated to T2DM and as a rare cause of MODY has also been described which appears to be linked to insulin promoter factor-1 (IPF-1). Mutations in the IPF-1 gene result in reduced binding of the protein to the insulin gene promoter and decreased insulin gene transcription in response to hyperglycaemia (Stoffers *et al* 1997; Macfarlane *et al* 1999). All of these mutations present as specific descriptions of genetic defects of beta cell function which are associated with abnormal patterns of glucose stimulated insulin secretion (Froguel *et al* 1992; Hattersley *et al* 1992; Horikawa *et al* 1997).

A genome wide search in affected sibling pairs has identified a locus encoding the cysteine protease calpain-10 on chromosome 2 (NIDDM1) that appeared to confer major susceptibility to T2DM in Mexican-Americans (Hanis *et al* 1996). Two genes associated to late onset T2DM have been reported both of which have been mapped to the long arm of chromosome 12. Firstly, NIDDM2, and secondly a locus between markers D12S1693 and D12S326. The identity of this NIDDM2 gene located on the long arm of chromosome 12 is unknown. However, the region on chromosome 12q has been linked to T2DM subjects from the Botnia region of western Finland, and also in a single pedigree from Australia (Mahtani *et al* 1996; Shaw *et al* 1998). Genome scans in other populations, including

Finland and an autosomal dominant T2DM U.S. population failed to detect linkage (Bektas *et al* 1999). A possible T2DM locus has also been reported to exist 50cM centromeric to NIDDM2 on chromosome 12q15 between markers D12S1693 and D12S326 (Bektas *et al* 1999; Bektas *et al* 2001).

The association of T2DM with deafness in families where inheritance occurred through the mother identified a point mutation in position 3243 of the mitochondrial gene encoding tRNA Leu, but this has been found to be <1% prevalence in diabetics (van den Ouweland 1994; Kobayashi *et al* 1997; Tawata *et al* 1998). Polymorphisms have been identified in the insulin receptor substrate 1 (IRS-1) gene and these are a common substrate for insulin receptor tyrosine kinases, but suggestions are that they are not pathogenic mutations (Almind *et al* 1993; Withers *et al* 1998). Initial observations in humans has suggested that a mutation in the gene for the β3-adrenergic receptor which is involved in regulating lipolysis in visceral fat may be associated with obesity and the onset of T2DM (Walston *et al* 1995; Widen *et al* 1995). These findings support the hypothesis that multiple abnormalities in genes controlling insulin action or secretion explain the non-mendelian inheritance and the variable penetrance of T2DM.

Non genetic risk factors of T2DM

The development of T2DM is also known to be influenced by exposure to different environmental factors, evidence of which has come from studies where the frequency of the disease in migrants is compared with the frequency in individuals remaining in the original environment (Kawate *et al* 1979). Observations have been made that the prevalence of impaired glucose tolerance and T2DM has increased dramatically in several ethnic groups whose lifestyle has become 'westernised' in the last few decades (Collins *et al* 1994). A study carried out by Ramachandran *et al* 1988 [b] looked at a population living

in Southern India and found that 5% tested positively for diabetes, when compared to the prevalence in Indians living in London and Fiji, this rose to 10%.

Reduced physical activity and central obesity has been found to be a major determinant of T2DM, prospective studies have shown that physical activity is associated with a reduced risk of T2DM. Obesity is known to decrease the sensitivity of the β-cells to glucose (Henry *et al* 1985) which is largely reversible by weight loss. The mechanisms by which obesity induces insulin resistance is poorly understood however several factors are thought to be important and these include; free fatty acids (FFA), tumour necrosis factor (TNF)-alpha, the pattern of fat distribution and the genetic abnormality in the β3-adrenergic receptor. An inverse relationship has also been determined between low birth weight and diabetes mellitus (Phillips *et al* 1994) where the relative risk of T2DM decreases with greater birth weight (Rich-Edwards *et al* 1999).

Complications of diabetes mellitus

The introduction of insulin in 1922 was of paramount importance in modern 20th century diabetic medicine. It provided a lifesaving treatment for many patients, but in doing so also unmasked the second consequence of diabetes which is the onset of late diabetic complications. Historically the complications of diabetes have been recorded for a long time, in as early as 1798 Rollo described pain and parasthesia in the legs of diabetic patients and was also the first to note eye changes which associated diabetes to the development of cataracts (Grenfell 1989; Mandrup-Poulsen 1998). Although most of the recognised features of diabetic complications had been described well before the introduction of insulin, it was not until patients survived for longer periods that the full extent and often-fatal nature of the complications were revealed. Diabetic patients live today controlled by insulin but still suffering the unremitting pain, amputation, blindness, dialysis and death from the long-term microvascular and macrovascular complications of their diabetes. Although T1DM and T2DM have different underlying pathogenic mechanisms, the chronic long-term microvascular and macrovascular complications affect both groups and are the major cause of morbidity and mortality in diabetes mellitus.

Presentation of diabetic complications

Although a great deal of research into diabetes has centred on susceptibility to diabetes, the major cause of morbidity and mortality result from the long-term microvascular, neurologic and macrovascular complications associated with the disease. In fact it is well recognised that a high proportion of patients with diabetes will develop one or more microangiopathic complications during the course of their disease. The complications of diabetes mellitus affect many types of tissue including nerves, skin, retina, kidney, heart and brain. In all of these tissues, the major cause of tissue damage is vascular disease affecting both the microvasculature and macrovasculature.

Microangiopathy of diabetes mellitus

The common microvascular complications of diabetes occur in the diabetic retina, the kidney, and the nerves, and lead to retinopathy, nephropathy and neuropathy respectively, each of which will be discussed here;

Diabetic nephropathy

The kidneys are one of the key organs to be affected by diabetes and diabetic nephropathy, which was first described by Kimmelstiel and Wilson in 1936, remains to be the most serious, life threatening, long-term complication of T1DM. The internal structure of the kidney consists of a cortex and a medulla which are enclosed within a capsule. The medulla contains pyramids, which drain into the pelvis collecting system. The histological and functional unit of the kidney is the nephron, and there are about 1 million in each kidney. Each nephron consists of a glomerulus and a tubule system. The glomerulus is a tuft of capillaries surrounded by very thin epithelial cells forming a rounded Bowmans Capsule. The thin walled tubule region of the nephron, namely the Loop of Henle consists of a proximal and a distal convoluting tubule, a collecting tubule and a collecting duct. Urine is the glomerular filtrate, which passes into the space of the Bowmans Capsule and so into the tubule system where it is modified by selective absorption and secretion. It is this precise mechanism which is greatly affected by diabetes and can lead to a range of complications categorised under the title of nephropathy.

The incidence of renal complications in T1DM patients has been reported to be 25-40% after 25 years duration of diabetes, a further 15-28% of patients are reported to have microalbuminuria (Andersen 1983). Borch-Johnsen and colleagues reported that proteinuria is strongly associated with death from uraemia and cardiovascular disease (Borch-Johnsen 1985). In Caucasoid subjects with T2DM the prevalence of progressive renal disease has generally been thought to be lower than in T1DM subjects (Cowie *et al*

1989). Recent evidence however, has suggested that the renal risk is equivalent in the two types of diabetes where the time to proteinuria from the onset of diabetes, and the time to end-stage renal disease from the onset of proteinuria were similar (Ritz and Orth 1999; Ritz and Stefanski 1996). Large epidemiological studies carried out to follow the progression of diabetic nephropathy in T1DM have shown that typically there is a lag phase from the time of diagnosis of diabetes to the incidence of development of nephropathy. Following this there is a rapid rise in incidence, which peaks after 15-20 years duration, and declines to 1% per year thereafter. Therefore the likelihood of developing nephropathy after 20 years duration of diabetes is very small (Krolewski 1985; Christensen 1985). The clinical progression and manifestation of nephropathy in T2DM subjects is less well defined, as subjects usually have a long duration of diabetes before clinical manifestation of the disease. However, it would appear to follow a similar course to that in T1DM subjects as described below (Nelson 1993; 1996). It has been suggested that T2DM subjects with normoalbuminuria or microalbuminuria may have impaired renal function and that cardiovascular risk factors are closely related to renal damage. As a result diabetic nephropathy is probably the most common indication for haemodialysis treatment and renal transplantation in the USA and the western world, and consequently it imposes a very high social and economic burden on the individual and society.

The clinical course of diabetic nephropathy can be divided into 5 key stages, which are comprehensively reported and reviewed by Mogensen 1984, 1985, 1996 [a], 1997; and Tuttle 1990. There are considerable patient-to-patient differences in the rate of progression of diabetic nephropathy, however certain stages such as the progression of renal failure are predictable (Jones 1979). Stage 1 of nephropathy occurs shortly after the onset of diabetes and is first manifested as an increase in glomerular filtration where the kidney shows hyperfiltration and kidney hypertrophy. Early structural glomerular changes in morphology include thickening of the glomerular basement membrane and accumulation of

extracellular matrix components (Ziyadeh 1993). Stage II, which may last for between 7 and 15 years is a 'silent stage', where albumin is maintained in the circulatory system and is not excreted. However, it is during this stage that the early structural glomerular changes such as the expansion of the mesangial matrix and renal tubular damage take place (Fletcher *et al* 1986; Yaqoob *et al* 1994). Stage III begins when microalbuminuria becomes evident, when urinary albumin excretion rate is greater than 20µg/min and less or equal to 200µg/min (table 3). Incipient diabetic nephropathy is suspected when microalbuminuria is found in 2 out of 3 urine samples collected consecutively. This is the point of established renal injury. Once constant microalbuminuria is established, and unless there is clinical intervention, progression to nephropathy is inevitable. Studies have shown that approximately 80% of patients with microalbuminuria progress to overt nephropathy in 10 years. Stage IV is established when overt diabetic nephropathy is suspected, when the urinary albumin excretion rate is greater than 200µg/min for at least 2 out of 3 urine samples collected within maximal 6 months. Almost all diabetic patients at this stage of nephropathy will also exhibit diabetic retinopathy. Once overt nephropathy is established, a relentless decline in renal function is seen, associated with an elevation in arterial pressure. At this stage anti-hypertensive treatment, particularly with angiotensin converting enzyme (ACE) inhibitors may be beneficial in reducing the rate of progression to renal failure (Raskin 1996; Bilous 1996; Mogensen 1996 [b]). Finally, stage V, otherwise known as end-stage renal disease is defined clinically by an increase in the excretion of larger plasma protein, β-2-microglobulin, with an albumin excretion rate of approximately 1000 µg/min, with a generalised glomerular closure leading to uraemia requiring dialysis or kidney transplantation.

It has been postulated that diabetic nephropathy occurs as a result of the interplay of metabolic and haemodynamic factors in the renal microcirculation. Pivotal studies, including the DCCT (DCCT 1993 [a], 1995 [a], 1998) have demonstrated the effects of

intensive blood glucose control and antihypertensive therapy on the progression of urinary albumin excretion. This implicates poor glycaemic control and hypertension in the progression from normoalbuminuria to microalbuminuria. It has been suggested that once urinary albumin concentration exceeds the normal range (4 ± 1 mg/l) the risk of developing diabetic nephropathy is increased. A large prospective clinic-based study of 1200 T1DM subjects which were followed for 4 years suggested that a urinary albumin concentration of 7.4 mg/l implicates a significantly increased risk for the development of nephropathy (Royal College of Physicians of Edinburgh Diabetes Register Group 2000; Walker *et al* 1999). Significant improvements have been made in the treatment of diabetic subjects and population based studies have shown a delay in the onset of renal disease probably due to improved glycaemic control and clinical intervention (Bojestig *et al* 1994; Krolewski 1996; Walker 2001; Hovind *et al* 2001 [b]). Studies have also suggested that unlike T1DM, where strict glycaemic control is the main preventative factor of diabetic nephropathy, in T2DM the control of hypertension, hyperlipidemia, obesity and hyperuricemia may have priority (Molnar *et al* 2000). The accumulation of advanced glycation end products (AGEs), the activation of isoforms of protein kinase C (PKC) and the acceleration of the polyol pathway may provide explanations of the effects of hyperglycaemia and nephropathy in T1DM and T2DM. Management strategies designed to modify progression from normoalbuminuria to microalbuminuria and early identification of patients at such risk should therefore be targeted.

Clinical nephropathy

Urinary albumin concentration (UAC).	> 300mg/l (30mg/dl)
Urinary albumin excretion rate (UAER).	> 300mg/24h
Urinary protein excretion.	> 500mg/24h

Microalbuminuria

UAC	20-30 mg/l
UAER	20-200µg/min (timed overnight collection) 30-300mg/24h (24 hour collection)
Albumin:Creatinine (ACR)	2.5-25mg/mmol- male 30-300mg/g- male 3.5-25mg/mmol- female 40-300mg/g- female

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Table 3. Definitions of nephropathy (adapted from recommendations by Bilous 1996).

- The diagnosis of clinical nephropathy is generally carried out using dipstick urinalysis for proteinuria. These tests detect albumin at a concentration of >300 mg/l, and a positive result on three or more consecutive occasions spread out over several months is the conventionally accepted definition of clinical nephropathy.

- The accuracy of the detection of microalbuminuria is improved by relating albumin and creatinine concentrations in an albumin:creatinine ratio (ACR).

- False positive results with concentrated urine, biological variation, posture/diurnal variation, exercise, urinary infection, other renal disease, and cardiac failure. False negative results with dilute urine or diuresis.

Diabetic retinopathy

Another key system to be affected by diabetes is the retina and there are several causes of blindness in diabetic patients including retinopathy, cataract and glaucoma, this study however focuses specifically on retinopathy. Retinopathy is a highly specific vascular complication that is debilitating in that it can lead to diminished visual acuity and blindness in both T1DM and T2DM patients. Early studies such as the 'Bedford Survey' indicated that impaired glucose tolerance carries minimal risk of eye disease, however, the risk increased dramatically upon diagnosis of diabetes (McCartney 1983). Retinopathy shows a clear pattern of progression, with incidence rising continually with increasing duration of diabetes. The incidence of retinopathy is relatively low in subjects with impaired glucose tolerance or newly diagnosed diabetes (Klein 1991). However, after a lag period of several years from the diagnosis of diabetes, the presence of diabetic retinopathy increases unremittingly, affecting approximately 90% of T1DM patients and >60% T2DM patients after 15-20 years duration of diabetes (Klein 1992). The 'Wisconsin Epidemiologic Study of Diabetic Retinopathy' (WESDR) evaluated the prevalence and incidence of retinopathy in a defined population in an 11 county region of Southern Wisconsin, USA (Klein *et al* 1984 [a and b]). Results from this study showed that one third to 86% of blindness in diabetic subjects was attributable to diabetic retinopathy (Klein 1998). Progression of retinopathy to the proliferative stage occurs in about 30% of T1DM subjects after 10 years of the disease, and in 60% after 40 years.

Retinopathy is primarily a vascular disorder, probably beginning with capillary dilation in the retinal capillary bed and promoted by chronic hyperglycaemia. Retinopathy involves both morphological and functional changes in the retinal capillaries, including basement membrane thickening, loss of pericytes, increased permeability and vascular dysfunction. Diabetic patients are routinely screened for retinopathy in the absence of any visual symptoms by pupilary dilation performed by an ophthalmologist. The progression of

retinopathy is orderly, advancing from mild non-proliferative abnormalities, characterised by increased vascular permeability and the formation of microaneurysms, to moderate and severe non-proliferative diabetic retinopathy, characterised by vascular closure. From this, pre-proliferative stage, retinopathy advances through to proliferative diabetic retinopathy which is characterised by the growth of new blood vessels on the retina and posterior surface of the vitreous (American Diabetes Association 1998; Aiello 1998). Vision loss due to diabetic retinopathy results from macular oedema or capillary nonperfusion, distortion or detachment of the retina, or through bleeding of new blood vessels.

Factors that have been associated with the development and progression of diabetic retinopathy include gender, HLA type, age at onset, duration of disease, degree of metabolic control, presence of pubertal development, growth hormone secretion and presence of proteinuria (D'Angio *et al* 2001). A follow up study carried out by Annunzio 1997, on patients with retinopathy who were treated with conventional therapy, suggested that poor metabolic control, age, and degree of pubertal development at diagnosis were the most important risk factors. Similarly, Kalter-Leibovici 1997 supported this view, reporting that poor glycaemic control was significantly and independently associated with an early progression to proliferative retinopathy. Cohen 1999 found that the levels of hyperglycaemia and diastolic blood pressure predicted progression of retinopathy in T1DM. The development of proliferative retinopathy has also been shown to be closely related to the development of proteinuria. This has been shown in a study by Pirart 1984, where 80% of patients with persistent proteinuria developed proliferative retinopathy. Intensive treatment of diabetes aimed at maintaining as near normoglycaemia as possible has been shown to significantly reduce the risk for the development and progression of diabetic retinopathy. Laser photocoagulation surgery has proved to be beneficial in reducing the risk of further visual loss, but not seen to be able to reverse already diminished acuity (Chantelau 2001). In the Diabetic Retinopathy Study (DRS) and the

Early Treatment of Diabetic Retinopathy Study (ETDRS) photocoagulation by argon laser or xenon arc light prevented new visual loss in patients with proliferative retinopathy and macular oedema and improved vision in some patients (ETRDS 1985; DRS 1978). Vitrectomy is beneficial in patients where visual loss is caused by proliferative retinopathy with vitreous haemorrhage, scarring, and retinal detachment. In the Diabetic Retinopathy Vitrectomy Study (DRVS), early vitrectomy improved the chance of good vision (DRVS 1985). Therefore, there are clear benefits in screening patients for the often-asymptomatic early stages of retinopathy such as clinically detectable capillary dilation and retinal capillary basement membrane thickening (RCBMT) (Glover *et al* 2000; Janghorbani *et al* 2001; Keen *et al* 2001 [a and b]). It is recommended that dilated eye examination and retinal photography should be included in the routine management of T1DM during the first 5 years to identify subjects at risk of developing vision-threatening problems (Malone *et al* 2001).

Diabetic Neuropathy

Peripheral and autonomic nerves are also significantly affected by the altered metabolism of diabetes mellitus, resulting in pathological change, functional disturbance and clinical morbidity. Approximately half of patients who have had diabetes for 20 years will have some evidence of neuropathic involvement. In diabetic subjects with end stage renal disease, the incidence of neuropathy varies from 60-90% (Parving 1988). The cumulative prevalence of neuropathy increases with duration of diabetes and neuropathy represents a concomitant or consequence of the diabetic state.

Diabetic neuropathy is a clinical state of nerve damage in which a patient complains of symptoms (pain, paraesthesiae) or is shown to have neurological deficit likely to lead to problems. Diabetic neuropathy is notably heterogeneous in its clinical presentation, encompassing several distinct syndromes that differ with respect to anatomical distribution

of neurological deficit and clinical course. Multiple aspects of neural function are currently being studied to enable accurate diagnosis of diabetic neuropathies, as early and accurate identification of this complication is essential for effective clinical intervention (Arezzo 1999; Hirai 2000). Sub-clinical neuropathy refers to the presence of evidence of impaired nerve function in the absence of clinical signs or symptoms of diabetic neuropathy. The most common form of clinical neuropathy associated with diabetes is distal symmetrical polyneuropathy with predominant sensory and autonomic involvement, and slow progressive loss of distal sensory, autonomic and motor fibres. Polyneuropathy is characterised by a loss of peripheral nerve function, which can cause sensory disturbances, motor weakness and autonomic dysfunction. Other forms of neuropathy are the asymmetric neuropathies involving one or more discrete cranial or peripheral nerves, which constitute roughly 15% of all diabetic neuropathies and are generally, restricted to older diabetic patients. The onset is usually acute and painful and the median, ulnar, deep peroneal radial, femoral and sciatic nerves are the most frequently affected. Mononeuropathy may involve single or multiple cranial nerves especially those innervating the extraocular muscles and the facial nerve. Diabetic neuropathy also underlies the development of diabetic foot ulcers, which can lead to lower limb amputations. Erectile impotence in male diabetic patients is also largely attributable to this disorder.

It is thought that diabetic neuropathy is conditioned by insulin deficiency and/or hyperglycaemia, although the specific pathogenic mechanisms that underlie this conditioning effect are not fully understood. It has been suggested that T1DM patients with autonomic neuropathy may have a degree of protection against the development of diabetic ketoacidosis as plasma fatty acids and ketone body concentration rises less rapidly in these patients following withdrawal of insulin (Krentz et al 1994). There is evidence based on animal work that there is a link with hyperglycaemia, through sorbitol accumulation, myo-

inositol depletion and Na^+-K^+-ATPase activity, with nerve dysfunction and damage (Greene 1984). Observations reported from experiments in rats and also in patients, looking at nerve blood flow suggest that hyperglycaemia induced blood flow reductions in sural nerve and resultant endoneurial hypoxia are important factors underlying nerve conduction deficits early in the development of diabetic neuropathy (Dyck 1989; Cameron 1991; 1994; Tesfaye 1994). An increase in platelet aggregation has also been observed in otherwise uncomplicated patients with neuropathy, which may have pathogenic implications (Jennings et al 1986). The EURODIAB IDDM complications study 1996 identified further associations with neuropathy which included elevated diastolic blood pressure, the presence of severe ketoacidosis, an increase in the levels of fasting triglyceride, and the presence of microalbuminuria. Further trials have also associated significant roles for height, cigarette smoking and female gender as risk factors for progression of distal symmetric polyneuropathy (Christen 1999).

Vasodilatory treatment aimed at improving blood flow, adrenoceptor blockade, aldose reductase inhibition and calcium channel antagonist treatment have all shown positive results, however as several metabolic abnormalities combine to produce deleterious changes in nerve perfusion multi-action therapy is important (Cameron 1997; Fedele 1997; Parry 1999). The mainstay treatment of the symptoms of painful neuropathies currently incorporates the use of tricyclic antidepressants (desipramine), or serotonin reuptake inhibitors (SSRIs), although the later has not proven to be as successful as the former. Gabapentin (neurontin) can also reduce the pain associated with polyneuropathy. However the only strategy shown to be consistently beneficial to the treatment of diabetic neuropathy is meticulous control of blood glucose by multiple injections or continuous subcutaneous infusion of insulin. It is imperative therefore that therapy is directed at early diagnosis, exclusion of other neuropathic disorders, prudent glucose control, and avoidance of secondary complications of neuropathy such as foot ulceration by aggressive foot care,

hygiene and patient education (Greene 1990; DCCT Research Group 1998). Physical therapy evaluation is important in patients followed with use of ankle-foot orthoses, splints, and walking assistance devices.

Macroangiopathy of diabetes mellitus

Macrovascular diseases occur in the large peripheral arteries of the lower limbs, in cerebral vessels and in coronary arteries, and are due to atherosclerosis of blood vessels, which result in reduced blood flow to tissues. Macrovascular complications of diabetes include coronary, cerebral and peripheral vascular disease such as angina, heart attacks, strokes, and amputations (Foley *et al* 1997). Diabetes and abnormal glucose tolerance is a major risk factor and is known to enhance the development of atherosclerosis and coronary heart disease (CHD) (Stamler *et al* 1993). Evidence has shown a significant relationship between long-term hyperglycaemia and diabetic macrovascular disease in T2DM (Kuusisto 1994). Results published in the DCCT 1993 [a and b] indicate that the risk of a cardiovascular event occurring in diabetic subjects was reduced by 41% by strict glycaemic control, albeit not a significant correlation. Atherosclerotic macrovascular disease accounts for more than 80% of all mortality in the diabetic population, with increased risk if diagnosed over 40 years of age or over 30 years duration of diabetes (Foley and Parfrey 1998; Morrish *et al* 2001). The risk for fatal and nonfatal CHD events in subjects with T2DM has been reported to be two to four times higher than in non-diabetic subjects. It is thought that dyslipidemia, hyperglycaemia, hypertension and obesity may account for the risk of developing atherosclerosis

Inter-linkage of diabetic complications

It can be seen that the common chronic complications of diabetes encompass multiple organ systems, and includes macrovascular diseases such as cardiovascular disease and atherosclerosis, and microvascular diseases such as retinopathy, neuropathy and

nephropathy (Rich 1997). Borch-Johnsen *et al* 1985 reported evidence for an association between microvascular and macrovascular diabetic complications where a large number of patient deaths due to macrovascular disease also presented with proteinuria. It has been suggested that microvascular complications are also interrelated with each other, for example, 90% of patients with nephropathy also have retinopathy. Studies by Florkowski 1988 and Parving 1988 and more recently by Aroca *et al* 2000 found that microalbuminuria was significantly associated with retinopathy, an association was also suggested between microalbuminuria and neuropathy in the absence of retinopathy. Jennings 1990 [a and b] reported an association between retinopathy and severity of neuropathy. However, non-concordance for the onset of the microvascular complications was reported in a follow up study carried out by Pirart 1977, which is illustrated in figure 1. A study carried out by Fong 1995 to determine prospectively whether cardiovascular autonomic neuropathy is a risk factor for proliferative diabetic retinopathy, showed evidence that there was an association. Some insight into the correlation between nephropathy and retinopathy came from a study carried out by Schwartz *et al* 1998 using T2DM subjects who underwent renal biopsy. This study indicated that severe retinopathy was more closely associated with Kimmelstiel-Wilson nodules than with mesangial sclerosis (p=0.0043), the reasons for which are not yet known. The relationship between diabetic nephropathy and retinopathy is less predictable in T2DM. For example studies of T2DM subjects found that subjects with marked proteinuria and retinopathy most likely have diabetic nephropathy, while those without retinopathy have a high incidence of non-diabetic glomerular disease (Parving *et al* 1992; Christensen *et al* 2000). It has been speculated that there is a common underlying cellular mechanism behind the diabetic complications, which leads to widespread microvascular disease and causes organ damage.
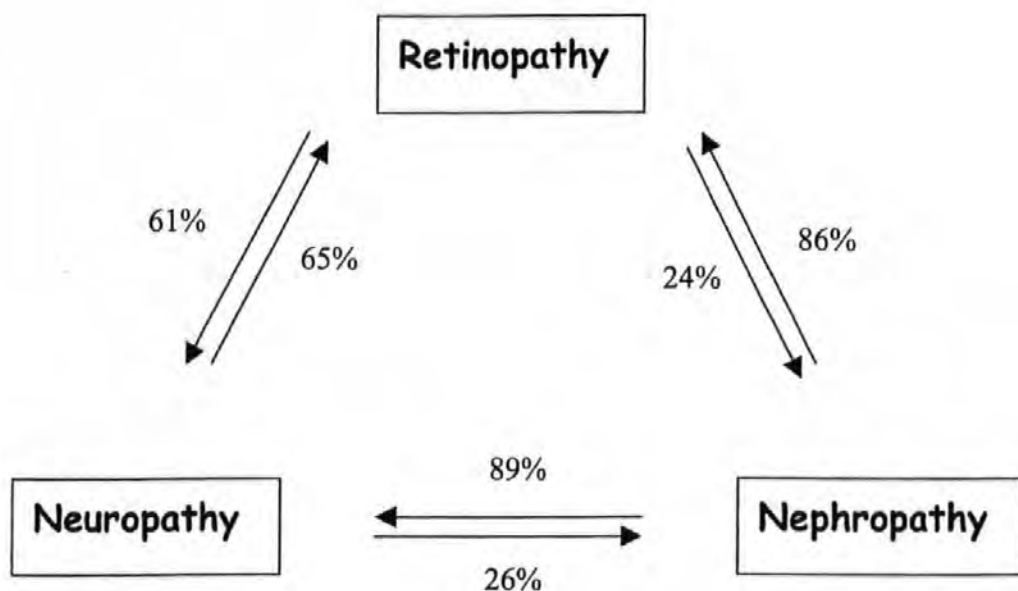
Figure 1. Concordance between diabetic microvascular complications in the follow up study carried out by Pirart 1984. For each new case of one given complication, the probability to display another complication is indicated by the percentage attached to the arrow directed to this complication (adapted with minor alterations from Pirart *et al* 1984).

Aetiology of diabetic complications

The development of diabetic complications in subjects with T1DM and T2DM is not by any means straightforward, and cannot as yet be confidently predicted. Some patients with diabetes are fortunate in that they can live through the duration of their diabetes without developing any sign of microvascular or macrovascular disease. Other patients on the other hand develop a whole handful of complications, which are both debilitating and significantly reduce the quality of life of the patient. A significant amount of research is currently underway to try to elucidate the mechanisms by which these complications take effect and to attempt to predict their onset in order to design appropriate preventative and treatment regimes to improve prognosis of the patient. To date several key components in the underlying aetiology of diabetic complications have been identified. These include various risk factors such as duration of diabetes, hyperglycaemia, hypertension and hyperlipidemia, as well as certain pathological features such as capillary basement membrane thickening and haemostatic abnormalities. Alterations in certain biochemical pathways as a result of the diabetic state have also been identified such as the increase in the synthesis of diacyglycerol (DAG), non-enzymatic glycation of proteins and an increase in the flux through the polyol/sorbitol pathway. An increase in the formation of reactive oxygen species (ROS), and Nitric Oxide (NO) has also been extensively examined. Alterations in the cell signal transduction pathways have also been investigated and implicate protein kinase C and mitogen activated protein kinases (MAPKs). Also gene expression promoters such as Transforming Growth Factors (TGF), Vascular Endothelial Growth Factors (VEGF) and Nucleotide Factor κB (NFκB) have attracted a great deal of interest. Numerous inter-related genetic and environmental factors have also been examined. These components (illustrated in figure 2) are reviewed here.
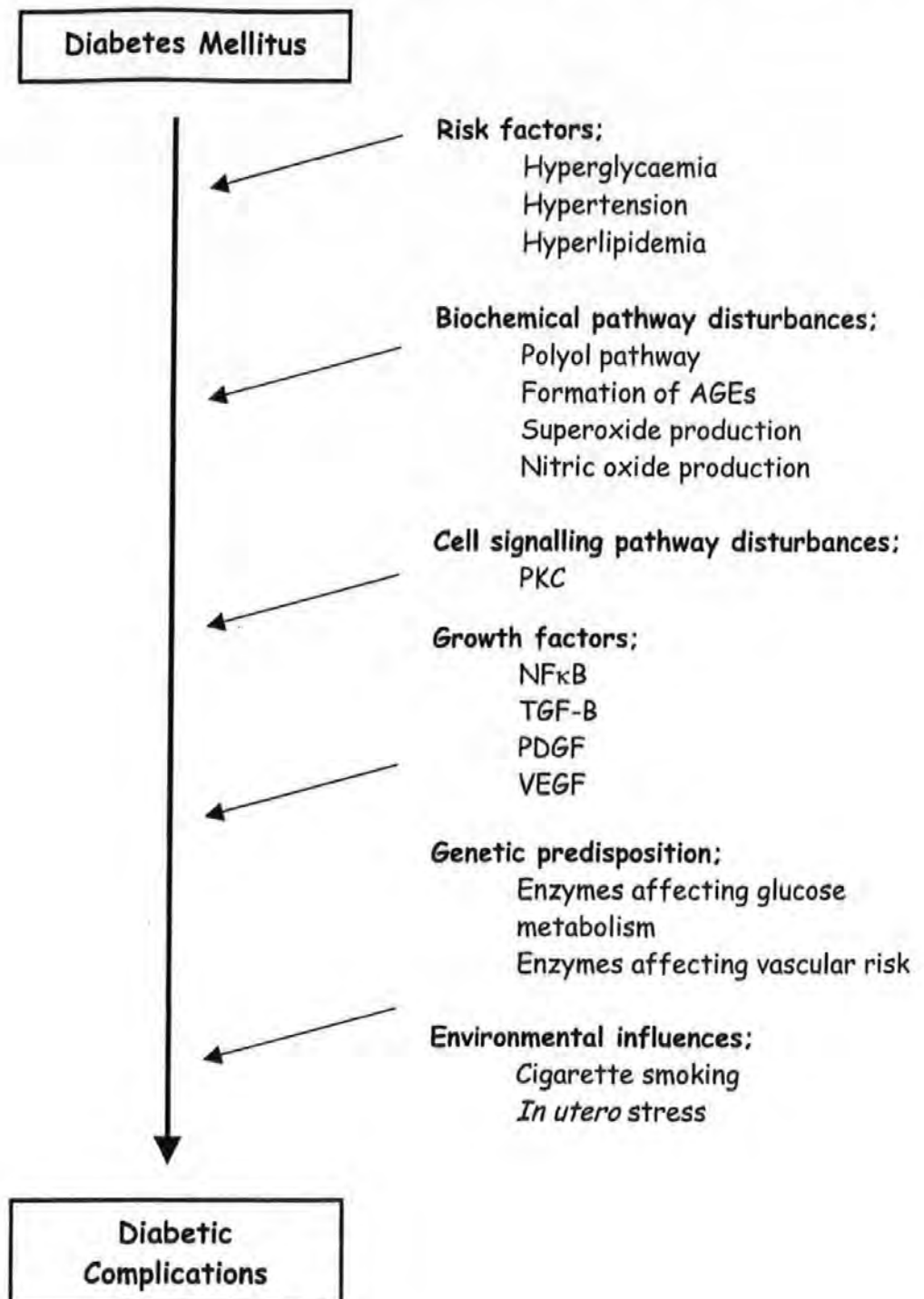
Figure 2. Factors affecting the onset and progression of diabetic microvascular and/or macrovascular complications (importantly, this is not a conclusive list).

Risk factors for diabetic microangiopathy

Various risk factors are thought to be involved in diabetic microvascular and macrovascular disease, and appear to include the duration of the disease, hypertension, hyperlipidemia and the quality of glycaemic control. Better metabolic control through anti-hypertensive therapy, and improved glycaemic control has been shown to be effective in decreasing the incidence of diabetic complications (Kofoed-Enevoldsen 1987; Hostetter 1994; Rossing 1998). Hyperlipidemia has also been associated with the development of microvascular complications although conclusive evidence from clinical trials is yet to be attained.

Hyperglycaemia and microangiopathy

Epidemiological, animal, and biochemical studies have strongly suggested that there is a relationship between hyperglycaemia and development and progression of diabetic microvascular complications involving the retina, glomeruli, and neuronal tissues. Early studies carried out by Jarret 1976, and also by Jean Pirart 1978 showed that poor control of glycaemia in T1DM subjects predisposes to chronic complications. Retrospective epidemiological studies have shown that nephropathy and retinopathy are more likely to occur in those patients with poorer glycaemic control (Krolewski 1988; Rosenstock and Raskin 1988; Molitch 1993; Dahl-Jorgensen; Barzilay 1992). Other studies, including the Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) and the Oslo Study of Diabetic Retinopathy have also demonstrated a strong consistent relationship between hyperglycaemia and the incidence and progression of microvascular and macrovascular complications in T1DM and T2DM populations (Brinchmann-Hansen *et al* 1992; Klein 1995; Leslie 1999 [b]). The United Kingdom Prospective Diabetes Study (UKPDS [b]) carried out a study of over 4000 patients with prolonged follow up, and suggested that strict glycaemic control results in a reduced risk of microvascular disease in patients with T2DM (UKPDS 1998 [b]). The DCCT research group provided conclusive evidence that

strict glycaemic control can both delay the onset of microvascular complications and slow the rate of progression of already established complications. They demonstrated in a multi-centre, randomised, prospective controlled clinical study that there was a 76% reduction in the development of retinopathy, a 54% lower incidence of nephropathy and 60% reduction of neuropathy with intensive therapy aimed at achieving glycaemic control as close to the non-diabetic range as possible. In the DCCT report intensive insulin therapy consisted of multiple daily injections or by continuous insulin administration through the use of an insulin pump. The DCCT also suggested that the total lifetime exposure to glycaemia was the principle determinant to the risk of developing retinopathy and other complications of diabetes, and that there was a continuous non-linear relationship between this risk and the mean level of HbA1c (DCCT Research Group 1993 [a], 1993 [b], 1995 [b]). A separate report by the DCCT looked at the development of diabetic nephropathy under intensive insulin therapy and found that there was a beneficial effect, where the onset of nephropathy was possibly prevented or at least delayed in its progression (DCCT Research Group 1995 [a]). The DCCT also found that there was no glycaemic threshold below which the risk of developing retinopathy was reduced (DCCT Research Group 1996). However, other authors have suggested a glycaemic threshold for microalbuminuria and for retinopathy exists, below which the risk of patients progression from microalbuminuria to overt proteinuria is reduced. The DCCT did show that the risk of development of microalbuminuria increases substantially when HbA1c increases beyond 8.8%. This is similar to findings from the Joslin Clinic (Krolewski 1995 [a]) and the Stockholm Study (Reichard 1995), both of which indicated that albumin excretion increases substantially when long term HbA1c is more than 8.8-9.0%. No cases of serious retinopathy were observed in patients over the period of observation when long-term HbA1c was less than 7.0%. To be reasonably sure that a patient will not develop any microvascular complications, the treatment goal should therefore be glycated haemoglobin within the normal range. Improved glycaemic control and maintaining an HbA1c level of below 8.5%

has therefore been shown to improve the prognosis of serious microangiopathy (Krolewski 1995 [a]; Hanssen 1995; Warram 2000).

A relationship between levels of chronic hyperglycaemia and diabetic microvascular and macrovascular disease in patients with T2DM has also been found in several studies (Ohkubo et al 1995; Kuusisto et al 1994; Colwell et al 1994; Kaiser et al 1993). As with T1DM patients, retrospective studies show a strong association between indexes of hyperglycaemia and the prevalence of diabetic retinopathy, nephropathy and neuropathy in T2DM (Klein 1995). A 6-year Japanese trial carried out to compare intensified and standard insulin treatment in T2DM and the progression of diabetic microangiopathy found a considerable risk reduction with intensive therapy, which was comparable to the DCCT study (Ohkubo et al 1995). In the DCCT, cardiovascular events were also reduced albeit not significantly by 41% in the intensively treated group. Several other studies have also shown a correlation between glycated haemoglobin and the development of cardiovascular disease (Andersson et al 1995; Abraira et al 1995).

Extracellular hyperglycaemia has therefore been conclusively related to the development of diabetic complications. Studies have looked at the cells that are damaged by hyperglycaemia, namely the vascular endothelial cells, and found that there is a reduction in the ability to down regulate glucose transport into the intracellular environment (Giardino et al 1996). Therefore it would appear that intracellular hyperglycaemia is the major determinant of diabetic tissue damage. Intracellular hyperglycaemia is thought to cause tissue damage by repeated acute changes in cellular metabolism and through cumulative changes in long lived macromolecules.

Hypertension and microangiopathy

A second risk factor, which has been implicated in the progression of diabetic complications in particular nephropathy, retinopathy and CHD, is hypertension (Baba 1997; Williams 1997; Mogensen 1999). Hypertension is a very common abnormality in diabetics, and in approximately 90% of patients with elevated blood pressure the cause is unknown. Among those with T1DM the incidence of hypertension rises from 5% at 10 years, to 33% at 20 years, and 70% at 40 years duration of diabetes (Epstein 1992). In the 'World Health Organisation Multinational Study of Vascular Disease in Diabetes' (WHO MSVDD), among T1DM subjects, hypertension (BP $\geq$ 140/90 mmHg or use of antihypertensive drugs) was associated with a relative risk for cardiovascular mortality of 1.7 in men and 2.7 in women at 12 years of follow up (Fuller *et al* 1996; Lee *et al* 2001 [b]). Hypertension has also been highly associated and inter-linked with microvascular complications, for example hypertension is thought to be a risk factor for development of retinopathy, and an aggravating factor of retinopathy in T1DM subjects with nephropathy (Klein *et al* 1989; Jnaka *et al* 1989; Norgaard 1991). An association has also been made between raised arterial pressure and the development and progression of diabetic nephropathy, which has indicated that local intrarenal alteration in haemodynamics or high glomerular capillary pressure may influence diabetic glomerular structural damage (Selby *et al* 1990; Burbury 1998; Agardh and Torffvit 1999). The association of blood pressure levels with progression of renal disease has been investigated in several observational studies. The Multiple Risk Factor Intervention Trial 1982 followed 332,544 men for 16 years and found a strong and independent association between both systolic and diastolic blood pressure and end stage renal disease, with an increased risk with higher blood pressure (Klag *et al* 1996). This was further supported by studies carried out by Perry *et al* 1995 through the Veterans Affairs Hypertension Screening and Treatment Program, and also Shulman *et al* 1989 through the Hypertension Detection and Follow-up Program. Raised blood pressure in patients with diabetic nephropathy was initially considered to be a

consequence of renal failure, however Viberti *et al* 1987 described elevated arterial blood pressure in parents of T1DM patients with proteinuria, which led to the hypothesis that susceptibility to diabetic nephropathy is linked to genetic predisposition to hypertension. Therefore high blood pressure in non-diabetic parents may also be a marker of susceptibility to clinical nephropathy in their T1DM offspring. Fagerudd *et al* 1998 also reported findings that familial predisposition to essential hypertension increases the risk of diabetic nephropathy and may also contribute to the development of systemic hypertension in patients with T1DM and diabetic nephropathy. Fogarty *et al* 2000 carried out a study to estimate heritibility of urinary albumin excretion (UAE) and blood pressure using a large family T2DM collection. The study showed a significant correlation between UAE and blood pressure in the presence of diabetes. These findings cumulatively indicate that high blood pressure and diabetic complications share a common genetic determinant.

Early identification and treatment of hypertension is therefore imperative in diabetic patients to prevent cardiovascular disease and to minimise progression of renal disease and diabetic retinopathy. There is clinical trial evidence in non-diabetic subjects that treating blood pressure decreases the risk of cerebral infarction (Collins 1990) and CHD events in older people (Thijs *et al* 1992; Beard *et al* 1992). Early treatment of hypertensive diabetic subjects should include nonpharmacologic methods, such as weight reduction, exercise, sodium restriction, and avoidance of smoking and excess alcohol ingestion. Several expert groups have made specific recommendations on the management of hypertension in diabetic patients. In accordance to recommendations published in the St Vincent Declaration (Krans *et al* 1995) the threshold for defining hypertension and the point at which clinical intervention using antihypertensive therapy should start is with a blood pressure at or above 140/90 mmHg. Other groups which include the 'American Diabetes Association Consensus Panel' 1993, 'The Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure-IV', and 'The National High

Blood Pressure Education Working Group' 1994 have also published similar recommendations. Intensive drug therapy has been shown to be unequivocally protective in all diabetics (Gall *et al* 1997; Gaede *et al* 1999; UKPDS Group 1998 [a]; Hansson *et al* 1998; Parving 1999; Collado-Mesa *et al* 1999). Parving 1996 [a]; 1999 reported results from a randomised, double blind parallel study, which revealed that the major cardiovascular disease rate was lowered by 34% for antihypertensive treatment compared with placebo. The study also showed that effective blood pressure reduction with ACE inhibitors and/or non-ACE inhibitors reduced albuminuria, delays the progression of nephropathy, postpones end-stage renal failure, and improves survival in diabetic nephropathy. Similar evidence has also recently been shown in the Heart Outcomes Preventative Evaluation (HOPE) study, where the incidence of cardiovascular events in diabetic subjects was reduced when treated with ramipril (Jones *et al* 2001; Patel *et al* 2001). Angiotensin converting enzyme inhibitors (ACE-I) such as ramipril (Patel *et al* 2001) and possibly angiotensin II-receptor blockers (ARBs) (losartan) can be used to lower blood pressure and lower the plasma glucose concentration by increasing responsiveness to insulin. ACE-I protect against the development of progressive diabetic nephropathy by lowering intraglomerular pressure, and appear to lower the incidence of adverse cardiovascular disease. Dietary salt restriction and diuretics are also likely to be effective in hypertensive diabetic patients for prevention of complications. Nondihydropyridine and dihydropyridine calcium channel blocker's such as diltiazem and verapamil, alpha-adrenergic antagonists and beta-blocker's are also commonly used therapeutic treatments for hypertension in diabetics, but the thiazide diuretics and beta-blockers have metabolic side effects which make them less appropriate as first line agents (Barnett 1994). Hovind *et al* 2001 [a] examined whether remission and regression of diabetic nephropathy are possible in T1DM subjects from a prospective observational cohort study, and found that aggressive anti-hypertensive treatment can induce remission and regression in a sizeable fraction of patients with diabetic nephropathy.

Hyperlipidemia and microvasculopathy

It has been suggested that an average or a below average cholesterol level may have an impact on coronary heart disease, and patients with diabetes mellitus are reported to be at an increased risk for cardiovascular disease (Haffner *et al* 1998; Colhoun *et al* 2001). Lipid abnormalities are common in patients with diabetes, and are thought to contribute to the increased risk of cardiovascular disease. In T1DM subjects the DCCT found that poor glycaemic control was associated with hypertriglyceridemia, high serum low-density-lipoprotein (LDL) cholesterol and low high-density-lipoprotein (HDL) cholesterol concentrations (DCCT Research Group 1992; Perez *et al* 2000). In T2DM subjects, insulin resistance, relative insulin deficiency, and obesity are associated with hypertriglyceridemia, low serum HDL cholesterol concentrations, and occasionally high serum LDL cholesterol and lipoprotein values (O'Brien *et al* 1998). The UK Prospective Diabetes Study (UKPDS [a]) showed that high LDL cholesterol and low HDL cholesterol levels were two of several potentially modifiable risk factors of diabetic cardiovascular risk in subjects with T2DM (Davis *et al* 2001).

Cholesterol-lowering intervention through the use of lovastatin which was first introduced in 1987, and other lipid lowering drugs such as simvastatin and atorvastatin which have since become available have been used primarily in the US and Europe in the treatment of hypercholesterolaemia (Pedersen and Tobert 1996; Stein *et al* 1997). Attention has since turned to the advantages of lipid lowering drugs in the treatment of some diabetic patients at risk of diabetic complications (Gall *et al* 1997). Studies have shown that lipid-lowering intervention in non-diabetic subjects decreased the risk of a cardiovascular event in both primary and secondary prevention studies. One such study was 'The Scandinavian Simvastatin Study' (4S) which consisted of 4444 patients with coronary disease and hyperlipidemia. In a subset group consisting of 202 patients with diabetes (mostly T2DM) those who were treated with simvastatin had a lower incidence of major cardiovascular

events (PyörŠIŠ *et al* 1997). Evidence therefore suggests that lipid-lowering therapy is worthwhile for secondary prevention in diabetics although no trials have yet been successfully completed specifically in these patients. One such study which is currently in process is 'The Lipids in Diabetes Study' (LDS), which is a five year, randomised, multi-centre, prospective primary intervention trial. Four thousand T2DM subjects were recruited nation-wide, randomised, and allocated to double-blind therapy with a fixed dose of 3-hydroxy-3-methylglutarly-CoA (HMG-CoA) reductase inhibitor (cerivastatin), and/or fibrate (fenofibrate). The aim of the study is to determine whether statin and fibrate lipid-lowering therapies are effective in preventing cardiovascular disease in people with diabetes. Following the rapid withdrawl of cerivastatin from the market by Baycol (Beyer) as a result of the serious adverse effect (SAE) involving an unacceptably high rate of rhabdomyolysis this has now been replaced with fluvastatin as pravastatin and fluvastatin are least likely to provoke muscle cell damage (Sica and Gehr 2002). The outcome of this study is yet to be determined. However, a recent study carried out over 2 years, as a randomised, double blinded placebo controlled pilot trial of simvastatin/diet vs. diet alone in T1DM subjects with overt diabetic nephropathy suggested that treatment with simvastatin may have beneficial effects on early nephropathy and diabetic neuropathy (Fried *et al* 2001).

Pathophysiological features of cell damage

It has been postulated that diabetic complications occur as a result of the interplay of metabolic and hemodynamic factors in the renal microcirculation (Cooper *et al* 1998; Cooper 2001). Several pathophysiological cellular and extracellular features of diabetic complications exist. This includes capillary basement membrane thickening and associated hemostatic abnormalities. The structural hallmark of diabetic microangiopathy is thickening of the capillary basement membrane in all tissues, including the glomerular basement membrane, resulting in increased capillary permeability, blood flow and

viscosity and disturbed platelet function (Mogensen 1979). The increase in basement membrane is found in the protein components (type IV collagen, fibronectin) and the carbohydrate content. High glucose has been found to up-regulate in a co-ordinated fashion the transcription of genes coding for basement membrane components through effects exerted intra-cellularly or at the cell matrix boundary (Cagliero 1991). Basement membrane thickening could cause vascular dysfunction by several mechanisms. First a decrease of negative charges could change the filtration properties of renal glomerular capillaries. Second, alterations in basement membrane components could affect vascular cell metabolism. Thirdly, non-enzymatic glycation of extracellular protein and its by-products have been reported to affect vascular cell metabolism. Vasodilation and increased blood flow are early characteristic vascular responses to acute hyperglycaemia and tissue hypoxia. In hyperglycaemic tissues these vascular changes are also linked to increased ratio of NADH/NAD+, and mediated by a branching cascade of imbalances in lipid metabolism, increased production of superoxide anion and possibly increased nitric oxide formation (Williamson *et al* 1993).

Brief overview of cellular pathways implicated in diabetes induced complications

Although we now know that the degree of hyperglycaemia is directly correlated to the extent of diabetic complications, we are still trying to elucidate the exact underlying mechanisms by which glucose disrupts cellular metabolism causing hyperpermeability and hemodynamic changes leading to vascular and neural dysfunction. However, it is likely that multiple pathways are involved because the metabolites of glucose can affect many metabolic pathways. It is also indicated from the vast array of literature, that metabolic anomalies contributing to the development of diabetic complications must in some way overlap with each other (Tomlinson 1999). Hyperglycaemia is causally related to a number of ultrastructural, biochemical and haemostatic processes, which culminate in tissue ischemia (reviewed by Barnett 1991, 1993; Ruderman 1992; King 1994; King 1996). The

mechanism by which a lack of glycaemic control predisposes to vascular disease is incompletely understood, however several contributing factors have been proposed (Larkins *et al* 1992). Considerable advancements have been made in our understanding of diabetic complications yet we still do not sufficiently understand the underlying pathogenesis of these complications to allow for effective preventive measures. The reason why only some patients with diabetes develop microvascular complications and not others is unclear, however they are generally thought to result from the interaction of multiple metabolic factors, which are briefly discussed here:

Increase of glucose transporters

High extracellular glucose concentration is known to lead to increased glucose uptake into the mesangial cell. The process by which glucose enters the cell involves facilitative integral membrane glycoprotein glucose transporters, of which several family members have been identified in renal mesangial cells (RMC). Glucose transporters have a low-Km, and a high affinity for glucose, and at physiological glucose concentration would be at or near saturation. It has been shown that increased glucose concentration (20mM) increases the glucose transporter 1 (GLUT1) expression [Heilig *et al* 1997(a); Heilig *et al* 1997(b)]. Experiments carried out by Henry *et al* 1999 reported that increased GLUT1 expression leads to a positive feedback of greater GLUT1 expression, increased AR expression and active PKCα protein levels, which leads to detrimental stimulation of matrix protein synthesis by diabetic mesangial cells.

Non-enzymatic glycation of proteins

Work carried out by Michael Brownlee and co-workers at the Joslin Diabetes Centre as well as other groups, have suggested that accelerated nonenzyme modification of serum and tissue protein macromolecules by glucose and other sugars plays a central role in the pathogenesis of diabetic complications (Brownlee 1991; Brownlee 1994; Vlassara 1996;

Hamada 1996; Hammes *et al* 1998). Initially, under hyperglycaemic conditions, reversible early glycosylation products are formed by non-enzymatic attachment of glucose to amino groups, a well-recognised example of which is the glycated haemoglobin, HbA1c. This attachment result in the formation of covalently bound Amadori products known as early glycation products, which are reversible, if normal glucose levels are restored. However, if hyperglycaemia persists these Amadori products are converted to irreversible advanced glycosylation end products (AGE's) via an Amadori rearrangement. The formation of irreversible AGE's originate from the glycohaemoglobin modification, the 1-amino-1-deoxyketose product, forming at a rate directly proportional to the glucose concentration. The glycohaemoglobin products go on to generate a variety of fragmentation products, particularly highly reactive carbonyl compounds such as 3-deoxyglucosone. It has been shown that the 3-deoxyglucosone reacts either oxidatively or non-oxidatively with different free amino groups to form a heterogeneous group of AGEs, for example the N-(carboxymethyl) lysine (CML) is formed oxidatively, and the imidazolone-type AGE (AG-1) is non-oxidatively formed. It is not yet known how many types of AGE's there are, however AGE localisation in complications varies according to AGE structure (Horie 1997). For example Hammes 1999 [a] reported that there appeared to be differential accumulation of AGE's in the course of diabetic retinopathy, indicating that oxidatively formed N-(carboxymethyl) lysine (CML) increases in diabetic neuroglial and vascular components and imidazolone-type AGE are restricted to microvessels and spread over the entire retina during the later stages of retinopathy.

Evidence for the accumulation of AGE's comes from enzyme linked immunosorbent assay (ELISA) techniques using AGE-specific antibodies which have shown that diabetic renal cortex samples have 10 to 45 times more AGE's than non-diabetic samples after 5 to 20 weeks of diabetes (Mitsuhashi 1993). Publications by Brownlee *et al* 1984, 1992 and 1994 comprehensively reported that three principle mechanisms are involved in accelerated

AGE production and diabetic complications. Firstly, AGE's alter signal transduction pathways by changing the structure and function of the extracellular matrix components such as collagen, vitronectin and laminin. AGE formation on intact matrix also effects biological functions important to normal vascular tissue integrity. Secondly, AGEs alter the levels of soluble signals such as cytokines, hormones and free radicals through interaction with AGE-specific cellular receptors i.e. the receptor for advanced glycation end products (RAGE). It is suggested that interaction of AGE's with their cellular receptors induces procoagulatory changes such as preventing the activation of protein kinase-C pathway, and also induces the increased production of the vasoconstrictor peptide endothelin-1, which together result in focal thrombosis and excessive vasoconstriction. Signal transduction by the AGE receptor also appears to involve generation of oxygen free radicals and leads to oxidant stress that results in potentially damaging changes in gene expression. For example, Galectin-3 (Gal-3) is a multifunctional AGE-binding protein that is associated with other AGE-binding receptor components and it is thought that up-regulation of Gal-3 is associated with diabetic nephropathy. Thirdly, intracellular glycation by glucose, fructose, and metabolic pathway intermediates occurs at a much faster rate than glucose derived extracellular AGE formation, and can directly alter protein function in target tissues, or have deleterious effects on gene expression. Basic fibroblast growth factor (bFGF) is the major AGE modified protein in endothelial cells, and its mitogenic activity is reduced 70% by AGE formation.

Enzymes such as glyozalase I detoxify AGE precursors and prevent AGE formation in endothelial cells and the ability to enzymatically detoxify AGE-intermediates may be genetically determined. Pharmacologic agents that specifically inhibit AGE formation such as aminoguanidine have been used in diabetic animal models, and studies found a considerable reduction of diabetic acellular capillaries (Hammes 1991[b]; Brownlee 1991, 1986, 1995), and AGE accumulation in the renal glomerulus was also prevented (Soulis-

Liparota 1995). The decrease in peripheral nerve conduction velocities in diabetic rats was also prevented by treatment with aminoguanidine (Cameron 1992 [b]). Over the past decade, a great deal of research has gone into studying the efficacy of aminoguanidine in blocking or slowing the progression of diabetes related organ damage. It has been proposed that aminoguanidine acts as a glucose competitor for the same protein bond that becomes the link for formation of AGE's, and also in improving action of nitric oxide (Corbett *et al* 1992). Primary prevention with aminoguanidine has been successfully employed to prevent diabetic retinopathy in the rat (Hammes *et al* 1995). A number of multi-centre clinical trials into the effects of using aminoguanidine in T1DM and T2DM diabetic subjects are in progress (Friedman 1999). Other studies have also looked at the effects of a novel inhibitor of AGE formation, NNC39-0028 (2,3-diaminophenazine), and a breaker of already formed AGE cross links, N-phenacylthiazolium bromide (PTB) in STZ-diabetic female Wistar rats. This study demonstrated that a pharmacological inhibition of collagen solubility alterations in diabetic rats without affecting diabetes-induced pathophysiology such as the increase in UAE or albumin clearance, and also that treatment with PTB had no effect (Oturai *et al* 2000). The prevention of diabetic retinopathy, nephropathy and neuropathy by pharmacological inhibition of AGE formation in animal models suggests that aminoguanidine and other AGE inhibitors have a potential therapeutic role in the treatment of diabetic complication in patients.

Increased flux through the polyol/sorbitol pathway

One of the theories of the proposed effect of hyperglycaemia and microvascular complications involves an increased flux of glucose through the polyol pathway. This pathway is an accessory pathway in glucose metabolism, and was first recognised over 30 years ago (Gabbay *et al* 1966). At elevated glucose levels, glucose metabolism via the polyol pathway accounts for 33% of glucose consumption by the lens and 10% by human erythrocytes (Cheng and Gonzalez 1986; Travis *et al* 1971). This increased flux through

the polyol pathway has been reported to be associated with a decrease in myoinositol uptake, decreased sodium/potassium adenosine triphosphatase activity (NaK-ATP), and increased production of vasodilatory prostaglandins in some target tissues. It is also associated with an alteration of the NADH/NAD+ ratio. It is this pathway that is the main focus of this thesis, therefore its mechanisms have been comprehensively reviewed in chapter 2.

Depletion of myoinositol/Na+/myo-inositol cotransporter (SMIT)

Myo-inositol has been identified as one of the major osmolytes in various tissues and types of cells (Nakanishi *et al* 1988). Animal studies have previously shown that myo-inositol is the only osmolyte found in substantial amounts in the cortex and the outer medulla of the kidney (Wirthensohn *et al* 1989). Under hypertonic stress Madin-Darby canine kidney (MDCK) cells have been shown to accumulate myo-inositol through Na+/myo-inositol cotransporter (SMIT), the transcription of which is also increased in response to hypertonicity (Yamauchi *et al* 1993). A study by Kitamura *et al* 1998 reported that an inhibitor of myo-inositol, 2-O,C-methylene-myo-inositol (MMI) led to extensive injury of the tubular cells of the outer medulla and also in the renal cortex. Further to this administration of myo-inositol was seen to prevent acute renal failure in the rat suggesting that myo-inositol plays a crucial role in osmoregulation of the cell under hyperglycaemic conditions. A depletion of not only myo-inositol but also taurine and other amino acids was observed in the sciatic nerve of a galactosemic rat. Treatment of the galactosemic rats with sorbinil, an AR inhibitor was also found to protect against the loss of myo-inositol, taurine and other amino acids (Nishimura *et al* 1987).

Increased formation of reactive oxygen species (ROS)

Hyperglycaemia increases intracellular reactive oxygen species (ROS), lipid peroxidation and attenuates anti-oxidative mechanisms. Oxidative stress is defined as a tissue injury

induced by increase in ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), and hydroxyl radical ($\cdot OH$) (Baynes and Thorpe 1999). ROS causes strand breaks in DNA, which has been shown to be increased in diabetic subjects and might contribute to the pathogenesis of diabetic complications (Ha et al 1994, 1995; Dandona et al 1996). Hinokio et al 1999 reported increased oxidative DNA damage in diabetic subjects compared to control subjects. The major source of oxidative species in the diabetic state is thought to be from the increase in glucose autoxidation and non-enzymatic glycation (Wolff and Dean 1987; Sakarai and Tsuchiya 1988; Hunt et al 1990; Williamson et al 1993). It has however, also been demonstrated by Lee and Chung 1999 that the flux of glucose through the polyol pathway is also a major source of diabetes associated oxidative stress in the ocular lens. It has been shown that in cells affected by diabetic complications such as aortic endothelial cells, 30 mmol/L glucose increases ROS formation by 250% within 24 hours, and resultant lipid peroxidation by 330% by 168 hours (Nishikawa et al 2000 [a]). This increase in ROS has been shown to be prevented by an inhibitor of the electron transport chain complex II by an uncoupler of oxidative phosphorylation, by uncoupling protein-1 and by manganese superoxide dismutase. A consequent reduction of glucose-induced activation of protein kinase C, formation of advanced glycation end-products, sorbitol accumulation and NF kappa B and AP-1 transcription factor activation were all seen with each of the above agents (Nishikawa et al 2000 [b]). Data has shown that exposure to high glucose concentrations induces an antioxidant defence in skin fibroblasts from normal subjects, in T1DM subjects with nephropathy however, this defensive mechanism is defective. On the other hand it has been shown that T1DM subjects without complications or non-diabetic nephropathic patients have an intact antioxidant response to glucose-induced oxidative stress (Ceriello et al 2000). A pathogenic role of ROS in diabetes is also strongly supported by the observations that antioxidants suppress high glucose-induced extracellular matrix protein synthesis in mesangial cells (Trachtman et al 1993; Trachtman et al 1994; Ha et al 1997). Mitochondria is also a target of oxidative stress consuming approximately 90% of

oxygen, where superoxide anions are produced from electron transport system and are scavenged by an intramitochondrial enzyme manganese superoxide dismutase (MnSOD). The major biochemical pathways of hyperglycaemic vascular damage and hyperglycaemia induced activation of NFkB have recently been shown to result from a single common mechanism; hyperglycaemia-induced overproduction of superoxide by mitochondria (Nishikawa *et* al 2000 [a]; Du *et al* 2000). The formation of reactive oxygen species (ROS) has been proposed to be the single unifying mechanism for the development of diabetic complications. In fact it has been demonstrated that the increased production of ROS serves as a causal link between elevated glucose levels and each of the three major pathways responsible for diabetic tissue damage; activated polyol pathway, PKC and AGE formation (Nishikawa *et al* 2000 [b]). It is suggested that ROS may be an integral component of membrane receptor signalling in mammalian cells. The production of ROS has been detected in various cells stimulated by cytokines, growth factors and transmembrane receptor agonists (Ha and Lee 2000). It has been reported by Hammes *et al* 1999 [a] that monitoring of intracellular concentrations of ROS causes increased oxidative stress in long-lived CD45RA+ lymphocytes by markers such as Nepsilon-(carboxymethyl)lysine possibly identifies a subgroup of patients of high risk for microvascular complications.

Decreased nitric oxide synthase (NOS) activity

Nitric oxide (NO) is a signalling molecule in blood vessels which maintains vasodilation and blood flow and acts as a potent inhibitor of platelet aggregation and adhesion to the vascular wall. Endothelial nitric oxide controls the expression of proteins involved in atherogenesis, decreasing expression of chemoattractant protein (MCP-1) and of surface adhesion molecules such as CD11/CD18, p-selectin, VCAM-1 and ICAM-1. It is also known to have a role in immune defence where activated macrophages synthesise large amounts of NO to destroy microorganisms and cancerous cells. NO is formed by the

oxidation of arginine by nitric oxide synthase (NOS) in endothelial cells (ecNOS), macrophage/inducible (iNOS) or neuronal cells (ncNOS). eNOS is activated by the phosphorylation of serine 1177 by the protein kinase Akt/PKB (Fulton *et al* 1999; Dimmeler *et al* 1999). NO is a highly reactive molecule that can interact with a variety of cellular components. It has been demonstrated that NO can cause damage to the nuclear DNA of beta-cells (Fehsel *et al* 1993). Wilson *et al* 1997 also demonstrated that mtDNA is vulnerable in NO-induced damage. Endothelium dependent vasodilation is impaired in both microcirculation and macrocirculation during acute hyperglycaemia in normal and diabetic subjects (Luscher *et al* 1993; Makimalti *et al* 1996), suggesting that NOS activity may be impaired in diabetic subjects. Decreased Nitric Oxide (NO) production has been associated with the development and progression of diabetic nephropathy in diabetic spontaneously hypertensive rats (Wessels *et al* 1997). It is a distinct possibility that diabetic microangiopathy in humans may be, in part, due to defective endothelial NO production (Anggard *et al* 1994; Snyder and Bredt 1992; Kolb and Kolb-Bachofen 1992). Du *et al* 2001 reported that hyperglycaemia inhibits eNOS activity in cultured bovine aortic endothelial cells (BAECs) by activating the hexosamine pathway via mitochondrial over-production of superoxide, which increases eNOS modification by GlcNAc and decreases eNOS serine phosphorylation. Earle *et al* 2001 investigated T2DM patients of African-Asian and White origin with microalbuminuria under euglycaemic conditions. The study measured glomerular filtration, renal plasma flow and clearance of the stable metabolites of nitric oxide, and found significant differences whereby the percentage clearance of nitrate was higher in the white compared with the African-Asian groups. The differences in the incidence of end-stage renal failure would therefore appear to involve defective nitric oxide production or indeed its bioavailability. Another similar study by Hiragushi *et al* 2001 looked at the relationship between diabetic glomerular hyperfiltration and the NO system in Japanese T2DM patients. Creatinine clearance and urinary NO excretion was measured in T2DM subjects and non-diabetic controls, and found

significantly higher levels of urinary NO in the T2DM subjects. These results suggested that NO might contribute to the pathogenesis of glomerular hyperfiltration in Japanese T2DM patients. Studies using pharmacologic modulators of nitric oxide in proteinuric rats have also yielded interesting findings. Rangan *et al* 2001 investigated in rats, the effect of continuous oral administration of an NO donor (molsidomine), NO precursor (L-arginine), or selective inhibitors of inducible NO synthase (iNOS; aminoguanidine, L-NIL) on the progression of tubulointerstitial inflammation. The study found endogenous NO to have a protective role against tubulointerstitial injury. Ido *et al* 2001 designed experiments using skin chambers mounted on the backs of Sprague-Dawley rats, and exposed an inhibitor of NOS to the granulation tissue. Albumin permeation and blood flow were significantly attenuated implicating nitric oxide in mediating permeability and blood flow changes in the diabetic milieu. Reduced levels of NO may therefore play a key role in the development and progression of microvascular disease in diabetic subjects.

Increase in cell adhesion molecules (CAM)

Increasing evidence indicates that hyperglycaemia increases the formation of free radicals by glucose auto-oxidation, which stimulate cytokine release and consequently induce expression of adhesion molecules (AM) on the endothelial cell surface (Pigott *et al* 1992). AM's are thought to be involved in chemotaxis of circulating monocytes and binding of leukocytes and platelets to endothelium. Raised concentrations of circulating AM's have been shown to exist in a variety of disorders including T1DM. They reflect endothelial activation and stimulation of leukocytes in diabetes and have a potential involvement in diabetes associated micro- and macrovascular disease. A study by Fasching *et al* 1996 showed that higher concentrations of vascular cell adhesion molecule-1 (cVCAM-1) was observed in T1DM subjects with retinopathy (+18%, $P<0.05$), and micro- and macroalbuminuria (+26%, $P<0.05$) than in those without. Similar findings were seen in T1DM patients with nephropathy (Schmidt *et al* 1996 [b]), and also neuropathy (Jude *et al*

1998). Other studies have indicated an increase in expression of VCAM-1 and intracellular adhesion molecule-1 (cICAM-1) on retinal and choroidal tissue of diabetic patients with retinopathy (Tang *et al* 1994; McLeod *et al* 1995). It is therefore possible that elevated concentrations of cVCAM-1 as an indicator of widespread endothelial damage may serve as a risk marker for the presence and progression of diabetic microangiopathy in T1DM. A study by Park *et al* 2000 measured the effect of high glucose on the expression of intercellular adhesion molecule-1 and vascular adhesion molecule –1 in mesangial cells and found that high glucose can up-regulate ICAM-1 but not VCAM-1 through osmotic effect. Hyperglycaemia increased activation of cell adhesion molecules may therefore be an important factor in the development of diabetic microvascular disease.

Alterations of intracellular signalling pathways

It is likely that glucose and its metabolites mediate their adverse effects by altering the various signal transduction pathways, which are used by vascular cells. Several mitogen-activated protein (MAP) kinase signal transduction pathways have so far been characterised. These pathways are activated by multi-step phosphorylation cascades after ligand-cell surface receptor binding, and transmit signals to cytosolic and nuclear targets. The MAP kinases are activated through Ras-dependent signal transduction pathway by hormones and growth factors, leading to cellular proliferation and differentiation by stimulating transcription factors that induce the expression of growth responsive genes (Igarashi *et al* 1999). Intracellular signalling pathways are therefore an important target for studying the diabetic cellular milieu leading to damage. Intracellular signalling pathways are discussed in more detail in chapter 2.

Increased de novo synthesis of diacylglycerol (DAG) and protein kinase C (PKC)

Hyperglycaemia increases the synthesis of diacylglycerol (DAG) by enhancing the metabolism of glucose to DAG precursors through glycolysis. DAG is an important

cellular metabolic regulator in vascular cells, involved in the activation of the protein kinase C (PKC) signal transduction pathway. This elevation of DAG is the presumed mechanism for the elevated protein kinase C activity observed in several tissues obtained from diabetic animals, or exposed in vitro to high glucose concentrations (Lee *et al* 1989; Koya and King 1998). Hyperglycaemic activation of the PKC pathway has been shown to alter several parameters in vascular metabolism and function. Such findings include a decrease in the sodium potassium ATPase activity in peripheral nerves and vasculature through increased phospholipase A2 (cPLA) activity, altered regulation of gene expression of many proteins including those involved in vascular contractility and those found in the basement membrane (Craven *et al* 1990).

Protein kinase C (PKC) is a family of serine-threonine kinases that influence a range of functions including cellular proliferation, blood flow and vascular permeability. A novel pathway for glucose transport has also been suggested involving PKC-λ/ξ (Bandyopadhyay *et al* 2001). Early suggestions of a role for PKC in the pathogenesis of diabetic complications have been made from the results of animal studies in diabetic rats. Studies have found that PKC is activated in glomeruli isolated from diabetic rats (Craven *et al* 1989), and activation of PKC by exposure to a PKC agonist is seen to reproduce the vascular abnormalities induced by diabetes and high glucose levels (Shiba *et al* 1993; Wolf *et al* 1991). Mesangial cells cultured in high concentrations of glucose (27.8 mmol/L) for five days have increased PKC and mitogen activated protein kinase activity (Haneda *et al* 1995), and it is thought that activation of the PKC system is an important pathway involved in diabetic glomerulopathy (DeRubertis and Craven 1994). PKC also increases levels of mRNA encoding matrix components in glomeruli isolated from streptozotocin-induced (STZ) diabetic rats (Ziyadeh *et al* 1995). Activation of PKC has also been found to further activate intracellular signal transduction systems such as extracellular regulated kinase (ERK) forming a DAG-PKC-ERK chain. It is thought that therapies aimed at

lowering the PKC levels may be beneficial and diabetic rats treated with d-alpha-tocopherol, which inhibits PKC activation has been seen to prevent glomerular hyperfiltration and minimise the development of proteinuria (Koya *et al* 1997; Way *et al* 2001). Haneda *et al* 2001 examined the DAG-PKC-ERK pathway and found that thiazolidinedione compounds could inhibit PKC activation by activating DAG kinase. Glucose altered content, cellular distribution and activity of diacylglycerol-sensitive PKC-alpha, -beta, -delta, and –epsilon isoforms has also shown to be dependent on polyol pathway (Kapor-Drezgic *et al* 1999), and could be prevented using aldose reductase inhibitors tolrestat and ARI-509. Insulin treatment also normalises membrane PKC isoforms content (Babazono *et al* 1998). Such findings highlight the importance of the DAG-PKC-ERK pathway in the development of glomerular dysfunction in diabetes. Early trials of PKC inhibition indicate the potential benefits of treatment against end-stage renal disease with PKC inhibitors.

Transcription factor nuclear factor kappa B (NFκB)

Nuclear factor kappa B (NFκB) is a member of the Rel family proteins and is a eukaryotic transcription factor that is involved in mediating the immune response (LeBeauet *et al* 1992; Baeuerle 1998; Brand *et al* 1996). Studies using glomerular mesangial cells have shown that NFκB activation occurs as a response to hypoxia, cytokines, angiotensin II and AGE proteins (Khachigan *et al* 1995). Animal studies have shown higher renal expression of the NFκB system in diabetic animals compared with healthy control animals (Bierhaus *et al* 1997 [a and b). It has also been shown that increased activity of NFκB is present in the peripheral mononuclear cells isolated from T1DM and T2DM patients with diabetic nephropathy (Mohamed *et al* 1999; Hoffman *et al* 1999). Romeo *et al* 1999 found that increased activation of NFκB is the first molecular abnormality detected in situ in retinal pericytes of diabetic donors. Characterisation of the biosynthetic program driven by NFκB in vivo, and its inhibition, will clarify the role of the transcription factor in diabetic

complications. Studies carried out using porcine vascular smooth muscle cells have shown that hyperglycaemia activates NFκB (Yerneni *et al* 1999). This has been shown to be true in ex-vivo isolated peripheral blood mononuclear cells (PBMC's) of T1DM patients, where poor glycaemic control induces the activation of NFκB (Hoffman *et al* 1998). Kumar *et al* 1999 demonstrated that hyperglycaemia-induced activation of NFκB in vascular smooth muscle cells was inhibited by a protein kinase C inhibitor, calphostin. Studies by Hoffman *et al* 1999; Du *et al* 1999 and Bierhaus *et al* 1997 [a] demonstrated that NFκB activation is in part dependent on oxidative stress as α-Lipoic acid was seen to reduce NFκB binding activity (Muller *et al* 1997). It has therefore been suggested that hyperglycaemia induced activation of NFκB may be involved in the susceptibility to diabetic microvascular complications and be a key mechanism for accelerated vascular disease observed in diabetics.

Transcription factor activating protein-1 (AP1)

Activating protein-1 (AP1) is a sequence specific transcription factor involved in regulating the expression of several genes including those involved in mediating growth, inflammation and differentiation. AP1 is reported to be regulated in response to hyperglycaemic conditions and resultant intracellular redox imbalances (Sen and Packer 1996; Nishio *et al* 1998; Natarajan *et al* 1999). Wilmer *et al* 1998 demonstrated increased binding of AP1 to in human mesangial cells (HMC) cultured in high glucose environment (30mM d-glucose). AP1 may therefore be activated in the diabetic state and involved in the development of microvascular disease. Weigert *et al* 2000 have reported evidence that AP1 proteins mediate hyperglycaemia induced activation of the human TGF-B1 promoter in mesangial cells.

Role of growth factors/cytokines in microvasculopathy

High glucose and its various metabolites such as nonenzymatic glycation products and hemodynamic changes can all stimulate the synthesis and release of a host of factors which stimulate proliferation of hypertrophy of cells and the production of extracellular proteins (Wolf and Ziyadeh 1999), some of which are discussed here;

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a heparin-binding homodimeric protein of 46-kDa, and at least five different molecular species with varying amino acid number have been shown to exist (Tisher *et al* 1991). So far four VEGF family members have been identified, VEGF-A, VEGF-B (Olofsson *et al* 1996), VEGF-C (Joukov *et al* 1996) and an endocrine derived ED-VEGF (LeCoute *et al* 2001). VEGF has been shown to be an angiogenesis and vasopermeability-inducing molecule and its expression has been shown to be increased in many hyperproliferative disorders. VEGF is a principle mediator in diabetic retinopathy and also appears to play a central role in diabetic vasculopathy in many other organs, although its precise involvement is not understood. VEGF expression is increased in mesangial cells cultured in high glucose media, and glomerular VEGF levels and urinary VEGF are increased in patients with diabetes (Aiello and Wong 2000).

VEGF mediates its action through transmembrane autophosphorylating tyrosine kinase proteins of high VEGF binding affinity. These receptors are expressed predominantly in endothelial cells although they have been identified in renal mesangial cells, monocytes, hematopoietic cells and the retina. Evidence suggests that nitric oxide mediates some of VEGF's mitogenic effects on coronary endothelial cells and that VEGF mRNA expression is dramatically regulated by oxygen tension, with markedly increased VEGF gene expression under hypoxic conditions (Shweiki *et al* 1992). It is suggested that VEGF might regulate neovascularisation in hypoxia induced diabetic retinopathy leading to increased

retinal vascular permeability and new retinal vessel growth. In animal models specific inhibition of VEGF by soluble VEGF receptor chimeric proteins, reduced neovascularisation in 95% to 100% of animals tested (Aiello *et al* 1995). Clinical studies have also confirmed that there is elevated VEGF concentrations in vitreous and aqueous samples of eyes in patients with proliferative retinopathy (Aiello *et al* 1994). Tilton et al 1999 showed that intravenous infusion of VEGF can acutely impair endothelial cell barrier functional integrity and relax resistance arterioles in ocular tissues and brain. Inhibition of VEGF action might therefore provide novel therapeutic approaches to diabetic vasculopathy.

Transforming Growth Factor Beta (TGF-β)

Cellular signalling pathways involved in Transforming Growth Factor Beta (TGF-β) induced renal injury are being extensively investigated. TGF-β is activated by the extracellular signal-regulated kinase (ERK) pathway and also p38 mitogen activated protein kinase (MAPK) system. TGF-β has been shown to have antiapoptotic effects in macrophages, and is implicated in type 1 collagen synthesis in mesangial cells, and induces fibronectin and plasminogen activator inhibitor (PAI-1) expression in mesangial cells. TGF-β also down regulates inositol 1,4,5-triphosphate (IP3) receptors which may also mediate vascular dysfunction in diabetes. Down regulation of ECM degradation is thought to be through a TGF-β dependent mechanism through glucose induced down regulation of matrix metalloproteinases (MMP) which may contribute to the pathogenesis of diabetic glomerulosclerosis. Lovastatin, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor has been shown to suppress glomerular TGF-β expression and ameliorate diabetic nephropathy in STZ induced diabetes in rats (Del Prete *et al* 1998; Lee *et al* 2000).

Other growth factors and chemokines

A whole variety of growth factors, cytokines, chemokines and vasoactive agents which have not already been mentioned have also been investigated and implicated in the pathogenesis of diabetic microvasculopathy. Such agents include; the cytokines- platelet derived growth factor-B (PDGF-B) (Kasuya *et al* 1999 [a]), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF); the chemokines- interleukin-8 (Il-8) and monocyte chemotactic peptide-1 (MCP-1); and the vasoactive substances, angiotensin II (AngII), andothelin-1 (ET-1) and the prostanoides such as thromboxane (Flyvberg *et al* 1998; Abboud 1997). These factors will however not be discussed in detail as they are beyond the scope of this thesis.

Interactions between hyperglycaemia altered pathways

Many lines of evidence indicate that there is a multifactoral pathogenesis of diabetic complications, and that several pathways are affected by diabetes, which lead to the onset and progression of microvascular disease. These pathways are thought to be interconnected and overlap with each other as illustrated in figure 3. It has been shown that increased sorbitol pathway metabolism and non-enzymatic glycation products are involved in the pathogenesis of vascular and neural dysfunction associated with diabetes. A study carried out by Ido 1996 using animal models looked at the vascular dysfunction induced by elevated glucose levels, and found that increased blood flow and increased albumin permeation was reduced by inhibitors of the sorbitol pathway, nitric oxide synthesis and of prostaglandin synthesis respectively. These findings suggest that the vascular dysfunction induced by increased sorbitol pathway metabolism and by products of non-enzymatic glycation are mediated by a common final pathway. Increased superoxide production is important in mediating vascular dysfunction and it is likely that increased flux of glucose via the sorbitol pathway increases superoxide production as a consequence of hypoxia like reductive stress resulting from reduction of NAD+ to NADH coupled to oxidation of

sorbitol to fructose. The final common pathway between the various biochemical abnormalities would therefore appear to involve the increase in oxidative stress (Cappiello 2000).

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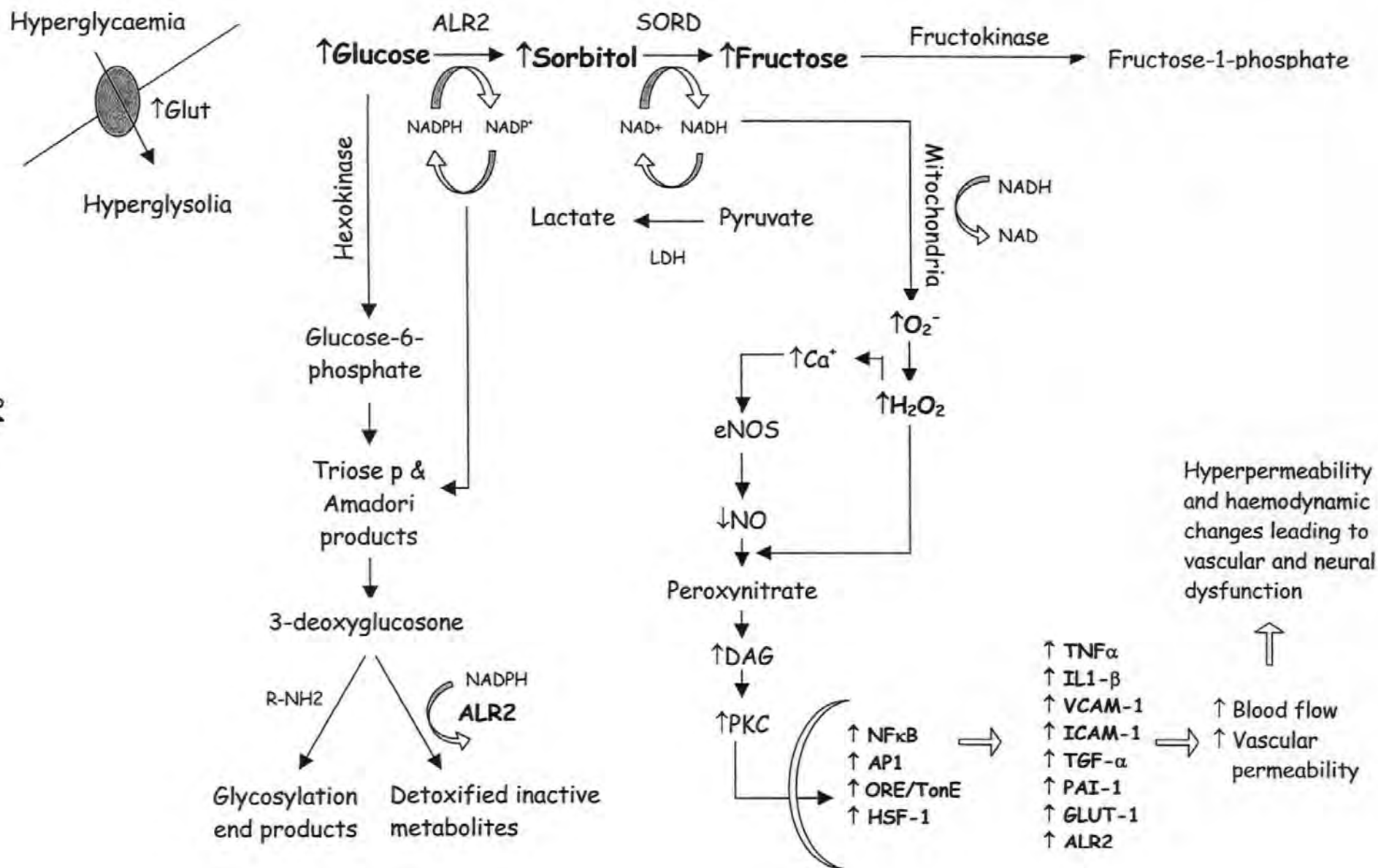
Figure 3. Diagrammatic representation of metabolic perturbations induced by increased flux through the polyol pathway as a result of hyperglycaemia.

Genetic susceptibility to diabetic complications

There is substantial evidence to support the involvement of genetic factors in the development and progression of certain diabetic microvascular complications. This evidence comes from genetic epidemiological studies of diabetic complications (Rich *et al* 1997). Molecular characterisation of genetic susceptibility is imperatively important and would not only increase our understanding of the pathogenesis of diabetic complications but would also provide diagnostic tools for identifying susceptible patients so that they could be targeted for intensive control of hyperglycaemia. Such information would also assist in the development of new preventative and therapeutic programs. Possible genetic models for the interaction between the diabetic milieu and susceptibility to complications have been proposed where genes may have a major or minor effect. These include, an additive genetic model where the genetic influence serves to regulate the progression of the disease, an interactive model where genes in conjunction with poor glycaemic control lead to the development of disease, and a mixed model where some genes influence development whereas other genes influence progression of disease (Krolewski *et al* 1992; Krolewski 1999). The suggestion that there is an interaction between genes could also help towards explaining why some patients with well-controlled diabetes still develop complications, whereas there are others with poorly controlled diabetes who 'escape' any complications. This may also suggest that it will be possible to predict the beneficial effect of different targeted therapeutic intervention (Cooper 1998). However despite promising findings that suggest a role for various genetic factors in the pathogenesis of complications, genetic screening cannot yet be considered appropriate as part of routine clinical practice in diabetic patients.

Genetic epidemiology of nephropathy

Transracial studies (Cowie *et al* 1989; Burden *et al* 1992), family studies (Seaquist 1989; Pettitt *et al* 1990; Borch-Johnsen 1992; Quinn *et al* 1996; DCCT 1997; Fioretto *et al* 1999)

and large epidemiological studies (Anderson *et al* 1983) have all shown marked differences in the incidence of diabetic nephropathy. These studies collectively indicate that only a subgroup of diabetic subjects (33%) are susceptible to diabetic nephropathy, that there is familial aggregation for renal disease, and that there are marked inter-racial differences in the disease prevalence, all indicating a genetic basis for nephropathy.

A large epidemiological study consisting of 1475 T1DM subjects diagnosed before 1953 and before the age of 31 years was conducted, all patients were diagnosed with diabetes at the Steno Memorial Hospital, Denmark (Anderson *et al* 1983). Patients were followed until 25 years duration of diabetes or until death. The study found that 41% of patients developed nephropathy, 3% had proteinuria due to other causes and 57% did not develop persistent proteinuria. Variation has been shown to exist in the prevalence of diabetic nephropathy in different racial groups. Asian, Hispanic, Native American or Afro Caribbean ethnic origin have increased rates of diabetic renal disease as compared to matched groups of Caucasoid patients (Pugh 1988; Cowie 1989; Collins 1989; Pettitt 1990; Reddan *et al* 2000; Bennett *et al* 2001). A trans-racial study that looked at the incidence of end-stage renal disease in diabetic subjects found an increased incidence in African-Americans compared to Caucasoids (Brancati *et al* 1992). The family study carried out by Seaquist *et al* 1989 demonstrated an increase in the incidence of diabetic nephropathy in siblings of T1DM probands with nephropathy compared to siblings of normoalbuminuric T1DM subjects, concluding that nephropathy occurs in familial clusters. Similar findings were also reported from a study carried out by Borch-Johnsen 1992 who found a significant increase in renal complications in T1DM siblings of probands with nephropathy than in T1DM diabetic siblings of probands without nephropathy. Studies in Pima Indians with T2DM showed that there was familial aggregation for proteinuria among diabetic siblings and that nephropathy was observed in 46% of diabetic offspring if both patients were proteinuric, 23% if one parent had proteinuria and 14% if neither parents had

proteinuria (Pettit *et al* 1990). Further evidence for familial aggregation of nephropathy was found in a study of siblings with T1DM. This study found that for probands and siblings combined the cumulative incidence of advanced diabetic nephropathy after 30 years duration of diabetes was 35%, however if the proband had persistent proteinuria the risk to the sibling rose to 71.5% (Quinn *et al* 1996). In 1997 the DCCT research group published the results of a large study investigating familial clustering of diabetic microvascular complications. The study aimed to determine familial associations and clustering of the severity of complications. The data with respect to nephropathy suggested that there was an increased risk of nephropathy in relatives of nephropathy positive versus nephropathy negative subjects and suggested that possible genetic factors influenced the development of diabetic nephropathy (DCCT 1997). A study by Fioretto *et al* 1999 examined the lesions of diabetic nephropathy among T1DM siblings and determined that there was strong concordance for the severity and patterns of glomerular lesions among the sibling pairs, suggesting familial concordance in the risk for nephropathy. Complex segregation analysis has been performed in diabetic members of Pima Indian families to determine whether familial aggregation of nephropathy reflects the action of a single major gene. Analysis of prevalent cases in this study supported evidence for the existence of a gene with a major role in the susceptibility to diabetic nephropathy in Pima Indians. This was shown where-by the hypotheses of no major effect and of no transmission of a major gene effect were rejected (P=0.00001; P= 0.003) (Imperatore *et al* 2000). These observations suggest that one or more hereditary causes may be involved in the pathogenesis of this complication, and that the proposed difference in risk suggests a major gene effect.

Genetic epidemiology of retinopathy

The DCCT 1997 report investigating familial clustering of diabetic microvascular complications indicated that the risk of severe retinopathy in the relatives of retinopathy

positive versus negative subjects was statistically significant. Correlation's for the severity of retinopathy which were computed from log-adjusted retinopathy scores were 0.187 (all family members), 0.327 (parent-offspring), 0.249 (father-child), 0.391 (mother-child), and 0.060 (sib-sib) and all were statistically significant (P<0.05). The WHO Multinational Study of Vascular Disease in Diabetes (WHO MSVDD) designed a study to compare the vascular complications of diabetes in different ethnic groups which included an estimate of visual function (Diabetes Drafting Group 1985; Miki *et al* 2001). This provided the opportunity to ascertain incidences and progression of visual impairment and its risk factors, which were found to include baseline systolic pressure and cholesterol levels. In the United States and the UK approximately 12% of all new blindness was attributable to diabetic retinopathy (Klein 1995). The prevalence rate in the WHO MSVDD study showed significant variation of visual impairment prevalence rates among centres with high total rates, American Indian Oklahoma (33.4%) and Pima (26.3), contrasting with lower European rates. These data provide evidence that the severity of diabetic retinopathy is influenced by familial (possibly genetic) factors (DCCT 1997), and to some degree ethnicity.

Genetic epidemiology of neuropathy

Diabetic neuropathy is the most common form of peripheral neuropathy in the western world and is a common complication in both T1DM and T2DM subjects. Much of the data on prevalence and incidence of neuropathy comes from the Rochester Diabetic Neuropathy study which was a population based longitudinal study of participants from Rochester, Minnesota (Dyck *et al* 1991). This study revealed that the prevalence of neuropathy did not differ greatly between T1DM (66%) and T2DM (59%) subjects or by clinical subtype. Another study, the San Louis Valley Study found a much lower prevalence of neuropathy (28%) in T2DM subjects of Anglo and Mexican/American heritage, although there were no indications of ethnic difference in incidence (Franklin *et al* 1990). The failure of studies

such as the San Louis Valley study to observe ethnic differences indicated that genetic effects do not play a major role in the aetiology of neuropathy as ethnic differences in disease are often a hallmark of genetic effects. Therefore, the observed factors such as duration of diabetes, hyperglycaemia and hypertension are suggested to may play a major role in neuropathy risk (DCCT 1993 [a and b]).

Genetic epidemiology of macrovascular disease

Epidemiological data indicate that diabetes and a familial predisposition are independent risk factors for cardiovascular disease. Research has identified evidence of familial aggregation of early coronary heart disease (Hopkins *et al* 1988), and a familial correlation for CHD risk factors (Glueck *et al* 1974). Excess mortality from stroke among diabetic patients is at least two-fold compared with the non-diabetic population (Garcia 1974). In the Honolulu Heart Program, the rate of thromboembolic stroke for diabetic men was 62.3/1,000 vs. 32.7/1,000 for non-diabetic men (Abbott *et al* 1987). In the Framingham Study the risk of stroke for T2DM subjects was 2.6 fold greater for men and 3.8 fold greater for women than for non-diabetic individuals (Stokes *et al* 1987). Tarnow *et al* 2000 has shown that cardiovascular morbidity and early mortality clusters in parents of T1DM subjects with nephropathy.

The search for susceptibility genes

The positional cloning of multifactoral disease genes is a major challenge in human genetics. Several forms of genetic mutations are known to exist and are identifiable using various molecular genetic techniques. Genetic variations include single nucleotide polymorphisms (SNPs), dinucleotide repeats and microsatellites. Several of the polymorphisms are located in the promoter region of the gene and affect transcription or translation, and not infrequently determine the level of expression of the protein product. Single nucleotide polymorphisms involve the substitution of one nucleotide for another,

and some can be identified using restriction endonuclease digestion. As a consequence of the Human Genome Project effort is underway to develop a dense set of biallelic markers (SNP's) throughout the human genome (Collins *et al* 1998). Technology to detect SNP's includes the use of a gene chip, a glass wafer, to which is bound high-density arrays of pre-pooled primers for multiplex PCR assays. It is anticipated that up to 100,000 SNP's scattered throughout the genome will become available (Wang 1998). The finishing of human genome sequence determination, improvements in informatics and large-scale identification of SNP's will undoubtedly increase the feasibility of identifying polymorphisms that predispose to diabetic complications (Johnson and Todd 2000). Dinucleotide repeats consist of continuous repeating units of a dinucleotide sequence, for example (CA)n, and similarly Variable Number Tandem Repeats (VNTR) consist of repeating units of more than two base pairs. Microsatellites are tandemly repeated arrays of one to six nucleotides and these sequences are both ubiquitous and highly polymorphic. Microsatellites are repeated 10-60 times and they are dispersed throughout the genome on average every 10^5 bp (Weber and May 1989; Love 1990). When they are mutated they are also an important cause of human disorders. Microsatellite markers possess high levels of allelic variation so that parental chromosomes often bear different alleles enabling chromosomes to be tracked from parents to affected offspring. Studies in a wide variety of systems have shown that replication slippage is the predominant means by which the number of repeats in a microsatellite array changes, and that alterations most commonly consist of insertion or deletion of one or two repeat units (Schlotterer and Tautz 1992). Microsatellite mutation rates are increased in cells deficient for mismatch repair (Strand *et al* 1993). It is suggested that alteration in repeat number occurs in distinct classes (rare, large changes and common, single or two step ones) (Freimer and Slatkin 1996). It is also possible that there is a constraint on the mutation process that would cause allele size to remain within a certain limit. Valdes *et al* 1993 showed that there was no obvious relationship between the variance in repeat number, which should be proportional to the

mutation rate, and the average allele size. Microsatellites are flanked by unique sequences to which PCR primers can be designed to allow amplification of the microsatellite region. Variations in the length of flanking sequences could affect the overall size of the PCR product lengths and obscure a relationship between the mean and the variance in allele size. A number of lines of evidence indicate that the total length of the microsatellite may have less of an effect on the mutation rate than the length of uninterrupted or perfect repeats. Direct sequencing data through semi-automated mapping has now become a widely used technique in analysing microsatellite polymorphisms (Reed *et al* 1994).

Whole genome scanning for susceptibility loci

A whole genome scan is the analysis of about 100 evenly spaced markers in mouse pedigrees, or about 300 markers in human families in which there are affected and non-affected progeny. The aim is to identify chromosome regions that are inherited by affected progeny more often than expected from Mendelian random segregation. Using large data sets, a map with dense markers, and multipoint programs, which make the map even more informative, can reduce false positive linkage results. One approach to detect linkage of genes has been based on the squared sib pair trait difference that is regressed on the estimated proportion of marker alleles shared by descent. The test for linkage is then based on the significance of a negative slope. For all pairs of affected concordant, unaffected concordant and discordant sib-pairs, the mean proportion of marker alleles shared can be estimated. The power of the sib-pair method depends on the proportion of the phenotypic variance due to segregation at the major locus and the sample ascertainment (Risch *et al* 1995). This strategy of whole genome scanning has been applied to screening for determinants of microvascular disease related to diabetes. For example Imperatore *et al* 1998 [a and b] used sib-pair linkage analyses to identify loci influencing susceptibility to microvascular complications in Pima Indians with T2DM. The study suggested that a genetic element on chromosomes 7 and 20 may influence susceptibility to nephropathy and

a genetic element on chromosomes 3 and 9 may determine susceptibility to both nephropathy and retinopathy. In this study chromosome 7 showed the strongest evidence for linkage, and contains the candidate gene aldose reductase (ALR2). A further study using T1DM sib-pairs discordant for diabetic nephropathy found a susceptibility locus on chromosome 3q which contains the candidate genes involved in angiotensin II sub-type 1 receptor (AT1R) (Moczulski *et al* 1998).

Fine mapping of susceptibility loci

A disease mutation will be flanked by microsatellite markers with certain alleles which can be typed and provide a recognisable signature to the chromosome region containing the disease mutation. If a marker is very close, within 1 centimorgan (cM) (Jorde 1995) to a disease mutation, then despite opportunities for recombination during meiosis between the marker and the disease mutation, there will still be a strong chance that the marker and the mutation will be in linkage disequilibrium. In regions of the genome that show some evidence of linkage to disease detected in the genome scan testing markers every centimorgan should yield a marker that shows linkage disequilibrium with disease. A study carried out by Herr *et al* 2000 empirically tested the utility of the available polymorphic microsatellite map to locate the already identified T1DM locus IDDM1. Allelic association of each of 13 evenly spaced polymorphic microsatellites markers was carried out using 385 affected sib-pair families. The study demonstrated that fine mapping of a multifactoral disease gene is possible with high accuracy even in a region with high linkage disequilibrium. By characterising new markers in the 1cM region flanking the disease associated marker, the disease mutation can be located to regions smaller than 1cM (Vyse and Todd 1996). The candidate gene approach in detecting disease mutation has to some extent dominated the genetic analysis of diabetic complications. However, candidate genes are numerous and have in many cases been reported but not confirmed.

Candidate genes investigated in diabetic complications

The candidate gene approach in detecting susceptibility genes for diabetic complications has also proved to be fruitful. This strategy has so far focused on target tissues that are susceptible to diabetic complications and biochemical pathways affected by altered glucose metabolism. The search for candidate genes has so far involved searching gene polymorphisms of the enzymes that drive glucose metabolism and also gene polymorphisms affecting background vascular risk (Marre 1999). Studies have looked at polymorphisms within candidate genes involved in the pathogenesis of complications and have found certain genotypes to have a progression promoter effect (table 4). Such studies include human leukocyte antigen (HLA) loci, genes of the renin-angiotensin system, polyol/sorbitol pathway, lipid metabolism and glucose transporter genes (Chowdhury *et al* 1995; Doria *et al* 1995; Doria *et al* 1998; Krolewski and Warram 1995 [b]; Chowdhury *et al* 1999 [a and b]; Marre *et al* 2000). It is thought to be worthwhile testing polymorphisms within each component of these various regulatory systems to determine their possible role in the development of diabetic complications in the event that gene polymorphisms are associated with variable levels of expression of the concerned protein.

Human Leukocyte Antigen (HLA) gene polymorphisms

Several groups, with somewhat conflicting results have extensively examined the human leukocyte antigen (HLA) locus and the insulin gene locus. Both positive and negative associations have been made for HLA A2, B8, DR4 and DR3/4 and diabetic nephropathy and retinopathy (Barbosa and Saner 1984; Stewart *et al* 1993). Previous examination of HLA identical non-diabetic siblings of T1DM probands showed basement membrane expansion, which was not observed in HLA non-identical siblings, which suggested that even in the absence of diabetes microangiopathy may occur due to HLA-related factors (Barbosa *et al* 1980). However, other association studies of HLA and insulin gene loci in large cohorts of T1DM subjects with and without nephropathy found no such associations.

It has therefore been suggested that the HLA and insulin gene loci are unlikely to have a major role in the susceptibility to nephropathy in Caucasoid T1DM subjects (Chowdhury *et al* 1998; Chowdhury *et al* 1999 [b]).

Receptor for advanced glycation end products (RAGE) gene polymorphisms

AGE's can bind to several binding sites including receptor of advanced glycation end-products (RAGE). Kankova *et al* 2001 investigated polymorphisms in the RAGE gene (G82S, 1704G/T, 2184A/G and 2245G/A) in T2DM subjects and found statistically significant differences in allelic frequencies between T2DM subjects and non-diabetic controls. Hudson *et al* 2001 [a] carried out a study of the –429 t/c and –374 t/a RAGE promoter polymorphisms in diabetic and non-diabetic subjects with macrovascular disease. The study evaluated the effects on transcriptional activity of functional polymorphisms and suggested that polymorphisms involved in differences in RAGE gene regulation may influence the pathogenesis of diabetic vascular complications.

Renin-angiotensin system (RAS) gene polymorphisms

Studies have demonstrated that a family history of hypertension or cardiovascular disease in association with poor glycaemic control greatly increases the risk of nephropathy in both T1DM and T2DM patients (Earle *et al* 1992; Barzilay *et al* 1992; Takeda *et al* 1992). Genes of the renin-angiotensin system (RAS) have therefore also been targeted as potential candidate genes for nephropathy. Prorenin, renin, angiotensin converting enzyme (ACE) and angiotensin II levels have all been reported to be elevated in diabetic nephropathy, and are thought to be genetic determinants for hypertension and cardiovascular disease (Parving *et al* 1996 [b]; Hallab *et al* 1992). The angiotensinogen gene (AGT) has also been reported to contribute to susceptibility to complications in some affected sib-pair studies (Jeunemaitre *et al* 1992; Caulfield *et al* 1994), and family based studies (Rogus *et al* 1998). Studies of the Insertion/Deletion polymorphism within intron 16 of the angiotensin I-

converting enzyme gene (ACE/ID) have found conflicting results. One positive study carried out by Doi *et al* 1996 found a significant correlation between the ACE gene polymorphism and nephropathy, but not retinopathy in Japanese patients with T2DM. On the other hand Utsugi *et al* 1999 found that the ACE polymorphism could be a risk factor for both nephropathy and retinopathy in Japanese patients with T2DM. This study suggested that patients with the D allele were at a higher risk of severe retinopathy than subjects without. Tarnow *et al* 2000 carried out a study using T1DM subjects from Denmark and found evidence for a protective effect of the homozygous II genotype and myocardial infarction. However studies have cumulatively shown the association of the ACE/ID polymorphism with nephropathy to be small, and consequently it is not thought to be a useful marker of nephropathy (Doria *et al* 1994; Marre *et al* 2000). The EURODIAB study has however shown that the ACE gene polymorphism can predict the beneficial effect of ACE inhibition (Penno *et al* 1998), where renoprotective therapy has been seen to be differentially effective according to ACE genotype (van Essen *et al* 1996). A further polymorphism of the angiotensin gene (M235T) has also initiated interest and has been shown to be in linkage with essential hypertension (Caulfield *et al* 1994). However, conflicting results have been seen in association with diabetic nephropathy (Fogarty *et al* 1996; Chowdhury *et al* 1996; Schmidt *et al* 1996 [a]; Ittersum *et al* 2000) and it was reported by Doria *et al* 1996 that the angiotensinogen polymorphism M235T might influence susceptibility to nephropathy in T1DM subjects, but its effect, if any, is small and independent of hypertension. A polymorphism of the angiotensin II type 1 receptor gene (A1166C) has also been linked to essential hypertension (Bonnardeaux *et al* 1994), and microalbuminuria (Doria *et al* 1997), although two large studies into diabetic nephropathy have suggested no association (Chowdhury *et al* 1997; Tarnow *et al* 1996).

Genetic studies of hereditary hypertension in the rat, and genome-wide scans have identified several loci involved in blood pressure regulation (Hilbert *et al* 1991; Jacob *et al*

1991; Dubay *et al* 1993; Pravenec *et al* 1995; Schork *et al* 1995; Brown *et al* 1996). Brown *et al* 1996 studied crosses involving the fawn-hooded rat, an animal model of hypertension that develops chronic renal failure to determine if there are susceptibility genes involved. They reported the localization of two genes, Rf-1 and Rf-2 responsible for about half the genetic variation of renal impairment. They found that Rf-1 strongly affects the risk of renal impairment, but has no significant effect on blood pressure. Their results suggested that susceptibility to a complication of hypertension is under at least partially, independent genetic control from susceptibility to hypertension itself. One of the principle loci identified in spontaneously hypertensive stroke-prone (SHR/SP) and Dahl salt-sensitive hypertensive rats has been localised near the angiotensin 1 converting enzyme (ACE) on rat chromosome 10 (Nara *et al* 1991; Deng and Rapp 1992). Based on comparative mapping data, the human homologues of the corresponding gene should reside within human chromosome 17, which contains the ACE gene. Julier *et al* 1997 carried out a study using affected sib-pair human hypertensive families to explore the whole homology region for a putative susceptibility locus for hypertension. Using microsatellite markers the study found nominal evidence of linkage to hypertension, from which the strongest markers were D17S183 and D17S934, that map 18cM proximal to the ACE locus. Baima et al 1999 used stroke-prone spontaneously hypertensive rat models to investigate linkage to blood pressure regulatory genes, and found evidence for an association with rat chromosomes 2, 10 and X, whereby rat chromosome 10 is syntenic to human chromosome 17. The study investigated five microsatellite markers spanning the chromosome 17 blood pressure quantitative trait loci (QTL), spanning a 12cM region and found significant evidence for linkage in a 5-cM interval between D17S946 and D17S932. Levy *et al* 2000 used a genome wide scan approach and found strong evidence for a blood pressure (BP) quantitative trait locus on chromosome 17. These results indicated that chromosome 17q could contain a susceptibility locus for human hypertension (Dominiczak *et al* 2000).

Aldose Reductase (ALR2) gene polymorphisms

The enzyme aldose reductase has been implicated in a number of microangiopathic complications of diabetes. Using the strategy to search for gene polymorphisms of the enzymes that drive glucose metabolism, a dinucleotide repeat polymorphism was found at the 5' end of the aldose reductase gene. Certain studies have found the polymorphism to be associated with early onset of retinopathy and also nephropathy (Heesom *et al* 1997; Demaine *et al* 2000) in diabetics. Other polymorphisms have also been identified and include a C to T polymorphism –106bp upstream of the aldose reductase start site, and an A to C polymorphism within intron 8 of the ALR2 gene itself. It is possible that aldose reductase, an enzyme able to affect glucose metabolism within target tissues of diabetic microangiopathy may effect microvascular prognosis for T1DM patients as a result of effects on variable, genetically determined levels of its activity. Polymorphisms and altered genetic expression of the aldose reductase gene form the main thread of this thesis and are therefore discussed in more detail in chapter 2.

Glucose Transporter (GLUT) gene polymorphisms

A recent study carried out by Hodgkinson *et al* 2001[a] reported that a polymorphism within the glucose transporter 1 (GLUT1) gene was significantly associated with susceptibility to diabetic nephropathy in Caucasoid T1DM subjects, this was also found to be in association with the 5'ALR2 polymorphism. The glucose transporters mediate the facilitative uptake of glucose into cells and within the glomeruli of the kidney the GLUT1 is known to be the most important (Heilig *et al* 1995; Heilig *et al* 1997 [b]). The results of this study suggest that those individuals with the 1.1 kb GLUT1 allele and the Z-2 5'ALR2 allele would be at a much higher risk of developing nephropathy than those without.

Apolipoprotein E (APOE) gene polymorphisms

Studies into the genes affecting lipid metabolism have shown a positive association of the $\in 2$ allele of the apolipoprotein E gene polymorphism, this is a particularly significant finding as physiological studies have implicated lipid abnormalities in the pathogenesis of diabetic nephropathy (Araki *et al* 1998; Chowdhury *et al* 1998). It has been suggested that T1DM subjects with the $\in 2$ allele have a reduced creatinine clearance and elevated albumin excretion rate compared to subjects without the $\in 2$ allele (Werle *et al* 1998).

Endothelial nitric oxide synthase (eNOS) gene polymorphisms

Polymorphisms in the endothelial nitric oxide synthase (eNOS) gene may be implicated in the development of nephropathy in patients with T1DM. A study by Zanchi *et al* 2000 looked at four eNOS polymorphisms and found that two were associated with diabetic nephropathy in a case-control comparison and also a family based transmission disequilibrium test (TDT). These were a T to C substitution in the promoter at position – 786 and the a-deletion/b-insertion in intron 4. Their findings demonstrate that sequence differences in eNOS influence the risk of advanced nephropathy in T1DM. Asakimori *et al* 2001 also investigated the intron 4 polymorphism of the ecNOS gene and found a higher frequency of the a-allele of intron 4 in both non-diabetic and diabetic hemodialysis patients, although this was not supported in a study using T2DM subjects (Taniwaki *et al* 2001).

Plasminogen Activator Inhibitor-1 (PAI-1) gene polymorphisms

Plasminogen activator inhibitor type 1 (PAI-1), is an inhibitor of fibrolysis and an important and independent cardiovascular risk factor which has been shown to be elevated in obesity and T2DM (Mertens *et al* 2001). The 4G/5G polymorphism in the promoter region of the PAI-1 gene has been related to plasma levels of PAI-1, the main inhibitor of fibrinolysis. PAI-1 is higher in T1DM subjects with microalbuminuria. Pucci *et al* 1999

investigated this polymorphism in 375 Caucasoid T1DM subjects and found that the presence of the 4G/4G genotype to be associated with a higher risk of proliferative retinopathy, but not with a higher risk of raised AER.

Paraoxonase gene (*PON*) polymorphisms

There is increasing evidence to suggest that paraoxonase (*PON*) protects tissues from oxidative stress. *PON* is an enzyme, which is exclusively bound to high-density lipoprotein (HDL), and it has been shown by in vitro studies that it protects low-density lipoprotein from oxidative stress. It has been shown that the serum activity and concentration of *PON* is lower in subjects with cardiovascular disease than those without (Heinecke *et al* 1998; Sanghera *et al* 1998). Two separate studies by Araki *et al* 2000 in T1DM subjects and by Ikedo *et al* 1998 in T2DM subjects looked at three common polymorphisms of PON-gene-1. These polymorphisms, namely T(-107)C in the promoter, Leu 54Met and Gln192Arg have previously been associated to cardiovascular disease (Ruiz *et al* 1995; Garin *et al* 1997). The studies did not find any significant relationship between any of these three polymorphisms and the development of nephropathy in either T1 or T2DM patients. Another study however, did find an association between diabetic nephropathy and the Gln191Arg polymorphism (Jenkins *et al* 2000). The human genome contains two other similar genes to *PON1* (*PON2* and *PON3*), located on chromosome 7 and it is hypothesised that these should also be considered as candidate genes for the susceptibility to diabetic nephropathy. Pinizzotto *et al* 2001 has reported an association between diabetic nephropathy in T2DM subjects and two polymorphisms in *PON2* that cause amino-acid substitutions (Ala148Gly and Ser311Cys). This correlation was however, not supported in a further study carried out by Canani *et al* 2001 using T1DM subjects. Thus concluding that the *PON2* polymorphisms are associated with diabetic nephropathy exclusively in subjects with T2DM, or that the results are spurious and require further studies in different populations.

Nuclear transcription factor B (NFkB) polymorphisms

The gene coding for NFkB located on chromosome 4q24 has been shown to be up regulated under hyperglycaemic conditions in porcine vascular smooth muscle cells and PBMC's from diabetic subjects with nephropathy. A polymorphic dinucleotide (CA) repeat has been identified close to the human NFkB gene (Ota *et al* 1999). A recent study by Hegazy *et al* 2001 reported that the A10 allele may contribute to susceptibility to T1DM, however no association was found with the NFkB allele or genotypes with either presence or absence of diabetic microvascular complications in T1DM subjects. It is still possible that there may be a minor gene effect with NFkB and diabetic complications, and further studies in different populations are required.

Vascular endothelial growth factor (VEGF) polymorphisms

It has been suggested that the cytokine vascular endothelial growth factor (VEGF) may play a role in the pathogenesis of diabetic complications. Studies have identified polymorphisms in the promoter region of the VEGF gene. A study carried out by Yang *et al* 2001 looked at an insertion polymorphism at position –2578 in the promoter region of the VEGF gene in T1DM subjects with and without diabetic complications. The study found that there was a significant increase in the frequency of the CC genotype in patients with microvascular disease compared to those without. This increase was particularly pertinent in the nephropathy subjects compared to the diabetic controls. The C allele was also significantly increased in the nephropathy subjects compared to the controls. The results of this study suggest that polymorphisms in the promoter region of the VEGF gene may be associated with the pathogenesis of microvascular disease.

Mitochondrial gene polymorphisms

Mitochondria are cytoplasmic organelles found in all Eukaryotic cells. They contain the enzymes of the Krebs cycle, carry out oxidative phosphorylation, and participate in fatty

acid biosynthesis. Mitochondria posses their own genome and ribosomes, both of which are distinctive from their counterparts in the nucleus and cytoplasm of the cell. Cells depending on their energy demand posses hundreds to thousands of mitochondria, each containing 2-10 mitochondrial DNA molecules. Mutations of mitochondrial DNA (mtDNA) are a well-described genetic cause of diabetes mellitus, and have also been found to be involved in the pathogenesis of diabetic microvascular diseases. Both mtDNA point mutations and rearrangements have been identified in families with diabetes (Van Den Ouweland 1994). Mitochondrial DNA has a number of interesting properties including exclusive maternal transmission, the ability to replicate in post mitotic cells, a high mutation rate and an extremely compact molecular architecture with no introns and no large non-coding sequences. Unlike the nuclear DNA, which is linear, the human mitochondrial genome consists of a closed double stranded, circular DNA molecule, and consists of 16569 bp of DNA, which have been fully sequenced (Anderson *et al* 1981).

Although the mtDNA encodes highly conserved proteins it exhibits a very high mutation rate which is 10-20 times more susceptible than the nuclear genome. Reasons for this are that the mt genome evolves 5-10 times faster than single copy nuclear DNA genes. Its half-life is between 6-10 days compared to nuclear DNA, which has a half-life of about 100 days. The mutability may reflect the relatively high insertion error-rate of the mitochondrial DNA polymerase-γ of about 1/7000 bases, resulting in 2-3 mismatched nucleotides per round of replication of the 16.6 kilobases (kb) mt genome. A change in phenotype however, only becomes apparent when the proportion of mutant DNA exceeds a threshold level, which varies for each organ. A critical threshold level of mutant mtDNA must be exceeded before the genetic defect is expressed. This threshold effect is a direct consequence of the maintenance of wild-type copy numbers by relaxed replication of mtDNA (Wallace 2001). The high mutation rate also derives from the fact that mtDNA is more exposed to chemical attack than the nuclear DNA. The mt genome is vulnerable

because it is not protected by histones, absence of DNA repair mechanisms, its highly compact structure, and the production of highly energetic oxygen radicals from the electron transport chain. Continuous hyperglycemia induces to generate several oxygen radical species and consequently result in developing diabetic microvascular complications. Oxidative damage is of particular relevance to mtDNA, since a large fraction of reactive oxygen species (ROS) in cells is thought to originate by leakage of electrons from the electron transport system. The proximity of mtDNA to the ROS generator suggests that there may be a high rate of endogenous oxidative damage to mtDNA.

Early observations of mtDNA were that pyrimidine dimers introduced into mtDNA were not repaired (Clayton *et al* 1974), suggesting that mammalian mitochondria were incapable of repairing their DNA. Despite the importance of each mitochondrial gene, mitochondria are relatively deficient in DNA repair mechanisms (Lightowlers *et al* 1997). However we know that mtDNA mutations occur too frequently for them to go completely un-repaired. The emerging pattern is that mitochondria in higher organisms are reasonably well equipped to conduct base excision repair (BER) but appear to be deficient in nucleotide-excision repair (NER) and mismatch repair (MMR) (Bogenhagen 1999). In a healthy cell, the number of mtDNA moleculas is tightly regulated (Lightowlers 1997). By contrast, mtDNA proliferation is one of the hallmarks of heteroplasmic mtDNA mutations involving tRNA genes, There are increasing numbers of publications showing that certain deletions, insertions or point mutations of the mtDNA may occur and are associated with distinct diseases (Gerbitz *et al* 1996). Individuals with pathogenic mtDNA defects (deletions and point mutations usually harbor a mixture of mutant and wild type mtDNA within each cell (Clayton). The percentage level of heteroplasmy varies between different organs and also between adjacent cells in the same organ. There is increasing evidence that the level of mutant mtDNA in non-dividing tissues may change during a human lifetime (Chinnery *et*

al 1999). A number of age related neuromuscular degenerative diseases have been associated with mutations in mitochondrial DNA (mtDNA), and progressive accumulation of oxidative damage in mtDNA from neuronal tissues over time has been shown. Recently distinct point mutations of the mitochondrial DNA mutations have been implicated in a wider range of clinical disorders including alzheimers, diabetes and nerve deafness (Lien *et al* 2001).

Recently it was reported in a Japanese population by Gong *et al* 1998 that a polymorphism (mt5178A at nucleotide 5178 within the NADH dehydrogenase subunit 2 gene is associated with longevity. The study identified mitochondrial genotypes associated with longevity, being an C- to – A transversion at nucleotide position (np) 5178 within the ND2 gene causing a Leu to Met replacement; a C- to –T transition at np 8414 within the ATP8 gene, causing a Leu to Phe replacement; and a G- to A transition at np 3010 within the 16S rRNA gene. These, being more frequently observed in the centenarians than in the controls. They focused their study on mt5178 A/C transversion and found the frequency of Mt5178A to be significantly higher in the centenarians (62%) than in the blood donors (45%). Further to this they evaluated the effect of MtDNA variations on the occurrence of disease by analyzing the frequency of Mt5178A and Mt5178C in 338 randomly selected patients. They found the ratio of Mt5178 A/C to be significantly lower in old patients than in both the centenarians and the healthy controls. This suggests that to carry a MtDNA genotype predisposing resistance to adult onset disease is one of the genetic factors for longevity. Therefore the longevity genotype Mt5178A would appear to be more resistant than Mt5178C genotype against oxygen radicals, and those individuals with Mt5178C genotype are more susceptible to adult onset chronic disease than those with Mt5178A.

Miura *et al* 1999 furthered this line of research and examined whether or not this polymorphism influences development of diabetic complications, in particular diabetic

nephropathy. They selected a random population of long standing Japanese T1DM patients with diabetic duration of more than 20 years and carried out PCR-RFLP after Alu1 digestion to distinguish Mt5178A and Mt5178C. Their findings suggested that T1DM patients with Mt5178C are more predisposed to diabetic nephropathy than those with Mt5178A. Uchigata *et al* carried out further studies on Japanese patients, to examine whether or not this polymorphism influences development of T1DM or T2DM and its complications. They analyzed the frequencies of Mt5178A/C by PCR-RFLP with *Alu* I in T1DM and T2DM patients. They found the frequency of Mt5178C to be significantly higher in the T1DM patients than in the healthy controls. They also found that in the T1DM patients with diabetic duration of more than 20 years, the Mt5178C frequency in nephropathy group was statistically higher than in the no nephropathy group. There was however no significant difference in the Mt5178A/C between T2DM patients and healthy controls. These results suggest that Mt5178C genotype predisposes to T1DM and diabetic nephropathy significantly more than those with Mt5178A genotype.

Other gene polymorphisms

Other studies have looked at inherited differences in the ability to enzymatically detoxify AGE intermediates such as 3-deoxyglucosone which may be one important genetic factor responsible for determining the impact of a given level of hyperglycaemia on diabetic complications. Other studies suggest that differences in the response of glomerular filtration rate in response to hyperglycaemia may be involved. This is thought to implicate enhanced nitric oxide synthesis by nitric oxide synthase (ecNOS) in afferent arterioles and glomerular endothelial cells and increased expression of insulin-like growth factor (IGF-1) receptor and cause glomerular hyperfiltration (Rudberg 1992; Makino 1999). These findings, along with observations that nephropathy susceptibility groups having large glomeruli, which may be a marker of nephropathy (Striker 1993) suggest multiple genetic components to be involved in the aetiology of diabetic complications. After hypertonic

stress transcriptional activation of heat shock proteins as well as several other genes occurs. This facilitates the accumulation of non-toxic intracellular osmoles. The induction of these genes by hypertonicity involves the activation of members of the MAP kinase family, including c-Jun NH2-terminal kinase (JUNK) and p38 MAP kinase (Wojtaszec *et al* 1998: Sprague 1998). Prostaglandin forming cycloxygenases (COXs) may represent an additional class of genes that promote medullary interstitial cell viability. A search for polymorphisms of COX-1 and COX2 genes may be important in the search for susceptibility genes to microvascular disease (Hao *et al* 2000).

Candidate Gene	Locus	Site	Polymorphism	Reference
Human Leukocyte Antigen-A	HLA-1	6p21	DQA1 and DQB1	Chowdhury *et al* 1998 Chowdhury *et al* 1999 [b]
Aldose Reductase	ALDR1	7q35	(CA)n	Heesom *et al* 1997 Moczulski *et al* 1999 Demaine *et al* 2000
Receptor for Advanced Glycation End products	RAGE	6p21.3	G82S, 1704A/G, 2184A/G, 2245G/A	Kankova *et al* 2001 Hudson *et al* 2001 [b]
Tumor Necrosis Factor (α and β)	TNF	6p21.3	G(-308)A	Hawrami *et al* 1996
Angiotensin	AGT	1q42-q43	M235T	Caulfield *et al* 1994 Doria *et al* 1996
Angiotensin II Type 1 Receptor	AGTR1	3q21-q25	A1166C	Bonnardeaux *et al* 1994 Doria *et al* 1997 Tarnow *et al* 1996
Angiotensin Converting Enzyme	ACE	17q23	I/D	Marre *et al* 1994 Chowdhury *et al* 1996 Doi *et al* 1996 Tarnow *et al* 2000
			AGT-M235T	Ittersum *et al* 2000
Apolipoprotein E	ApoE	19q13.2	Exon 4	Araki *et al* 2000 Chowdhury *et al* 1998 Werle *et al* 1998
Endothelial Nitric Oxide Synthase	eNOS	7q36	T(786)C a(Intron 4)b	Zanchi *et al* 2000 Asakimori *et al* 2001
Plasminogen Activator Inhibitor	PAI-1	1p31-p22	4G/5G	Pucci *et al* 1999
Paraoxonase	PON1	7q21.3	T(-107)C Leu54Met Gln192Arg	Araki *et al* 2000 Sanghera *et al* 1998 Jenkins *et al* 2000
	PON2	7q21.3	Alu148Gly Ser311Cys	Pinizzotto *et al* 2001 Canani *et al* 2001
Glucose Transporter-1	GLUT1	1p31.3-p35	G/T	Hodgkinson *et al* 2001 [a]
Nuclear Factor Kappa Beta	NFkB	4q24	(CA)n	Hegazy *et al* 2001 Ota *et al* 1999
Vascular Endothelial Growth Factor	VEGF	6p12	(-2578)C	Yang *et al* 2001
Hypertension-linked	HYT-1/ D17S934	17q	(CA)n	Julier *et al* 1997

Table 4. Candidate genes so far investigated and implicated in the onset and progression of diabetic vascular complications.

Environmental influences

Cigarette smoking has been identified as an important factor in the development and progression of diabetic renal complications in both T1DM and T2DM subjects (Biesenbach *et al* 1997; Reddy *et al* 1996; Orth *et al* 1997). Also compared to normal subjects the prevalence of smoking in diabetics has been seen to be significantly higher (27 vs. 33%, p<0.0001) (Dierkx *et al* 1996). The overall incidence of microvascular complications in particular retinopathy and increased urine albumin excretion, have been shown to be more common and more severe in T1DM subjects who smoked and more so in heavy smokers (Sinha *et al* 1997). Smoking has been shown to increase the risk of microalbuminuria and progression of proteinuria in both T1DM and T2DM subjects where the prevalence of increased albumin excretion rates was 2.8 times higher in smokers than non-smokers. Albuminuria has been seen to improve significantly when diabetic subjects ceased smoking indicating that it is an independent risk factor of early diabetic renal damage (Chase *et al* 1991). The link between smoking and diabetic renal microangiopathy is thought to be through mechanisms such as increased platelet aggregation, accentuated tissue hypoxia and haemodynamic or metabolic effects of repeated noradrenaline release (Norden and Nyberg 1984).

Low birth weight is a reflection of adverse effects on development in utero and it therefore thought to be partly environmentally influenced. Many studies have also shown that low birth weight (LBW) is strongly associated with hypertension, stroke and myocardial infarction. It has been shown that retarded intrauterine growth is associated with a significant reduction in nephron number, and that there are strong correlations between glomerular number and size with LBW. It has hence been postulated that decreased nephron numbers may be a risk factor for hypertension and the progression of renal disease as it is more vulnerable to damage from a range of pathological processes (Manalich *et al* 2000; Lackland *et al* 2000). Correlation's have also been made between low maternal

education and smoking during pregnancy and the risk of developing incipient nephropathy in offspring with T1DM (Rudberg 2000). Recently studies have also shown a significant association between end-stage renal disease and low birth weight in both African Americans and Caucasoids (Lackland *et al* 2001). Similar studies have been carried out to examine the relationship between LBW and early onset of diabetic retinopathy, however no significant correlation has been found. These results indicate that foetal growth is not a factor of major importance for the development of diabetic retinopathy (Agardh *et al* 2000).

Chapter 2

Aldose Reductase and the Polyol Pathway

Hyperglysolia and the polyol pathway

It has been observed that tissues susceptible to developing diabetes-linked abnormalities are in general not dependent upon circulating insulin for the uptake of glucose (Nathan 1996). Therefore, under extracellular hyperglycaemic conditions these tissues also exhibit hyperglysolia (elevated intracellular glucose levels) (van den Enden 1995). This observation suggests that in selected tissues an increase in glucose concentration of the cellular cytosol may perturbate intracellular metabolic patterns. One of the principle intracellular biochemical mechanisms that has been implicated in the pathogenesis of diabetic microvascular complications is the increased flux of glucose through the polyol pathway under diabetic hyperglycaemic conditions. Under euglycaemic conditions glucose is metabolised by three key pathways, primarily by a hexokinase dependent phosphorylating pathway to form glucose 6-phosphate, which then enters the glycolytic pathway to form lactate, or the hexose monophosphate shunt to form pentose-phosphate. Secondly, glucose may be oxidised to gluconic acid via an NAD^+-dependent glucose dehydrogenase. Thirdly, non-phosphorylated glucose may enter an accessory pathway of glucose metabolism known as the polyol pathway, which is comprised of a series of enzyme dependent reactions, namely aldose reductase (ALR2 or *AKR1B1*) (alditol: $NADP^+$ oxidoreductase [EC 1.1.1.21]) and sorbitol dehydrogenase (SORD) (L-iditol: NAD+2-oxidoreductase [EC 1.1.1.14]), metabolising glucose to sorbitol and fructose respectively. Throughout this thesis aldose reductase will be referred to as ALR2, however the most recent abbreviation is *AKR1B1*. The polyol pathway is an alternative route of glucose metabolism, which is illustrated in figure 4. Under physiological conditions, this polyol pathway plays a minor role in glucose metabolism, except in the kidney where ALR2 may regulate osmolytes (Bagnasco *et al* 1988).

The polyol pathway was first identified in the seminal vesicle of the sheep by Hers 1956 in a report demonstrating the conversion of blood glucose into fructose, an energy source of

sperm cells. As already mentioned, the metabolism of glucose by the polyol pathway involves two reactions resulting in the conversion of D-glucose into sorbitol and D-fructose, catalysed by two rate-limiting enzymes, namely aldose reductase (ALR2) and sorbitol dehydrogenase (SORD). In the first rate-limiting reaction of the polyol pathway ALR2 sequentially catalyses the NADPH-mediated reduction of glucose to the organic osmolyte sorbitol. This NADPH dependent enzyme reduces a carbonyl oxygen to a hydroxyl ion in an ordered 'bi-bi' mechanism, in which NADPH is bound first and $NADP^+$ released last after the catalytic conversion of the aldehyde to the alcohol (Kubieski *et al* 1992; Grimshaw *et al* 1990). This reduction is stereospecific with respect to the coenzyme (Boghosian *et al* 1981; Grimshaw *et al* 1995). In the second step of the polyol pathway SORD oxidises sorbitol to fructose using NAD^+ as the co-factor.

The catalytic efficiency of aldose reductase for D-glucose is relatively low with a Michaelis-Menton Constant (Km) of ≈ 100 mM, a 20-fold higher concentration to normal glycaemic levels of ≈ 5 mM (Bohren 1989). Under normoglycemic conditions the majority of the glucose is therefore phosphorylated to glucose 6-phosphate by hexokinase and the flux through the polyol pathway is minimal (Boel *et al* 1995). Under hyperglycaemic conditions however, the hexokinase pathway becomes saturated with ambient glucose and the increased glucose level in the tissues activates the polyol pathway. In hyperglycaemic states, metabolism of glucose by the polyol pathway has been reported to account for up to 33% of total glucose utilisation in the rabbit lens and 11% in human red blood cells (Kinoshita *et al* 1988; Morrison *et al* 1970).
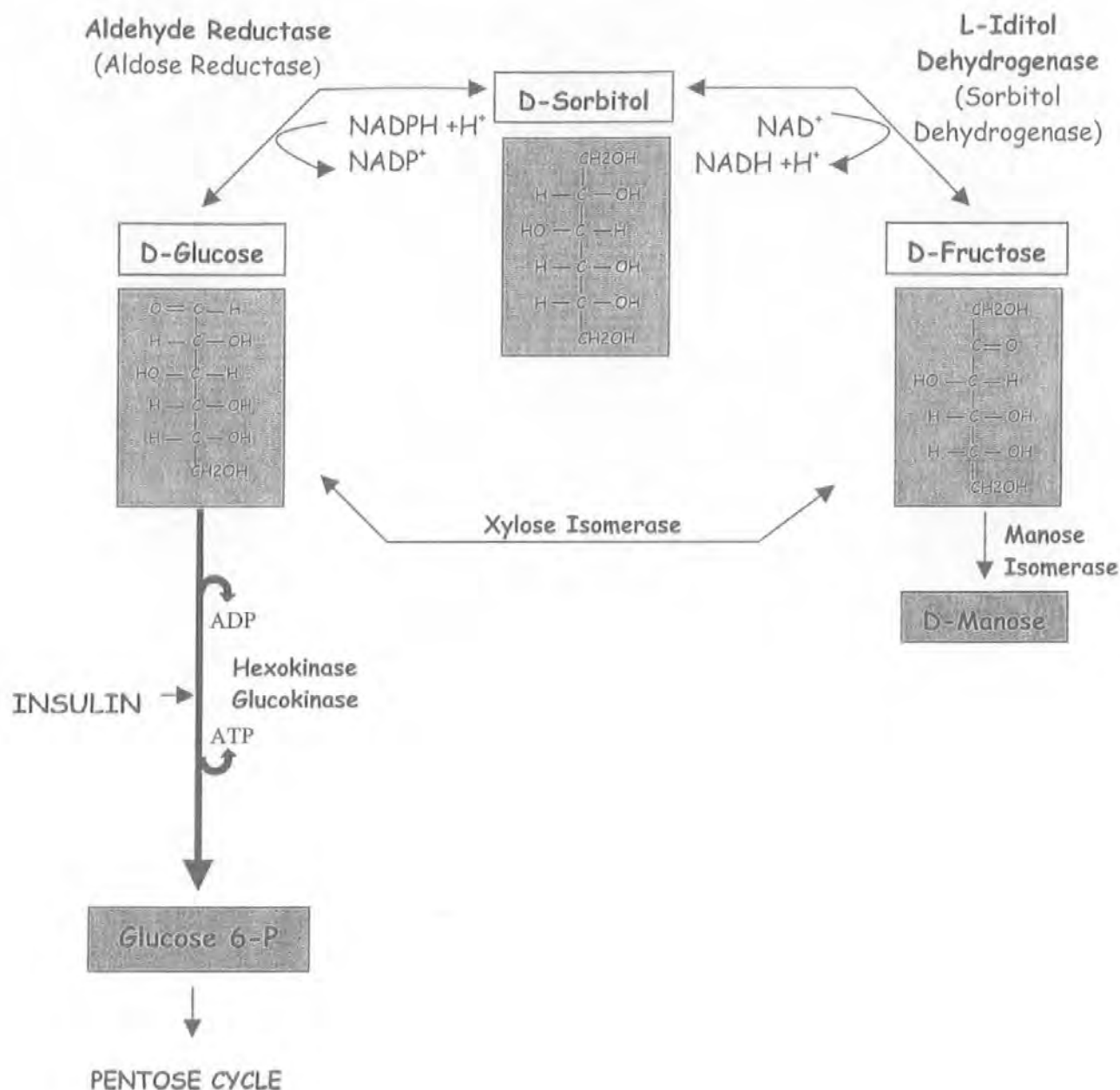
Figure 4. The sorbitol (polyol) pathway (adapted from; Tomlinson 1994), converts glucose into fructose using the enzyme aldose reductase and sorbitol dehydrogenase. Aldose reductase catalyses the conversion of D-Glucose into sorbitol in an NADPH dependent reaction and sorbitol dehydrogenase oxidises sorbitol to D-Fructose using NAD+ as a cofactor. There is also an interdependence of ALR2 and the hexose monophosphate shunt (HMPS).

Aldose Reductase (ALR2)

Aldose reductase is a member of the aldo-keto reductases, which constitute a superfamily of monomeric oxidoreductases. There are three main enzymes within this group namely, aldehyde reductase (ALR1), aldose reductase (ALR2) and carbonyl reductase (ALR3). ALR1 is involved in the detoxification of a variety of different aldehydes, and reduces isocorticosteroids during steroid catabolism. The primary physiological role of ALR1 is the reduction of D-glucoronate to L-gluconate. ALR3 is also an oxidoreductase mainly for ketones but has broad substrate specificity. ALR2 is the rate-limiting enzyme of the polyol pathway and is believed to be of primary importance in the development of severe degenerative complications of diabetes mellitus, through its ability to reduce excess D-glucose to D-sorbitol in non-insulin dependent tissues. Importantly, ALR2 has also been shown to efficiently reduce by-products of glucose and cellular metabolism such as methyglyoxal, 4-hydroxynonenal and 3-deoxyglucosone suggesting that it may be responsible for detoxification of these harmful metabolites (Vander Jagt *et al* 1992; 1995). Another postulated function of ALR2 is osmoprotection in the kidney as sorbitol is an inert compound and reaches levels in certain tissues great enough to affect osmotic pressure (Bagnasco *et al* 1987).

ALR2 is a small monomeric NADPH-dependent oxidoreductase composed of 315 amino acid residues (M_r 35,900) (Petrash *et al* 1994). The sequence is well conserved from one tissue to another and also between species, for examples the ALR2 gene sequence from the rabbit kidney has 84% homology with ALR2 gene sequence from bovine lens and 89% homology with rat lens (Garcia-Perez *et al* 1989). Several biochemically-modified forms of ALR2 have been identified, however tissue specific isozymes have not yet been confirmed in humans (Yabe-Nishimura 1988; Grimshaw and Lai 1996), however Yang *et al* 2000 identified a renal-specific oxido-reductase (RSOR) in newborn diabetic mice. Structural studies by crystallographic analysis have been performed on ALR2 which have

revealed that AR folds as a $(\beta\text{-}\alpha)_8$ barrel and that its catalytic sites lie at the bottom of a deep hydrophobic cleft (figure 5). ALR2 catalyses the reduction of a wide variety of carbonyl compounds to the corresponding alcohol's. Interestingly, glucose is not the preferred substrate for ALR2, in fact it is more efficient in reducing various aromatic and aliphatic aldehydes such as glyceraldehyde (Inazu *et al* 1994).

The catalytic cycle and kinetics of ALR2

The catalytic cycle of ALR2 follows an ordered reaction mechanism whereby binding of NADPH induces a first conformational change in the enzyme that occurs before substrate binding (figure 6). NADPH binds at the carboxy-terminus of the enzyme in an extended conformation. Then ALR2 stereospecifically transfers the 4-*pro*-R hydrogen from the C4 of the nicotinamide ring to the *re* face of the carbonyl carbon of the substrate. The substrate active site contains Tyr 48 as the proton donor during aldehyde reduction. After aldehyde reduction, the enzyme undergoes a second conformational change, which is associated with the dissociation of the enzyme-oxidised NADP+ complex (Constantino *et al* 1999; Varnai *et al* 1999). It has been suggested that the enzyme conformational change is the limiting factor of the overall reaction. Findings have shown that ALR2 from diabetic tissue displays linear kinetics with a V_{max} two or three times higher than normoglycemic tissue (Srivastava *et al* 1985). Grimshaw *et al* 1995 reported transient kinetic data to estimate the rate constants for the ALR2 catalysed reaction: E•NADPH $\leftrightarrow$ E•NADPH $\leftrightarrow$ *E•NADPH•RCHO $\leftrightarrow$ *E•NADP$^+$•RCH$_2$OH $\leftrightarrow$ *E•NADP$^+$ $\leftrightarrow$ E•NADP$^+$ $\leftrightarrow$ E.. The proposed kinetic mechanism for ALR2 suggests that the isomerisation of E•NADPH complexes may be the rate-limiting step of the reaction. It has hence been proposed that under certain conditions, such as ionic stress or non-enzymatic glycosylation of the enzyme, ALR2 is able to assume an active conformation.

Aldose Reductase (EC 1.1.1.21)
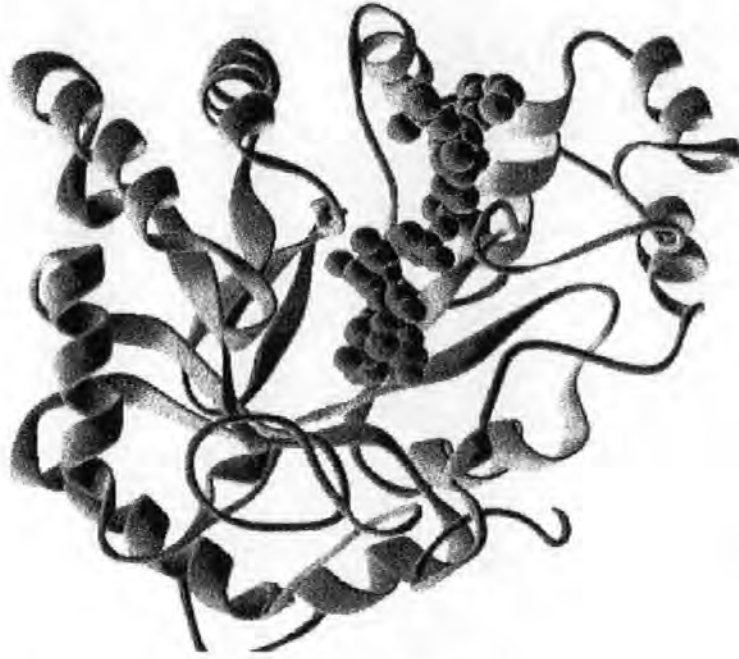
Figure 5. Schematic of the 3-dimensional x-ray crystal structure of AR in complex with its cofactor NADPH (Purple). It consists of >2,500 atoms and folds in the beta/alpha barrel formation. http://www.biochem.szote.u-szeged.hu/astroian/protein1.htm
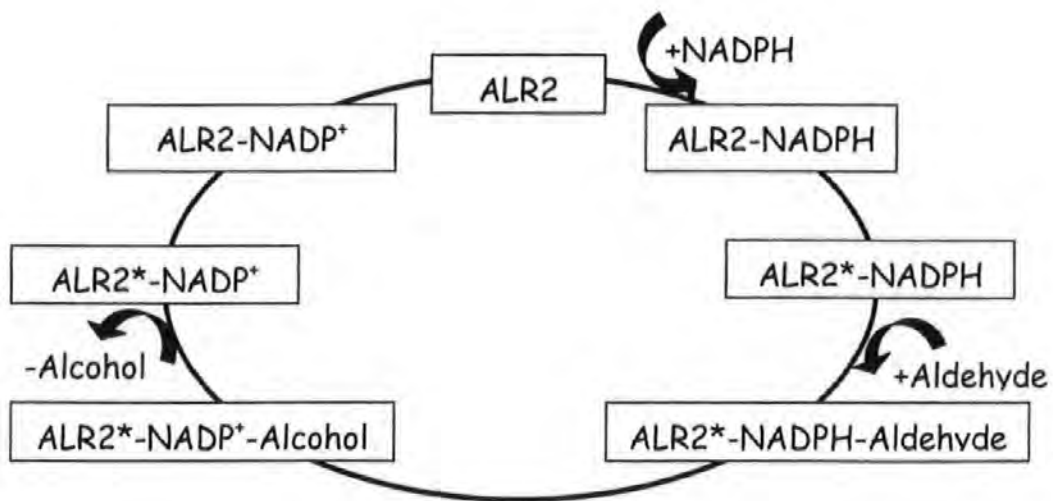
Figure 6. The catalytic cycle of ALR2 adapted from Constantino *et al* 1999. The free enzyme binds to NADPH first and undergoes a conformational change. Next the aldehyde substrate binds which reacts to form the alcohol product and NADP+. The alcohol product is released and the enzyme undergoes another conformational change releasing NADP+.

Sorbitol dehydrogenase (SORD)

Sorbitol dehydrogenase (SORD) is a zinc-containing enzyme and is the second enzyme of the polyol pathway. SORD is involved in the conversion of sorbitol to fructose by oxidation using NAD+ as a cofactor. Little is known about the physiological function of the enzyme SORD, however, its activity has been found in a number of tissues, for example, kidney, liver, lens, testis, prostate, placenta, erythrocytes, brain, spinal cord and peripheral nerves (Gabbay and O'Sullivan 1968; King *et al* 1996). SORD displays moderately broad substrate specificity, catalysing the conversion of xylitol to D-xylulose, ribitol to D-ribulose, and iditol to L-sorbose, as well as sorbitol to D-fructose. The K_m for sorbitol of SORD is ~6 mM. Elevated levels of ATP, lactate, and high NADH/NAD+ ratios that accompany hyperglycaemia are known to inhibit the action of SORD. The human SORD protein was reported to be 355 amino acids long and to have a Mw of 38,067 daltons (Karlsson *et al* 1989). SORD is a member of the long chain alcohol dehydrogenase gene superfamily which interconvert alcohol's to the corresponding ketone or aldehyde. Lee *et al* 1994 first cloned the human SORD gene and used fluorescence in situ hybridisation to determine its chromosomal location. Carr *et al* 1995 and Iwata *et al* 1995 also reported the position, structure and expression of the human SORD gene, and described a range of polymorphic variants. By in situ hybridisation the SORD gene was found to be located on chromosome 15q21.1, with two transcription sites. A SORD-related pseudogene was also found to exist which shows a high degree of similarity with the SORD1 gene (Carr *et al* 1997). A deficiency in SORD activity has been previously linked to cataract formation in non-diabetics (Vaca *et al* 1982; Shin *et al* 1984). Studies to investigate the role of SORD have been carried out using SORD inhibitors (SDHI) (Geissen *et al* 1994; Tilton *et al* 1995; Cameron *et al* 1997; Oates *et al* 1998). However, there have been contradictory findings, whereby some studies have shown that SDHIs reduce nerve conduction deficit induced by diabetes in rats (Tilton *et al* 1995). Other studies using diabetic rats treated with SHDI or SORD deficient diabetic mice, however

have not found this to be the case (Cameron *et al* 1997; Ng *et al* 1998). Obrosova *et al* 1999 investigated the use of SORDI upon diabetic neuropathy using STZ-diabetic rats, the findings of which were that inhibition of SORD resulted in adverse neural dysfunction effects. The activity of SORD by mRNA expression has been shown to be modestly increased in diabetic subjects with diabetic complications (Hodgkinson *et al* 2001 [b]).

The polyol pathway and diabetic complications

When hyperglysolia occurs the hexokinase pathway for glucose metabolism becomes saturated due to undefined regulatory mechanisms and the relatively high affinity of hexokinase for glucose. Hyperglycaemia has hence been shown to activate the polyol pathway in tissues that are insulin independent and freely permeable to glucose such as the peripheral nerves, renal cortex and lens tissue (Dvornik 1987; Greene 1988 [a and b]; Pugliese 1991; Larkins 1992). An excessive flux through the polyol pathway has long been thought to be involved in the pathogenesis of diabetic microangiopathy (Ido 1996). There have been no findings so far of a metabolite feedback mechanism for lowering the activity of aldose reductase in reducing glucose to sorbitol although citrate and nitric oxide have been shown to inhibit ALR2 in vitro (Harrison *et al* 1994; Chandra *et al* 1997). Gabbay 1966 recognised that there was a potential link between the formation of sorbitol from glucose via the polyol pathway involving aldose reductase, and the onset of diabetic complications (Gabbay 1966; 1972 [a and b]; 1973). Under hyperglycaemic conditions there is an intracellular increase in sorbitol production and as sorbitol is not rapidly removed from the cell and the conversion to fructose is slow there is a significant intracellular accumulation of sorbitol (Sharma 1997). This is particularly true in tissues such as the renal cortex, peripheral nerve, lens and retina that exhibit polyol pathway activity and do not require insulin to facilitate the uptake of glucose and are therefore freely permeable to glucose. It is these insulin independent tissues that are greatly affected

by the microvascular complications of diabetes, indicating that the polyol pathway is an important mediating factor (Sato 1992; Dorin 1995).

Ruth Von Heyningen 1959 made early descriptions of an excess of sorbitol in the lens of diabetic rats. Similar cataracts have been found to develop even more rapidly in animals fed a diet containing a high percentage of galactose (30-50% by weight). The explanation for this being galactitol, the sugar alcohol galactose, cannot be reduced by sorbitol dehydrogenase, the second enzyme in the pathway, and therefore accumulates to much higher levels in susceptible tissues than sorbitol (Kinoshita 1965). Sorbitol has also been found to accumulate in the peripheral nerves of diabetic animals (Gabbay 1966) as does galactitol in the nerves of rats fed excess of galactose. The motor nerve conduction velocity in these animals is substantially slowed (Gabbay 1972 [b]) and studies have shown similar results in humans (Gregersen 1968). Aldose reductase has been shown to be present in the inner medulla, the loop of Henle, collecting tubules and glomerular podocytes of the rat kidney and also in the human glomeruli. The enzymatic activities of the human diabetic glomeruli have shown diminished hexokinase, elevated ALR2 activity and decreased SORD activity. Lee *et al* 1995 demonstrated that sorbitol accumulation was implicated in the development of diabetic cataract using transgenic mice expressing the ALR2 gene in the lens. In this study, when the SORD deficient mutation was also present there was a further increase in sorbitol accumulation and further acceleration of the diabetic cataract. The second half of the polyol pathway is therefore also thought to be important in facilitating the development of microvascular disease. Schmidt *et al* 1998 reported that the use of a sorbitol dehydrogenase inhibitor (SDI) CP-166,572, which interrupts the conversion of sorbitol to fructose resulted in markedly increased levels of sorbitol in the peripheral nerve in the diabetic rat model. Ho *et al* 2000 developed mice deficient in ALR2 gene and found that they exhibited a phenotype similar to that of nephrogenic diabetes insipidus. These factors cumulatively suggest that the polyol pathway

may be a contributing factor in the pathogenesis of human diabetic retinopathy, glomerulosclerosis and neuroaxonal dystrophy via the accumulation of intracellular sorbitol.

Hyperglysolia induced increase in expression and action of ALR2

An increase in glucose levels has been associated with high ALR2 containing cells. In the case of diabetic patients with and without microvascular complications many studies have been carried out to determine differences in the ALR2 levels between these groups. Increased amounts of ALR2 mRNA were found in rabbit inner renal medullary cells under hyperosmotic stress (Garcia-Perez et al 1989; Uchida et al 1989). The increased expression of aldose reductase under hyperosmotic stress has also been reported in a variety of cells of non-renal origin such as Chinese hamster ovary cells (Kaneko et al 1990). Other studies also showed similar findings in glomerular endothelial cells and cultured rat mesangial cells (Hohman et al 1991; Kikkawa et al 1992). A 132% increase in white cell ALR2 mRNA levels was reported in both T1DM and T2DM subjects compared to normal healthy controls (Kicic and Palmer 1996). Ratliff et al 1996 found that levels of ALR2 protein in mononuclear blood cells were twice as high in T1DM subjects with neuropathy than in T1DM patients without. Ito et al 1997; Ohnishi et al 1996; Nishimura et al 1994, 1997 and Takahashi et al 1998 also reported similar patterns in T2DM subjects with respect to neuropathy and also retinopathy, where the higher levels of erythrocyte ALR2 protein was significantly associated with microvascular disease. A significant report, which was published by Shah et al 1997, showed that the expression of the ALR2 gene is significantly increased in the lymphocytes of patients with diabetic nephropathy. The study measured ALR2 mRNA from peripheral blood monocytes and found a significant increase in T1DM patients with renal disease compared to T1DM subjects without or normal controls. Hamada et al 1998 used the two-site ELISA technique to determine the enzyme protein content of human ALR2 in erythrocytes from patients with T2DM and normal controls.

The study found a strong correlation between enzyme activity and an increase in enzyme content and also an increase in sorbitol and fructose as well as an elevated lactate-to-pyruvate ratio. They suggested that inter-individual variability of ALR2 content might contribute to the polyol pathway flux in diabetic patients. Recently, Hasegawa *et al* 1999 also measured ALR2 protein in peripheral mononuclear cells (PMCs) from patients with T2DM using two site ELISA using anti-human AR monoclonal antibody. The study found no differences between ALR2 levels and the presence or absence of diabetes. However a significant increase in ALR2 levels was found in diabetic subjects with microvascular disease than in those without.

Srivastava *et al* 1986 suggested that the properties of ALR2 were changed by hyperglycaemia. They showed that the activation of the enzyme in human erythrocytes incubated with glucose was also accompanied by changes in enzyme kinetics. They suggested that the ALR2 enzyme is activated by non-enzymatic glycosylation (Srivastava *et al* 1986; Srivastava *et al* 1985; Das and Srivastava 1985; Srivastava 1989). Ghahary *et al* 1989 reported the STZ-diabetic rats showed increased renal ALR2 activity. It has since also been shown that ALR2 protein and the activity of the enzyme is significantly increased in the erythrocytes and neutrophils of patients with both T1DM and T2DM and microvascular disease compared to those with no complications (Hamada *et al* 1991[a and b]; Nishimura *et al* 1994, 1997). Dent *et al* 1991 reported an increase in neutrophil ALR2 activity in T2DM subjects with complications compared to those without. Importantly, there were no significant difference found between patients without complications and normal controls. Hamada *et al* 1993 reported that there was an association between erythrocyte ALR2 activity and diabetic complications in T1DM subjects. In the T1DM population investigated the study found that patients with greater ALR2 activity were four times more likely to develop diabetic complications than those patients whose enzyme activity was close to that of non-diabetic individuals. These observations suggest that the

variability of ALR2 activity may be the cause of differences in susceptibility to diabetic complications. It has also been proposed that ALR2 is induced in human microvascular endothelial cells by advanced glycation end products. This is supported by a recent study carried out by Nakamura *et al* 2000 who investigated the effects of advanced glycation end products on the levels on ALR2 mRNA, protein and activity in human microvascular endothelial cells. Their results suggested that accelerated formation of AGEs in vivo might elicit activation of the polyol pathway, possibly via oxidative stress, to enhance endothelial cell damage leading to microvascular dysfunction. In conclusion there is strong evidence for an increase in ALR2 activity, protein levels and mRNA in diabetics with complications suggesting that its regulation through gene expression and altered conformation may be important factors in modulating the risk of developing microvascular disease.

Metabolic perturbations induced by increased flux through polyol pathway

The acceleration of the polyol pathway elicits various metabolic imbalances in those tissues that undergo insulin independent uptake of glucose for which there are several metabolic consequences. These include alterations of the intracellular redox state by decreased availability of NADPH and increased $NAD^+/NADH$ ratio (pseudohypoxia). Also implicated is osmotic stress resulting from lowered myo-inositol concentrations and sorbitol accumulation, protein kinase C activation, impaired phosphatidylinositol metabolism, glutathione and antioxidant depletion, reduced NO production and increased glycosylation (Williamson 1993; King and Brownlee 1996; Oates 1997). Additionally Morrisey *et al* 1999 reported that polyol pathway activation was also associated with fibronectin generation in the proximal tubule cells of the kidney, enhancing thickening of the tubular basement membrane. An increased flux through the polyol pathway would also implicate an increase in the production of fructose, which has been reported to be a 10-fold better substrate than glucose for glycosylation and formation of AGEs (Brownlee 1992). Such metabolic perturbations provoke the early tissue damage in the target organs of

diabetic complications, such as ocular lens, retina, peripheral nerves and renal glomerulus (Kinoshita 1988; Pugliese 1991) (figure 7).

The overflow of the products of the polyol pathway is associated with depletion in reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the oxidised form of nicotinamide adenine dinucleotide (NAD^+) the cofactors used in the pathway (Williamson 1993). This metabolic imbalance is thought to be related to abnormalities such as altered vascular permeability and increased synthesis of diacylglycerol (DAG) precursors through glycolysis and consequent PKC activity (Ishii and Isogai 1998). DAG is a rate-limiting co-factor for Ca^{2+}-dependent protein kinase C that is involved in the signal transduction pathway. This subsequently effects the gene expression of many proteins involved in cell growth and proliferation, vascular contractility and the synthesis of basement membrane proteins. The increased use of NADPH by the over-activity or ALR2 could also alter the pentose phosphate pathway by depleting the inhibiting factor (NADPH) and enabling constant passage of glucose to pentose phosphate pathway intermediates.

Intracellular sorbitol accumulation has also been associated with a decrease in the levels of myo-inositol in tissues including lens, nerve, retinal pericytes and the kidney glomeruli by competitively inhibiting its uptake into the cell (Greene 1987; 1988 [b]). This fall in myo-inositol may result from an osmoregulatory compensation for elevated sorbitol, by acting as a compatible osmolyte where abnormal accumulation of one osmolyte results in reciprocal depletion of others (Finegold *et al* 1983; Stevens 1993). Tissue myo-inositol is a precursor of phosphatidylinositol (PI) which activates sodium/potassium adenosine triphosphatase (Na+/K+ ATPase), a component of the sodium pump. This perturbation undermines the cells osmotic regulation by effecting cell permeability. Protein kinase C stimulation also becomes impaired by diacylglycerol and a reduction of Na+-K+-ATPase activity.

Hyperglycaemia

Increased flux through
polyol pathway

↑DAG/PKC   ↑NADH/NAD   ↓Myoinositol

Vascular   Gene expression   ECM protein
contractility   synthesis
and
permeability

Diabetic Complications
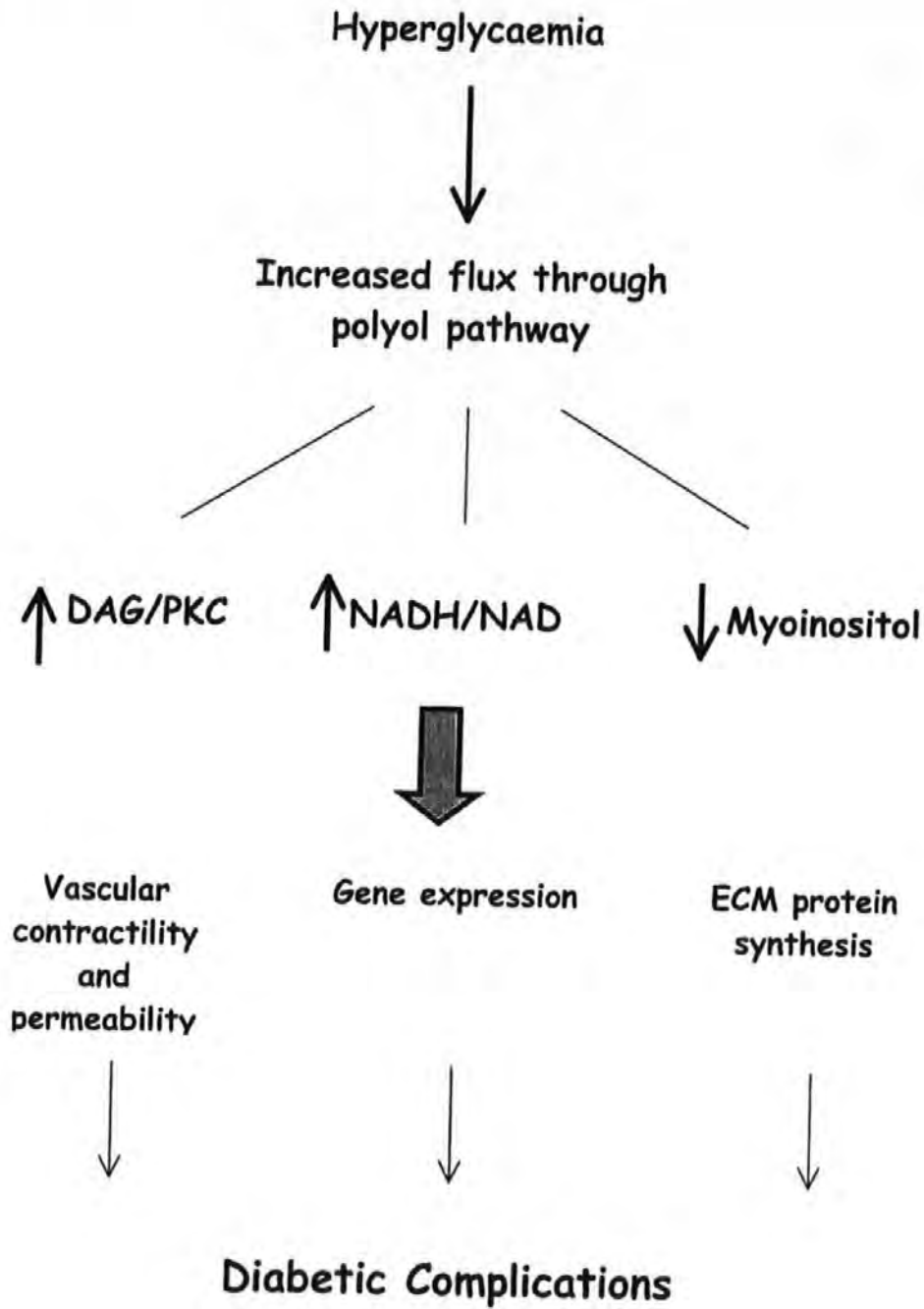
Figure 7. Diagrammatic representation of hyperglycaemia induced metabolic perturbations resulting from increased flux through the polyol pathway (adapted with alterations from King and Brownlee 1996).

Aldose reductase inhibitors (ARI's)

Reports from experimental studies carried out in the US and Europe on diabetic study populations, have associated ALR2 to elevated diabetic microvascular disease risk. Based upon these described observations, it is thought that inhibiting aldose reductase activity should reduce diabetic microvascular complications by minimising tissue injury without requiring the often difficult to attain euglycaemia (Dvornik 1996; Oates 1997; Oates *et al* 1999). The potential value of blocking ALR2 came from a Brazilian natural medicine for diabetes which is made from Myricia multi flora which contained myrciaitrin 1 and myrciaphenone B which are potent inhibitors of aldose reductase (Yoshikawa *et al* 1998). As previously mentioned structural studies carried out on ALR2 have determined the catalytic sites of the enzyme are at the bottom of a deep hydrophobic cleft. Various structurally diverse compounds have been observed to inhibit this enzyme site. Aldose reductase inhibitors such as sorbinil, ponalrestat, epalrestat, tolrestat, zopolrestat as well as other synthetic inhibitors have since been developed based upon these observations, as possible therapeutic agents for diabetic complications. However, the clinical efficacy of these inhibitors in diabetic patients has not been fully proved. These compounds have been grouped into four main classes according to their structure, firstly flavonoids (quercetin), secondly carboxylic acids (epalrestat, FK-366, AD-5467, tolrestat, TAT, NZ-314, zopolrestat), thirdly hydantoins (SNK-860), and fourthly other compounds (Dvornik *et al* 1987; Hotta 1997). Studies into specificity pockets and binding of inhibitors have shown that the ALR2 active site adapts itself to bind tightly to different inhibitors, indicating flexibility and explains the large variety of substrates (Urzhumtsev *et al* 1997; Lee *et al* 1998; Rogniaux *et al* 1999; Calderone *et al* 2000). El-Kabbani *et al* 1999 also found that certain structural features are responsible for differences in coenzyme and inhibitor specificity's of ALR2, whereby differences in structural changes required for the binding of ARIs are responsible for differences in the potency of ALR2 inhibition.

Many animal studies of aldose reductase inhibition have been carried out using streptozotocin (STZ) or alloxan-induced diabetic rats (Robinson *et al* 1990; Ido *et al* 1996). From the results obtained from animal studies of diabetic complications, ARIs have been shown to have a beneficial inhibitory effect on the development of neuropathy, retinopathy, nephropathy, keratopathy, cataract formation, infection and atherosclerosis. Additionally, Keegan *et al* 2000, have shown a protective effect of the ARI WAY121509 in the corpus cavernosum and mesenteric vessels in diabetic rats.

Kinoshita *et al* 1979 studied the effect of an ALR2 inhibitor CP-45,634 on the corneal epithelium of a diabetic rat, and found that the ARI treated epithelium showed faster regeneration than the untreated eye. Animal studies of ARIs have shown that they are capable of normalising some of the polyol-pathway linked biochemical imbalances in the renal cortex in rats. For example, Robinson *et al* 1990 showed that the aldose reductase inhibitor, sorbinil, minimises albuminuria and glomerular basement membrane thickening in STZ diabetic rats treated for 5 months. Ido *et al* 1996 using animal models of diabetes observed reduced vascular dysfunction by inhibition of the sorbitol pathway. It has also been reported that daily administration of epalrestat to STZ diabetic rats prevented gastric erosion and ulceration and normalised mucosal blood flow, suggesting that aldose reductase inhibitors act in part by blocking the action of induced nitric oxide (Suzuki *et al* 1999). Many animal studies have focused on the effects of ARI's on neuropathy, for example, Cameron and Cotter 1992 [a] conducted a study to examine the effects of two different doses of the ARI, ponalrestat on the peripheral nerve in diabetic rats. The study found that the administration of 8 mg/kg-1day-1 and 100mg/kg-1/day-1 both resulted in a reduction in sciatic nerve sorbitol content, however the flux through the polyol pathway still remained substantially elevated in the lower dose rats. Only the higher dose treated rats showed complete restoration in sciatic motor branches and sensory saphenous nerve. During the course of a 5-year study, nerve conduction velocity progressively decreased in

untreated diabetic dogs, and this decrease was prevented by treatment with an aldose reductase inhibitor (sorbinil) (Engerman *et al* 1994). The same study however showed no beneficial effects on renal structure or albuminuria (Kern and Engerman 1999). Several studies of ALR2 inhibition and vasodilator treatment have shown to improve nerve conduction velocities in STZ rats, and prevent the formation of diabetic cataracts (Nishimura *et al* 1994; Stevens *et al* 1994; Kador *et al* 1985). A comparative study by Mizuno *et al* 1999 investigated the effects of three ARIs, fidarestat, epalrestat and zenarestat on the slowing of sensory (SNCV) and motor (MNCV) nerve conduction velocities in STZ induced diabetic rats. The study found that fidarestat showed significant effects in lowering sorbitol content continuously up to 24 hours after administration, and improved slowing of SNCV, indicating that continuous inhibition of polyol pathway flux can improve diabetic neuropathy. Positive results have also been attained in the use of epalrestat in reducing morphological and biochemical deficits in galactose fed dogs where galactose is known to stimulate the polyol pathway and result in diabetic like microangiopathic defects (Kasuya *et al* 1999 [b]). Experimental studies in diabetic rats have also indicated a cardioprotective effect of ARI's (zopolrestat), where it has been shown that attenuation in the rise in Na and Ca during ischemia provides protection (Ramasamy *et al* 1999). It is important to add that there have been negative results obtained in certain animal studies, where aldose reductase inhibitors have not been seen to prevent or improve retinopathy, or reduce capillary basement membrane thickening in the retina, renal glomerulus or leg muscle in alloxan-induced diabetic dogs (Kern *et al* 1991; Engerman *et al* 1993).

Clinical trials carried out over the last decade have attempted to assess the efficacy of aldose reductase inhibitors in humans in the treatment of diabetic microvascular complications. Although clinical, experimental and pharmacological data indicate that there is an involvement of ARI's in slowing the progression of diabetic complications past

clinical trials have been disappointing. Importantly, sorbinil has been withdrawn from further use because of its severe and frequent toxicity, and tolrestat has shown elevated hepatic enzymes which return to normal when administration is discontinued (Foppiano and Lombardo 1997). Positive and negative findings have been made in the use of ARI's in the treatment of retinopathy. The use of epalrestat treatment of retinopathy over a three-year period has been shown to generate improvements in retinal structure and electroretinogram (Hotta *et al* 1990; Hotta *et al* 1990). Cunha-Vaz *et al* 1986 also showed positive findings for the use of sorbinil over a six-month period, finding a reduction in blood-retinal barrier alterations and a reduced incidence of microaneurysms of the retina. Negative findings have however also been made in the case of retinopathy. For example the Sorbinil Retinopathy Trial Research Group 1990 showed no significant differences between sorbinil treatment and placebo groups, and the same conclusions appeared to be true in similar studies using ponalrestat (Sorbinil Retinopathy Trial 1990; Tromp *et al* 1991). Although these studies appear to have conflicting findings there is still some degree of evidence for a positive role for ARI's in the treatment of diabetic retinopathy, indicating a strong requirement for further studies into the role of ARI's and retinopathy.

Aldose reductase inhibitors may improve diabetic nephropathy, which has been shown by its lowering of the glomerular filtration rate and an alteration in the course of microalbuminuria (Passariello 1993; Oates 1994). Studies have shown that ARIs have only a partial effect in ameliorating renal microvascular complications. For example, a six-month trial of ARI administration had an effect on hyperfiltration in the presence of normoalbuminuria (Pedersen *et al* 1991). However, many other studies of ARIs and nephropathy have also proven negative findings (Ranganathan *et al* 1993; McAuliffe *et al* 1998).

Many clinical trials of ARIs have also focused on diabetic neuropathy (Pfeifer *et al* 1997; Oates 1997). Sima *et al* 1988 carried out a double blind study in patients with diabetic neuropathy, and found exciting evidence for the efficacy of sorbinil against degeneration in surial nerve biopsies, which was also accompanied by a decrease in the nerve sorbitol level and an increase in the nerve conduction velocity. A study by Nicolucci *et al* 1996 found that treatment of diabetic neuropathy with tolrestat showed a reduced risk for developing nerve function loss compared with placebo treated patients. Robust inhibition of aldose reductase in human nerve has been shown to have a dose dependent effect on nerve structure and function and continuous inhibition of increased polyol pathway flux can improve diabetic neuropathy. However, several groups have suggested that in the case of neuropathy the use of ARI's such as tolrestat and epalrestat in it management act mainly to slow the progression of neuropathy rather than to reverse it (Gerven 1993; Goto *et al* 1993; Pfeifer *et al* 1997). Greene *et al* 1999 carried out a study to determine whether the ARI zenarestat improves nerve conduction velocity (NCV) and nerve morphology in diabetic peripheral polyneuropathy. This randomised, placebo-controlled, double-blinded, multiple-dose clinical trial found an improvement in NCV slowing and a reduction in small myelinated nerve fiber loss, but indicated the >80% suppression of nerve sorbitol content is required. In controversy to these previous studies Airey *et al* 2000 investigated the efficacy of ARI's in the prevention, reversal or delay in the progression of diabetic peripheral neuropathy. They used nerve conduction velocities as the trial end points to evaluate treatment with 4 different ARI's. They found a small reduction in the decline of median and peroneal motor nerve conduction velocities, but no clear benefit was seen in terms of median and sural sensory nerves.

In summary, enzyme inhibition as a direct pharmacokinetic approach to the prevention of diabetic complications resulting from the hyperglycaemia of diabetes has not been fully effective because of non-specificity of the inhibitors and some appreciable side effects.

Such negative findings may be partly due to the multiple cell types involved in diabetic complications, potential differences in ALR2 expression, the long time course for the development of complications and the relatively short length of human trials, and the variability in potency and penetration of ARI's (Dunlop 2000). Improvement has however been seen in human diabetic subjects who receive ARI's as compared to those on a placebo mainly in terms of motor, sensory and autonomic neuropathy. It has been suggested that promising therapy may come from the administration of two or more synergistic metabolic blockers, for example using aldose reductase inhibitors in combination with aminoguanidines (Boel *et al* 1995). Interestingly vitamin E and other antioxidants have been reported to delay or prevent cataract in diabetic animals (Creighton and Trevithick 1979). Furthermore, in vitro studies have indicated that ascorbic acid (vitamin C) is able to reduce sorbitol levels in humans by inhibiting ALR2 activity (Cunningham *et al* 1994; Lindsay *et al* 1998). It has also been suggested that the synthesis of new phthalazinyl derivatives may provide compounds to inhibit both ALR2 and SORD (Fourmaintraux *et al* 1999).

ALR2 as a candidate gene for diabetic microvascular complications

There is strong evidence for an involvement of a major genetic susceptibility component in the aetiology of diabetic complications. It is possible that variation within the genes coding for the enzymes of the polyol pathway explain why only some patients with diabetes are affected by diabetic complications. Variations in the gene encoding the enzymes of the polyol pathway may result in either the increased expression or increased action of the respective enzymes. Conversely variation may result in the under expression of the sorbitol dehydrogenase gene however both result in an accumulation of sorbitol in cells (Lee et al 1995) (figure 8).

$\uparrow$AR activity $\downarrow$SORD activity

$\uparrow$Glucose $\longrightarrow$ $\uparrow$Sorbitol $\longrightarrow$ Fructose
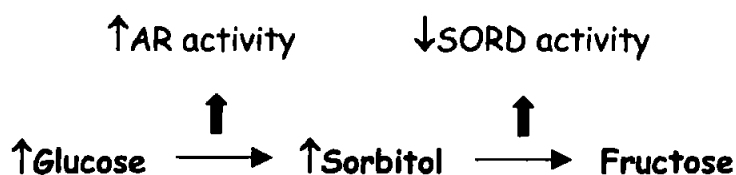
Figure 8. Illustration of the effect of polyol pathway enzyme expression and the accumulation of sorbitol.

The presumption that the involvement of the polyol pathway in diabetic complications, along with the presumption that genetic factors play an important role in the pathogenesis of diabetic microvascular disease has stimulated the search for candidate genes. The cloning of genes coding for aldose reductase (Graham 1991 [a]) and sorbitol dehydrogenase (Lee 1994) now enables the role of the polyol pathway to be studied at a molecular level. The sib-pair linkage analysis carried out in T2DM Pima Indians showed evidence that the strongest linkage with diabetic nephropathy was on chromosome 7 (Imperatore *et al* 1998 [a]). This has also been confirmed in a Caucasoid T2DM study population (Fogarty *et al* 1999). Interestingly this chromosomal location is in the region corresponding to the ALR2 region 7q35. Patients with T1DM and microvascular complications have been shown to have raised enzymatic activity of ALR2 and also

increased amounts of the ALR2 protein itself. It is a possibility that high ALR2 levels are genetically provided reflecting either heterogeneity of gene expression or the presence of a significant polymorphism of the structural ALR2 gene. These genetic alterations may modulate the risk of microangiopathy in association with various other metabolic, genetic and environmental factors.

The ALR2 gene and its promoter region (AR; EC 1.1.1.21)

The gene for ALR2 has been isolated, cloned and sequenced from a number of tissues in man (Petrash and Favello 1989; Bohren *et al* 1989; Chung *et al* 1989; Nishimura *et al* 1990). Graham and colleagues 1991[a and c], mapped the putative functional human ALR2 gene to chromosome 7 in the q35 region adjacent to the TCRβ loci, using somatic cell hybrids and in situ hybridisation. It has previously been shown that the ALR2 and adjacent TCRβ loci on chromosome 7q35 are strongly associated with diabetic nephropathy (Hibberd *et al* 1992; Patel *et al* 1993, 1996). The ALR2 gene has hence been proposed as a candidate gene for the development of late diabetic complications. Bateman *et al* 1993 used a complementary DNA clone encoding human ALR2 to further map the gene sequence to human chromosomes 1,3,7,9,11,13,14 and 18. They also used *in situ* hybridisation analysis to localise sequences to human chromosomes 1q35-q42, 3p12, 7q31-q35, 9q22, 11p14-p15, and 13q14-q21 which may represent other active genes, non-aldose reductase homologous sequences or pseudogenes. Cao *et al* 1998 identified a novel human protein that is highly homologous to ALR2 namely ARL-1, which shows 71% sequence homology to human ALR2, and 81% and 83% homology with mouse vas deferens protein and fibroblast growth factor-regulated protein. This protein was found to have similar enzymatic activities and to act on a similar spectrum of substrates, although there was a difference in the site of expression of the genes. Importantly, this study determined by Northern blot analysis that ARL-1 is not expressed in tissues prone to diabetic lesions. A study carried out by Ho *et al* 1999 investigated the genomic structures and chromosomal

locations of the mouse ALR2 gene and ALR2-like genes. Using a comparative approach they determined that the area of the mouse chromosome in which ALR2 (*Aldor1*) is mapped, corresponds to the q33-34 region of chromosome 7 of the human genome. The study also found that the mouse *Aldor1* gene is highly homologous to the fibroblast growth factor regulated protein gene (*Fgfrp*) and the androgen regulated vas deferens protein gene (*Avdp*). The importance of which is that there is a possibility that these closely related genes may have similar physiological function and may be implicated in the aetiology of diabetic complications.

The human ALR2 gene located on chromosome 7q35 extends over approximately 18 kilobases and consists of 10 exons which give rise to a 1,384 nucleotide mRNA (excluding the poly A tail). The ATG codon at nucleotide 37 encodes the first amino acid methionine, and the codon 985 signifies translation termination (Chung and LaMendola 1989). The human aldose reductase gene codes for a 316 amino-acid protein with a molecular mass of 35,858 Dalton's (Graham 1991 [a and b]). The size range of the exon's is 82 to 168 base pairs, whilst the size range for intron's is 325 to approximately 7,160 base pairs. Four Alu elements have also been found in the aldose reductase gene: two were found in intron 1 and one each in inton 4 and intron 9. An Sp1 sequence has also been shown to lie in the 5' untranslated region of exon 1. A major site for transcription initiation in human liver has been mapped to an A residue 31 nucleotides upstream from the A of the ATG initiation codon. The promoter region of the ALR2 gene has also been well characterised, firstly in the rat by Graham *et al* 1991 [a] and also in the human by Wang *et al* 1993. The promoter region of the ALR2 gene has been found to contain a regulatory TATA (TATTA) box and a CCAAT box which are located 37 and 104 nucleotides upstream, respectively, from the transcription initiation site (Graham *et al* 1991 [a]; Wang *et al* 1993). Wang *et al* 1993 characterised the human ALR2 gene promoter region and demonstrated that the basal promoter activity of the human aldose reductase gene is located between −192 and +31

upstream of the mRNA cap site. Several common *cis*-elements were isolated and point mutations in certain sequences such as the TATTTA sequence reduced promoter activity to 35% of the wild type DNA. This finding suggests that the TATA box is very important to the promoter activity of the ALR2 gene. The basal promoter region has also been found to contain a consensus sequence of an androgen response element (Wang *et al* 1993), three osmotic response element (ORE) sequences, a sequence homologous to a consensus Ap-1 site, and a microsatellite dinucleotide repeat sequence. A report by Ferraris *et al* 1994 investigated the rabbit ALR2 gene and found that a 235bp fragment within the promoter region of the ALR2 gene was able to drive the downstream reporter gene in transfected PAP-HT25 cells under isoosmotic conditions. Hyperosmotic stress induced a 40-fold increase in luciferase expression indicating an important role for osmotic response elements in diabetic complications. A number of genes have been localised to the 5'ALR2 region, these include endothelial nitric oxide synthase (eNOS), XRCC2 DNA repair, long QT2 interval (LQT2), skeletal muscle chloride channel and thromboxane synthase genes.

Polymorphisms of the ALR2 gene and its promoter region

Patel *et al* 1993 and Kicic *et al* 1993 investigated restriction fragment length polymorphisms at the human ALR2 gene locus. Patel *et al* 1993 used amplified regions of the ALR2 gene as probes to screen for restriction fragment length polymorphisms (RFLPs) in subjects with T1DM. Using 7 different restriction endonucleases (BamHI, SstI, HindIII, TaqI, MspI, EcoRI, and PstI) the study identified two polymorphisms. Firstly, a 3'ALR2/BamHI polymorphism within the ALR2 gene was found, however there was a complex hybridisation pattern as the probe detected both the functional and pseudo- ALR2 genes. Secondly, a PstI polymorphic site was found at the 5' end of the ALR2 gene in the non-coding region. No significant differences were found between any of the genotypes or alleles for 5'ALR2/PstI with either T1DM or microvascular complications. However, the 3'ALR2/BamHI polymorphism showed a statistically significant increase in frequency in

T1DM subjects with retinopathy compared to uncomplicated subjects (66.7% vs. 32.0% p<0.007), and nephropathy groups (66.7% vs. 34.9% p<0.006). This was also shown to be true when analysed in association with the previously identified TCRB polymorphism (Patel *et al* 1996).

To date there have been three reported biologically significant polymorphisms within the ALR2 gene and its promoter region. Firstly, a polymorphism which has provoked a great deal of interest has been the discovery of the (AC)n microsatellite dinucleotide repeat sequence polymorphism in the 5' region of the ALR2 gene. This CA dinucleotide repeat polymorphism designated 5'ALR2 is located approximately 2.1kb upstream of the initiation site of ALR2 close to the ORE sequences. Secondly, a C-106T polymorphism situated in the basal promoter region of the ALR2 gene has also been well investigated. Thirdly, and less well investigated, an A(+11842)C polymorphism within intron 8 of the ALR2 gene itself (figure 9). A fourth polymorphism has also been recently reported, this being a C(-12)G polymorphism reported in a Chinese population (Li *et al* 2002), although this has not been well investigated to date.
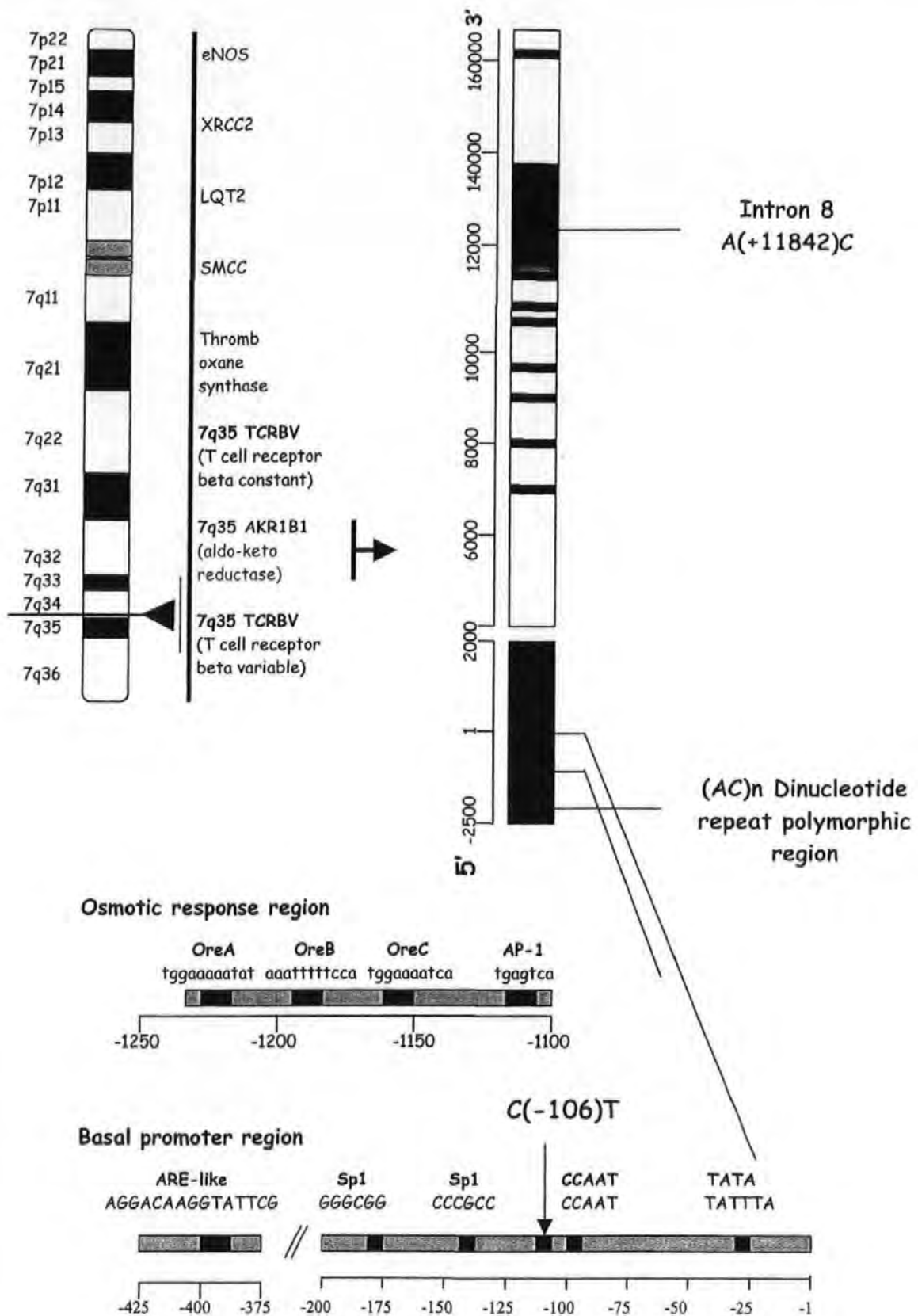
Figure 9. A schematic organisation of the promoter elements in the 5'ALR2 promoter region as described by Wang *et al* 1993

5'ALR2 (CA)n microsatellite

Ko *et al* 1995 studied the ALR2 gene promoter region and identified a highly polymorphic dinucleotide repeat marker (microsatellite) located 2.1 kilobases upstream of the transcriptional initiation site of the ALR2 gene as illustrated in figure 10.
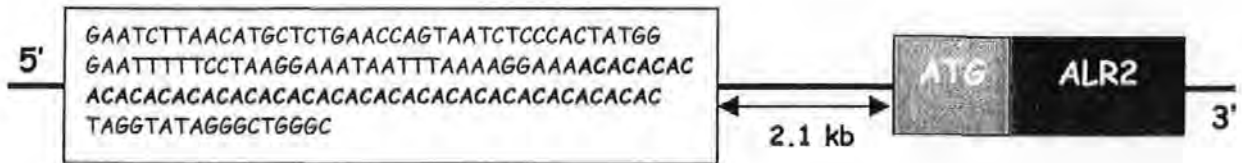
Figure 10. Chromosome 7q35 illustrating the location of the 5'ALR2 (CA)$_n$ microsatellite dinucleotide repeat polymorphism (Graham *et al* 1991 [c]; Ko *et al* 1995).

Many studies in various countries using population groups of varying ethnicity have investigated the 5'ALR2 polymorphism (table 5). From the published investigations between 7 and 12 alleles have been detected at the polymorphic site. The most common allele is the (AC)$_{24}$ repeat (138bp), designated as 'Z' allele. In some studies the (AC)$_{23}$ repeat (13xbp)has been associated with a high risk for diabetic complication, designated 'Z-2', and (AC)$_{25}$ repeat (13xbp) has been associated with a protective role, designated 'Z+2'. Discrepancies have however been reported to exist between studies, in particular between different ethnic groups and the different types of diabetes mellitus. The following section attempts to review the work that has been carried out to investigate this exciting polymorphism.

5'ALR2 (CA)$_n$ microsatellite and microvascular disease in T1DM

Investigations have been carried out to determine the relationship between the (CA)$_n$ polymorphism and microvascular disease in T1DM subjects. Diabetic retinopathy, nephropathy and neuropathy have been extensively investigated. Kao *et al* 1999 reported

that in a cohort of T1DM subjects of Caucasoid (Australian) origin which included 97 retinopathy subjects and 67 non retinopathy subjects the frequency of the Z-2 allele was significantly higher in those patients with retinopathy than those without retinopathy (P<0.0005). Negative findings have however also been made with regard to the onset of retinopathy in T1DM subjects. Chistyakov *et al* 1997 reported no associations of the ALR2 (CA)$_n$ polymorphism in T1DM subjects of Caucasoid (Moscow, Russia) origin and diabetic retinopathy. Yamamoto *et al* 1999 reported no association with retinopathy in T1DM subjects of Japanese (Oaska, Japan) origin. Iserman *et al* 2000 also looked at the (CA)$_n$ polymorphism in T1DM Caucasoid (Heidelberg, Germany) subjects with and without diabetic retinopathy. No significant differences were found in this study, however the Z+2 allele tended to be less frequent (11.5%) in subjects with retinopathy compared with T1DM subjects with no complications (17.8%), although this did not reach statistical significance.

Several studies have also investigated the (CA)$_n$ polymorphism in association with diabetic nephropathy in T1DM subjects and have found a statistically significant relationship. Importantly, a study carried out in our own laboratory using a collection of Caucasoid (Plymouth, UK) subjects with T1DM demonstrated a highly significant decrease in the frequency of the Z+2 allele in T1DM patients with nephropathy (Heesom *et al* 1997). The Z+2 5'ALR2 allele was significantly increased in subjects with no complications after 20 years duration of diabetes (uncomplicated) compared to those with nephropathy. This was accompanied by a significant increase in the Z-2 allele in the nephropathy group compared to those without. At least one copy of the Z+2 allele was found in 65% of the uncomplicated group to only 21.3% of the patients with nephropathy (odds ratio 7.1). These results indicate that the dinucleotide repeat polymorphism at 5' flanking region of the ALR2 gene might play a role in the regulation of the constitutive expression of ALR2 in T1DM and thus provide a useful genetic marker for diabetic microangiopathy. Another

groundbreaking study by Shah *et al* 1998 also found significant associations with this polymorphism in two different study cohorts. Firstly, in a Caucasoid (USA) cohort the prevalence of the Z-2 allele among T1DM subjects was increased in those with diabetic nephropathy where 60% of subjects with nephropathy v. 22% T1DM subjects without nephropathy were homozygous for Z-2. The second cohort of subjects with T1DM of Caucasoid/Hispanic origin (Milan, Italy) origin also showed the same trend. In this cohort the prevalence of the Z-2 allele was higher in the subjects with diabetic nephropathy than in the diabetics without nephropathy, where 81% of subjects with diabetic nephropathy had one or more copies of the Z-2 allele. This study did not find any association with the Z+2 allele and subjects without nephropathy. Moczulski *et al* 2000 also furthered this line of evidence by investigating the (CA)n polymorphism in Caucasoid (Boston, USA) T1DM subjects with and without diabetic nephropathy. The study showed that the Z-2 allele was significantly more frequent in cases than controls (38% vs. 28.8%, $P = 0.005$), and carriers of the Z-2 allele had a higher risk of diabetic nephropathy than non-carriers. Yamamoto *et al* 1999 also looked at this polymorphism in T1DM subjects of Japanese (Osaka, Japan) origin. Conversely this study found that there was a higher increase in the frequency of nephropathy in subjects with the Z+2 allele, and also that homozygosity for the Z+2 allele was significantly associated. This therefore leads to some discrepancy between positive association results.

Several other studies however, do not agree with the (CA)n results seen in the previously mentioned studies into diabetic nephropathy. Chistyakov *et al* 1997 also investigated the (CA)n polymorphism in a smaller cohort of Caucasoid (Moscow, Russia) T1DM subjects (29 no nephropathy vs. 20 diabetic nephropathy), and found no significant associations with diabetic nephropathy. Dyer *et al* 1999 also investigated whether the 5'ALR2 (CA)n microsatellite marker could be used as a genetic marker for susceptibility to diabetic nephropathy by analysing 442 British Caucasoids with T1DM. PCR was used to amplify

the DNA followed by size fractionation with a sequencer and genotyped. They detected nine alleles, and found no significant difference in allele frequencies between patient groups. Specifically they also found no significant difference in the frequency of the reported associated allele Z-2, and the protective allele Z+2. Isserman *et al* 2000 also looked at T1DM subjects of Caucasoid (Germany) origin with and without diabetic nephropathy and also found no significant increase in incidence of Z-2 (CA)23 allele between groups. The study did show a tendency that the Z and Z+2 alleles were slightly lower in the group with nephropathy compared to the group with no complications but no statistical significance was achieved. Furthermore, the Caucasoid cohorts (North of England and Belfast) T1DM subjects included in the study carried out by Neamat-Allah *et al* 2001 also showed no evidence of an association between the microsatellite marker (CA)$_n$ alleles and diabetic nephropathy. Additionally, stratification of the Belfast cohort in this study according to HbA1c also revealed no evidence of altered risk of diabetic nephropathy in Z-2 carriers vs. non Z-2 carriers. Ng et al 2001 carried out the same studies using Caucasoid (Australia) T1DM subjects and found no associations with diabetic nephropathy. Reporting that despite the study being relatively small with regard to the size of the cohort, the 5'ALR2 (CA)n polymorphism is not useful as a genetic marker for susceptibility to nephropathy in Caucasoid T1DM subjects.

A positive association has also been shown in Caucasoid (Plymouth, UK) T1DM subjects with diabetic neuropathy (Heesom *et al* 1998). The study again carried out by our laboratory found that there was a highly significant decrease in the frequency of the Z+2 allele in those patients with overt neuropathy compared to those with no neuropathy after 20 years duration of diabetes. The neuropathy group also had a significant decrease in the frequency of the Z/Z+2 genotype compared to subjects without diabetic neuropathy. This finding was not supported in the Caucasoid (Australia) neuropathy T1DM subjects investigated by Ng *et al* 2001.

5'ALR2 (CA)$_n$ microsatellite and microvascular disease in T2DM

There have been several studies reported which have found positive associations between the (CA)$_n$ polymorphic region and certain diabetic microvascular complications in T2DM subjects of varying ethnicity. Studies into the (CA)n polymorphism in T2DM and diabetic retinopathy have however lead to conflicting reports being published. Firstly, the pioneering study carried out by Ko *et al* 1995 suggest that the gene encoding aldose reductase (ALR2) may be involved in the early onset of diabetic retinopathy in T2DM patients. The study showed that the (AC)$_n$ dinucleotide repeat polymorphism 2.1kb upstream of the promoter region of the ALR2 gene is associated with early onset diabetic retinopathy in 44 unrelated Chinese (Hong Kong) patients with T2DM and nearly identical HbA1c values. They found seven alleles at this locus and observed a strong association between the (AC)$_{23}$ (also called 'Z-2') allele and early onset diabetic retinopathy in patients with T2DM (59%), compared with the group with no complications (9%). The results strongly suggest that the (AC)$_{23}$ allele was related to early appearance of retinal microangiopathy. This report therefore suggests that aldose reductase or a gene in the close vicinity might be involved in the pathogenesis of this diabetic complication in T2DM subjects of Chinese origin. Secondly, Fulisawa *et al* 1999 carried out a study of the 5'ALR2 microsatellite in T2DM subjects of Japanese origin. Eleven different alleles were observed in this study and it was reported that the length of the alleles was significantly different between the two groups. It was suggested that the frequency of the shorter alleles (equal to or shorter than Z-4) were more associated with retinopathy than the longer alleles (greater than Z-4). The molecular mechanism associating the length polymorphism is ill understood, although it has been suggested that the dinucleotide repeat length may directly effect ALR2 gene expression through enhancer/promoter interaction. Alternatively, the allelic variation may be in linkage disequilibrium with an unknown aetiological mutation, which may affect the expression or function of aldose reductase. The line of thought which is suggested is that the shorter form of the dinucleotide repeat is associated with disease

phenotype. Thirdly, a small study of 27 Spanish T2DM subjects was carried out by Olmos *et al* 1999 reporting that the absence of the $(AC)_{24}$ allele of the $(AC)_n$ polymorphic marker was associated to a five fold reduction of retinopathy appearance rate. A later report by the same group (Olmos *et al* 2000) also tested the hypothesis that the $(AC)_{23}$ allele of the ALR2 gene is related to an enhanced rate of progression of retinopathy in Chilean Caucasoid T2DM subjects. The longitudinal-retrospective study showed that the subjects with the $(AC)_{23}$ allele had a retinopathy progression rate that was 8.9 times higher than that of the diabetics having other alleles. A fourth study carried out by Ikegishi *et al* 1999 also investigated the $(CA)_n$ polymorphism in 27 T2DM subjects with retinopathy and 34 T2DM subjects without retinopathy and 96 controls of Japanese origin. The study identified 10 alleles and found a significant difference in the frequency of the Z-4 and Z-2 alleles. The frequency of the Z-4 allele was seen to be significantly greater in subjects with proliferative retinopathy, whereas the Z+2 allele was significantly greater in subjects without retinopathy. The study also found, using transfection experiments, that the Z-4 allele showed significantly higher transcription of the reporter gene. A fifth study by Ichikawa *et al* 1999 carried out an investigation using microsatellite PCR and direct sequencing methods, to assess the association of the 5'ALR2 (CA)n locus and diabetic microvascular complications in Japanese patients with T2DM. They were able to identify six alleles, and found no significant difference in allele distribution between diabetic patients and controls. The Z-2 allele frequency was however, significantly higher in subjects with diabetic retinopathy than those without retinopathy, suggesting that aldose reductase is involved in the development of diabetic retinopathy. In contrast however the microsatellite marker was not associated with diabetic nephropathy, peripheral or autonomic neuropathy. But they concluded that the discrepancy might be partly attributable to the low frequency of Z+2 allele in the Japanese subjects.

Contradictory to these findings however, several groups investigating the $(AC)_n$ polymorphic region in T2DM subjects with retinopathy have found no significant associations. Maeda *et al* 1999 studied a population of Japanese T2DM subjects and found no association between any of the alleles and diabetic retinopathy. Groves *et al* 1999 investigated 250 British Caucasoid T2DM subjects with retinopathy which were carefully matched with controls, and found no association with the $(CA)_n$ polymorphism. Lee *et al* 2001 [a] investigated 384 late onset T2DM subjects of Hong Kong Chinese origin and identified 10 alleles at the $(CA)n$ polymorphic region. The study found no significant differences in allelic distributions of the Z+2/ Z/ Z-2 allelic variants between subjects with and without diabetic retinopathy. However, the study did report an increased presentation of the Z-4 allele in T2DM subjects with retinopathy compared to those without (9% vs. 4%, $p < 0.05$).

Studies into T2DM and diabetic nephropathy have so far also produced positive and negative correlation's. Maeda *et al* 1999 studied a population of Japanese T2DM subjects and found no association between any of the alleles and nephropathy, although ALR2 protein content was increased in subjects with nephropathy. The T2DM subjects of British Caucasoid or Pima Indian origin investigated by Neamat-Allah *et al* 2001 showed no significant associations of either the Z-2 or Z+2 alleles with diabetic nephropathy. The study also carried out a meta-analysis of all the published data for the $(CA)n$ polymorphism and revealed no evidence for an effect of the microsatellite marker in T2DM subjects with nephropathy. The Olmos *et al* 2000 study investigated the nephropathy progression rate in T2DM Caucasoid subjects with and without the $(AC)_{23}$ allele. Although the mean nephropathy progression rate was higher in the $(AC)_{23}$ group no statistically significant differences were found. Moculski *et al* 1999 examined the association between the 5'ALR2 $(CA)n$ polymorphism and diabetic nephropathy in a large group of Caucasoid patients with T2DM which consisted of 179 normoalbuminuria

subjects, 225 microalbuminuria subjects and 70 proteinuria subjects. They also found no association between the Z-2 allele and nephropathy in Caucasoid T2DM subjects. Park *et al* 2002 investigated the relationship between the aldose reductase gene polymorphisms and microvascular complications in Korean patients with T2DM, and found no associations with the frequencies of the Z-2 and Z+2 alleles between subjects with and without diabetic nephropathy and retinopathy. A recent study however, carried out by Liu *et al* 2002 has found an association between the 5'ALR2 (CA)n polymorphism in the development of diabetic nephropathy in Southern Chinese with T2DM. In this study the normoalbuminuric subjects had the lowest Z-2/X genotype frequency 18.8% vs. 36.5% in microalbuminuric (p<0.001), and 38.5% in albuminuric patients (p=<0.02).

5'ALR2 C(-106)T polymorphism

A bi-alleleic polymorphism situated close to the osmoregulatory element (ORE) has also been identified and located to position −106. It has been found to be a C to T substitution (C-106T) at this position in the 5' flanking region of the ALR2 gene (figure 11).
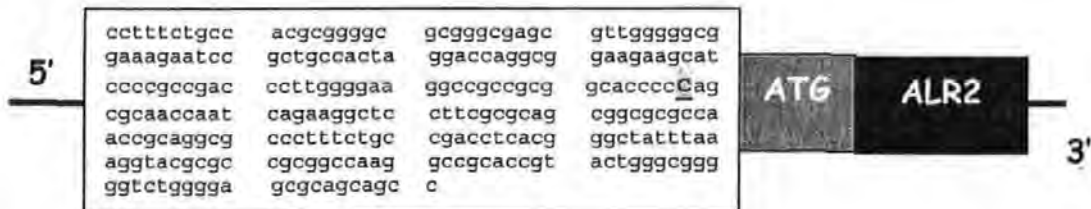
Figure 11. Chromosome 7q35 illustrating the location of the 5'ALR2 C(-106)T polymorphism (Graham *et al* 1991 [a and c]).

In a study by Kao *et al* 1999, young adolescent Caucasoid (Australia) T1DM subjects with and without diabetic retinopathy were investigated. This study suggested that the C(-106) allele was associated with the Z-2 allele in subjects with retinopathy, and conversely that both the C and the T alleles were associated with the Z+2 allele in the uncomplicated group. However, Moczulski *et al* 2000 showed, in a Caucasoid (North American) population of T1DM subjects with diabetic nephropathy, that the T(-106) allele was

associated with the Z-2 allele. The Z-2/T haplotype was also shown to have a higher incidence in the T1DM subjects with nephropathy than in T1DM subjects without nephropathy. A more recent study by Neamat-Allah *et al* 2001 reported a significant association between the T allele of the ALR2 –106 marker and diabetic nephropathy in both T1DM and T2DM subjects of Caucasoid (UK) origin. The Pima Indian cohort also investigated did not however show any statistical correlations. They also found that the Z-2 allele was positively associated with the T allele, and the Z allele was positively associated with the C allele in T1DM and T2DM subjects. Also the carriage of the combination of both Z-2 and –106T was increased in subjects with diabetic nephropathy compared to subjects without diabetic nephropathy, although not to statistical significance. Li *et al* 2002 have also carried out a study into the C(-106)T polymorphism using a population of Chinese subjects and controls. This study found that there was an association with the C(-106)T polymorphism and diabetic retinopathy in the Chinese population.

ALR2 A(+11842)C intragenic polymorphism

A single base substitution A(+11842)C has been previously described within intron 8 of the ALR2 gene (Kicic *et al* 1993; Patel *et al* 1996; Patel *et al* 1993). The A(+11842)C polymorphism is a silent substitution that creates a new Bam H1 restriction endonuclease site (figure 12). Kicic *et al* 1993 found an association between the (+11842)C allele and proliferative retinopathy patients with T1DM. Kao *et al* 1999 also examined DNA from 164 adolescents with T1DM for the A to C substitution at the 95th nucleotide of intron 8 of the ALR2 gene. The authors named the alleles as 'B' where the restriction enzyme cut and 'b' where it did not. Kao *et al* 1999 reported that this polymorphism was strongly associated with diabetic retinopathy in adolescent T1DM subjects of Caucasoid (Australia) origin.
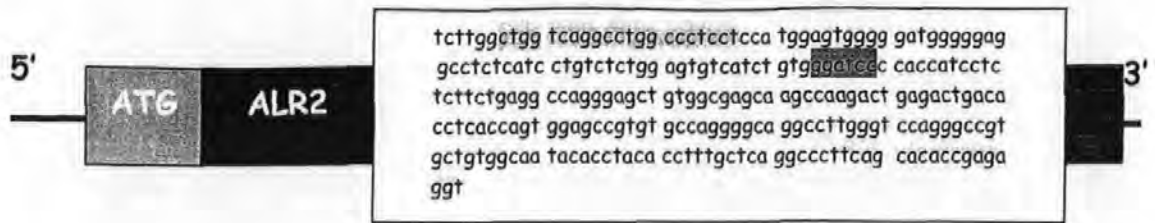
Figure 12. Chromosome 7q35 illustrating the location of the ALR2 A(+11842)C polymorphism (Graham *et al* 1991 [a and c]).

Previous studies by Patel et al 1993/1996 using restriction fragment length polymorphism analysis had studied this BamH1 site and found an association with microvascular complications. It is clearly important to understand the relationship and strength of the association of the A(+11842)C site to the 5'ALR2 locus and relevance to the susceptibility to diabetic complications.

Author	Population	Diabetes	Complication	Polymorphisms studied	Association
T1DM					
Isermann et al 2000	Caucasoid (Heidelberg, Germany)	T1DM	Retinopathy	(CA)n	-ive
			Nephropathy	(CA)n	-ive
			CHD	(CA)n	+ive
Ng et al 2001	Caucasoid (Australian)	T1DM	Nephropathy	(CA)n	-ive
			Neuropathy	(CA)n	-ive
Kao et al 1999a	Caucasoid (Australian)	T1DM	Retinopathy	(CA)n	+ive
				C(-106)T	+ive
Kao et al 1999b	Caucasoid (Australian)	T1DM	Retinopathy	(CA)n	+ive
				A(+11842)C	+ive
Heesom et al 1997	Caucasoid (Plymouth, UK)	T1DM	Nephropathy	(CA)n	+ive
Heesom et al 1998	Caucasoid (Plymouth, UK)	T1DM	Neuropathy	(CA)n	+ive
Dyer et al 1999	Caucasoid (Birmingham, UK)	T1DM	Nephropathy	(CA)n	-ive
Neamat-Allah et al 2001	Caucasoid (Belfast, UK)	T1DM	Nephropathy	(CA)n	-ive
				C(-106)T	+ive
	Caucasoid (N of E, UK)	T1DM	Nephropathy	(CA)n	-ive
				C(-106)T	+ive
Shah et al 1998	Caucasoid/Hispanic (USA)	T1DM	Nephropathy	(CA)n	+ive
	Caucasoid (Milan, Italy)	T1DM	Nephropathy	(CA)n	+ive
Chistyakov et al 1997	Caucasoid (Moscow, Russia)	T1DM	Retinopathy	(CA)n	-ive
			Nephropathy	(CA)n	-ive
Yamamoto et al 1999	Japanese (Osaka, Japan)	T1DM	Nephropathy	(CA)n	+ive
			Retinopathy	(CA)n	-ive
Moczulski et al 2000	Caucasoid (Boston, USA)	T1DM	Nephropathy	(CA)n	+ive
				C(-106)T	+ive
T2DM					
Groves et al 1999	Caucasoid	T2DM	Retinopathy	(CA)n	-ive
Neamat-Allah et al 2001	Caucasoid (N of E, UK)	T2DM	Nephropathy	(CA)n	-ive
				C(-106)T	+ive
	Pima Indian (Arizona, USA)	T2DM	Nephropathy	(CA)n	-ive
				C(-106)T	-ive
Moczulski et al 1999	Caucasoid (Boston, USA)	T2DM	Nephropathy	(CA)n	-ive
Olmos et al 1999	Caucasoid (Madrid, Spain)	T2DM	Retinopathy	(CA)n	+ive
Olmos et al 2000	Caucasoid (Madrid, Spain)	T2DM	Retinopathy	(CA)n	+ive
Ko et al 1995	Chinese (Hong Kong, China)	T2DM	Retinopathy	(CA)n	+ive
Lee et al 2001 [a]	Chinese (Hong Kong, China)	T2DM	Retinopathy	(CA)n	-ive
Li et al 2002	Chinese (Changsha, China)	T1DM	Retinopathy	C(-106)T	+ive
				C(-12)G	
Liu et al 2002	Chinese (Hong Kong, China)	T2DM	Nephropathy	(CA)n	+ive
Qingjie et al 2002	Chinese (Xiangya, China)	T2DM	Retinopathy	C(-106)T	+ive
				C(-12)G	+ive
Fujisawa et al 1999	Japanese (Osaka, Japan)	T2DM	Retinopathy	(CA)n	+ive
Ichikawa et al 1999	Japanese (Kurume, Japan)	T2DM	Retinopathy	(CA)n	+ive
			Nephropathy	(CA)n	-ive
			Neuropathy	(CA)n	-ive
Ikegishi et al 1999	Japanese (Yamanashi, Japan)	T2DM	Retinopathy	(CA)n	+ive
Maeda et al 1999	Japanese (Shiga, Japan)	T2DM	Nephropathy	(CA)n	-ive
Park et al 2002	Korean (Seoul, South Korea)	T2DM	Retinopathy	(CA)n	-ive
			Nephropathy	(CA)n	-ive

Table 5. Summary of published studies of the ALR2 polymorphisms within the ALR2 gene and its promoter region in T1DM and T2DM.

Family based studies

Moczulski *et al* 2000 was the first to report the investigation of the ALR2 promoter region polymorphisms in a family based study using the transmission disequilibrium test (TDT). Families used in this study had T1DM with or without persistent proteinuria or diabetic nephropathy, and were of Caucasoid (USA) origin. The study found that transmission of the Z-2 allele was 54% in case trios and 45% in control trios, which although show consistency with previous case control studies, did not reach statistical significance. Also the transmission of the T allele for the C(-106)T polymorphism was 54% in case trios and 37% in control trios, again not reaching statistical significance. The Z-2/T haplotype was transmitted from heterozygous parents 57% in the nephropathy trios and 47% in control trios. In should be noted however, that due to the small numbers involved in this study the power to detect any linkage was somewhat reduced and may have little efficacy in this study.

ALR2 promoter region polymorphisms and gene expression

Shah *et al* 1997 previously reported that aldose reductase mRNA levels in PBMC's were 3-fold higher in T1DM patients with diabetic nephropathy than in those without nephropathy, and also that mRNA levels were not elevated in non-diabetics with renal disease. Shah *et al* 1998 continued this investigation by exploring the possibility of a link between the 5'ALR2 microsatellite polymorphism Z-2, and increased levels of ALR2 expression in diabetic nephropathy. Their studies found that in both Caucasoid/Hispanic (USA) and Caucasoid (Milan, Italy) T1DM subjects, those with one or more copy of the Z-2 allele also had higher ALR2 mRNA levels than did diabetics without the Z-2 allele. In contrast, among non-diabetics the ALR2 mRNA levels were similar in subjects with and without one or more copy of the Z-2 allele.

These findings are extremely exciting because firstly, the 5'ALR2 marker is associated with diabetic nephropathy in those patients with T1DM and is linked to this region in T2DM. This demonstrates that the genetic lesion is independent of the cause of diabetes. Secondly, it occurs across ethnic groups, which is further evidence that ALR2 plays a critical role in the susceptibility to, and pathogenesis of diabetic nephropathy. Thirdly, the Z-2 allele is associated with up-regulation of ALR2 gene expression in patients with diabetic nephropathy. The mechanism in which these polymorphisms regulate aldose reductase gene expression is not known. However it was suggested that because the 5'ALR2 microsatellite is located in the 5' region of the gene, different populations might be associated with differential binding of transcription factors. It may also be that the expression of other genes near this locus may be altered and may also modulate the risk for diabetic complications. Several other sequence variants have also now been reported but have not been so extensively investigated (Moczulski *et al* 2000; Kao *et al* 1999). It is clear from this review of the literature regarding the polymorphisms within the ALR2 gene and its promoter region that more work is now required to confirm these results in family based studies, and in addition to establish whether the ALR2 polymorphisms has a functional role in diabetic complications.

Cellular osmotic regulation and diabetic complications

Changes in extracellular osmolality such as the effect of hyperglycaemia results in osmotic stress due to unequal rates of movement of water and solutes across the cell membrane. The osmotic balance between intracellular and extracellular compartments is critical for the maintenance of cellular homeostasis. Cells respond to osmotic stress by osmoregulation compensatory mechanisms such as changes in cell volume, water content, and intracellular solute concentration. An increase in extracellular glucose levels leads to increase in the flux through polyol pathway, and probably an increase in activity of aldose reductase leading to an intracellular accumulation of sorbitol. The pathway is driven by increased intracellular glucose availability leading to sorbitol accumulation (Gabbay *et al* 1973). Abnormally high levels of osmolytes can be deleterious to cells, for example an accumulation of sorbitol has been reported to be a cause of cataract formation (Lee 1995). In target tissues of diabetes such as kidney, nerve, and eye, sorbitol accumulation is thought to exert a hyperosmotic effect that contributes to some complications of diabetes (Bhatnagar and Srivastava 1992). Several studies have shown that sorbitol accumulates in renal medulla cells and other cell types when cultured in vitro under hypertonic conditions, this response reaction therefore extends and increases the sorbitol accumulation and adds to the risk of developing complications. Osmoregulatory mechanisms occur throughout the human body but are known to be predominantly located in the renal medulla, this is because renal medullary cells are routinely exposed to a greatly hyperosmotic milieu. Because of the urinary concentrating mechanisms, cells in the inner mammalian renal medulla are constantly exposed to steep osmotic gradients by varying concentrations of salt and urea that may reach molar level. Similar pathways have been observed in other non-renal cells in the body, suggesting that these are a common response to hyperosmotic stress. The epithelial cells lining the inner renal medulla of the kidney are protected from the osmotic effect of concentrated sodium ion and urea in the interstitium by several regulatory mechanisms described below: -

(a) Cell volume and ionic electrolye concentration regulation

Cells in the kidney can volume regulate in response to hypertonic challenge. Some are able to respond immediately by RVI, other cells require stimulation prior to exposure to hyperosmolarity to demonstrate RVI. An increase of intracellular osmolytes during RVI usually occurs by an increase of NaCl influx either via the activation of parallel Na(+)-H+ and Cl(-)-HCO3- exchangers, or Na(+)-K(+)-2Cl- co-transporters. In response to hypotonic challenge a RVD response occurs in kidney cells. The efflux of solute during RVD is usually via K+ loss by activation of conductance pathways. Stretch-activated K+ channels and Ca2(+) –activated –K+ channels have been shown to be stimulated in cells exposed in hyposmotic solutions (Montrose-Rafizadeh *et al* 1990).

(b) Regulation of urea concentration

The deleterious effect of urea on enzymatic activities has been demonstrated in vitro on several enzymes including aldose reductase (Burg *et al* 1999). A high level of expression of the urea transporters (UT-A) is normally observed in the renal medulla and is further expressed by water depravation (Bagnasco *et al* 2000), similar to the genes involved in the osmoregulation of inner medullary cells. This suggests that the same regulatory pathways contribute to maintain a steady level of expression of several genes in inner medulla under physiological conditions (Bagnasco 2000).

(c) Accumulation of compatible organic osmolytes

Exposure to anisotonic media initiates a response that counteracts volume perturbations by complex mechanisms involving changes in the intracellular concentrations of active organic solutes (osmolytes). Medullary and papillary cells use these organic solutes to increase the intracellular concentration. These cells are protected from the osmotic effect of concentrated sodium ion and urea in the interstitium by accumulating compatible osmolytes. This process enables normal cell volume to be maintained without a deleterious

increase in intracellular inorganic ion (electrolyte) concentration and enables the cell volume and osmolality to be preserved without perturbing macromolecular protein structure and function over a wide range of concentrations (Yancey *et al* 1982). The accumulation of such osmolytes is primarily dependent on changes in gene expression of enzymes involved in the synthesis of osmolytes or transporters that uptake them into the cell. Hyperosmotic stress has been shown to induce the transcription of a number of proteins, which include aldose reductase and the betaine, myo-inositol, and taurine transporters (Garcia-Perez and Burg 1991; Burg *et al* 1995). The organic osmolytes that rabbit and rat kidney cortex and medulla cells accumulate include sorbitol, glycine betaine, inositol, taurine, and glycerophosphocholine (GPC) and were identified by proton nuclear magnetic resonance, mass spectrometry, and chemical assays (Bagnasco *et al* 1986). By accumulating these osmolytes the renal medullary cells maintain both their volume and their intracellular medium unperturbed under hyperosmotic stress (Somero 1986; Martin *et al* 1989; Garcia-Perez and Burg 1991). Sorbitol is produced within the cell via the reduction of glucose by aldose reductase, betaine, myoinositol, and taurine are transported into the cells by betaine/γ-aminobutyric acid transporter (BGT1), Na+-dependent myoinositol transporter (SMIT) and taurine transporter respectively (Burg 1995).

Signal pathways involved in the gene expression of osmolytes

Regulation of gene expression in response to hyperosmolality has been well investigated in the prokaryotic organism's *Escherichia coli* and *Salmonella typhimurium*. Prokaryotes exhibit a variety of mechanisms responsible for osmosensory signal transduction. The prominent response to hyperosmotic stress is uptake of K^+ and compatible osmolytes. These organisms have three transport systems for the uptake of K^+, namely Trk, Kup, and Kdp, and two transporters with overlapping specificity's for proline and glycine betaine namely ProP and ProU (Csonka *et al* 1996). In response to hyperosmotic stress, there is an increase in the expression of the Pro-U operon, which encodes a high affinity betaine

transporter (Cairney *et al* 1985), resulting in an increase in the activity of the ATP-consuming betaine transporter and accumulation of betaine (Lucht *et al* 1994). The DNA binding protein (H-NS) acts as a transcriptional repressor of the Pro-U operon under isosmotic conditions. The inhibitory effect of H-NS is relieved by extracellular hyperosmolality resulting in transcription of the operon (Ueguchi and Mizuno 1993).

The mechanism of osmoregulation of gene expression in response to hyperosmolality is also better understood in yeast *(Saccharomyces cerevisiae)* which involves a two-component signalling transducer system as an osmolality sensor (Maeda *et al* 1994, 1995; Posas *et al* 1996; Kultz *et al* 1997). The yeast responds to osmotic stress by enhanced production and intracellular accumulation of glycerol as a compatible solute. When exposed to hyperosmotic medium the stress signal is transduced through the MAP (HOG1) kinase cascade to induce the synthesis of glycerol-3-phosphate dehydrogenase (GPD) (Albertyn *et al* 1994). Exposure of yeast to high extracellular osmolarity induces Sln 1p-Ypd1p-Ssk1p two component osmosensor to activate the mitogen activated protein (MAP) kinase cascade composed of the Ssk2p and Ssk22p MAP kinase kinase kinases (MAPKKK's), the Pbs2p MAPKK, and the Hog1p MAPK. A second osmosensor, Sho 1p, also activated Pbs2p and Hog 1p, but does so through the Ste 11p MAPKKK *(Posas and Saito 1997)*.

In mammals, extracellular hyperosmolality also tightly regulates the expression of several genes. Hypertonicity is known to induce the expression of a group of genes that are responsible for the intracellular accumulation of protective organic osmolytes (Burg *et al* 1997). Expression and accumulation of osmolytes within the cell is due to up-regulated expression of the betaine (BGT-1) gene and myo-inositol transporters (SMIT), and aldose reductase (ALR2) which occurs in response to extracellular NaCl and non-permeable osmotically active solutes (Bagnasco *et al* 1987). Hyperosmolality is known to increase the

transcription of these genes, leading to increased mRNA abundance, enzyme levels and accumulation of compatible osmolytes (Burg 1997). How the initial signal of external hyperosmolality is relayed to the nucleus to induce the expression of genes responsible to increase cellular osmolytes is still not completely clear. However some degree of progress has been made in the venture to elucidate this and will be discussed here. Signal transduction pathways relate signals from the cell membrane across the cytoplasm to the nucleus in order to initiate an appropriate transcriptional response. The osmotic stress of hyperglycaemia is the extracellular stimuli, which initiates the stress response pathways. Osmotic shock induces marked activation of mitogen activated protein kinase (MAPK's) in fibroblastic cells (Matsuda et al 1995). Activation of transcription response to hyperosmolarity has been described for three MAPK pathways; (1) mitogenic extra-cellular regulated kinase (ERK 1/2) cascade, and the less well understood pathways ERK3 and ERK5 cascades, (2) stress activated protein kinase (SAPK2) c-Jun N-terminal kinase (JNK), (3) p38 kinases. These MAPK cascade pathways function as signal transducers from the cell surface to the nucleus, and play an important role in transducting environmental stimuli to the transcriptional machinery in the nucleus. The MAP kinase cascade is highly conserved in all eukaryotes and is involved in numerous cellular responses (Wilson et al 199; Karin 1998). Activation of the MAPK cascade plays essential roles in many signal transduction pathways. Investigations by Reiser et al 1999 revealed that at least three events contribute to signal induced nuclear localisation of the MAP kinases; activation by phosphorylation, regulated nuclear import and export, and nuclear retention. Hypertonicity-induced activation and binding of transcription factors to the ORE are regulated by the p38 kinase and MEK-1 signalling pathways (Nadkarni et al 1999). MAPK has been demonstrated to phosphorylate and regulate numerous cellular proteins, including growth factor receptor, transcription factors, cytoskeletal proteins, phospholipase and other protein kinases. Evidence also suggests that acute activation of the MAPK

cascade promote G1 progression/S phase entry and that chronic activation of the MAPK cascade inhibits this process (Tombes *et al* 1998).

The growth factor signalling kinases include the ERK's. The mitogen-activated protein (MAP) kinases ERK-1/ ERK-2 are proline directed kinases that are themselves activated through concomitant phosphorylation of tyrosine and threonine residues (Han *et al* 1994). ERK activity was not found to be essential for transcriptional regulation of betaine and inositol transporters (Kwon *et al* 1995). The stress activated protein kinase (SAPK1) pathway transmitting cellular insult to the nucleus to influence gene expression consists of four levels of protein kinases. Unlike the structurally related MAPK pathway, the stress-induced kinases are not required for mitogenesis and instead induce growth arrest (Woodgett *et al* 1996). JNK's respond to several forms of cellular stress including inflammatory cytokines such as Interleukin-1 (Il-1) and tumour necrosis factor-alpha (TNF-alpha), heat and chemical shock, bacterial endotoxin and ischaemia/cellular ATP depletion (Wilson *et al* 1997). The signal transduction pathway c-Jun N-terminal kinase (JNK*)* cascade is known to be a cellular stress response to stimulation by UV light, oxidative stress and inflammation, and involves phospholipase C, Raf-1 kinase, mitogen activated protein kinase kinase and mitogen activated protein kinase (Guan 1994; Kyriakis *et al* 1994). JNK has three subforms coded 1, 2 and 3, and each subform has multiple isoforms; four for JNK1, four for JNK2 and two for JNK3 (Ip and Davis 1998). P54 is the principle JNK that is activated by cellular stress and tumour necrosis factor (TNF)-alpha (Itoh *et al* 1994). P38 is tyrosine phosphorylated after extracellular changes in osmolarity (Han *et al* 1994). Four subtypes have been cloned for p38; $\alpha,\beta,\gamma,\delta$ (Wang *et al* 1997). P38 kinase mediates the activation and/or binding of the transcription factors to the ORE (Nadkarni *et al* 1999). Nadkarni *et al* 1999 showed that the hypertonicity induced ALR2 mRNA increase in HepG2 cells is attenuated by the p38 kinase and MEK1 inhibitors. Transfection studies also showed that the specific locus of action of the inhibitors used is

the activation of the ORE in response to hypertonicity. They also demonstrated the involvement of p38 kinase in the binding of trans-acting elements to the ORE. Their studies therefore indicate that the hypertonic induction of ALR2 mRNA in HepG2 cells is regulated by p38 kinase and MEK1 and is mediated by the ORE.

Gene regulation of ALR2 under osmotic stress

Animal studies investigating the regulation of ALR2 gene expression by hypertonicity are numerous. Cowley *et al* 1990 carried out a study using Brattleboro rats and showed that an alteration in extra-cellular sodium, but not urea leads to a rapid change in aldose reductase mRNA. Russell 1990 studied a lens epithelial cell line from a transgenic mouse and Kaneko *et al* 1990 studied Chinese hamster ovary cells and both studies found similar results. Smardo *et al* 1992 investigated into osmotic regulation using a line of rabbit inner medullary cells (PAP-HT25) that accumulate large amounts of sorbitol under hyperosmotic conditions. They demonstrated that extracellular hyperosmolarity induces transcription of the ALR2 gene resulting in an increase in ALR2 mRNA followed by increased ALR2 protein synthesis rate and subsequent rise in sorbitol levels (Garcia-Perez *et al* 1989; Uchida *et al* 1989). Hypertonicity in PAP-HT25 cells has been shown to increase synthesis of ALR2 mRNA 15-fold in 24 hours without a detectable change in the rate of degradation of the ALR2 protein (Moriyama *et al* 1989). Grunewald *et al* 1998 studied the inner medullary collecting duct (IRMC) cells from the rat using an RT-PCR-based strategy, and found that increasing the extracellular osmolarity from 600 to 900 mosm/l resulted in a more than 4-fold increase in mRNA for ALR2 within 24 h. Recently, important findings were made by Meakawa *et al* 2001 who investigated ALR2 mRNA levels in rat Schwann cells cultured under hyperglycaemic or hyperosmotic conditions. This study showed that the expression of ALR2 mRNA was unaltered by hyperglycaemia (30mM), but that osmotic stress elicited significant increases in ALR2 mRNA.

An osmoregulatory role of ALR2 has also been suggested by studies in cell lines derived from human renal inner medulla showing that increases in the osmolality of the medium is associated with increases in cellular sorbitol levels, ALR2 activity and ALR2 gene expression. Bedford et al 1987 identified aldose reductase to be an osmoregulatory protein, which was induced, in the renal medullary cells by high extracellular NaCl. Similarly, Bagnasco et al 1987 reported on a line of renal medullary cells in which aldose reductase activity and intracellular sorbitol was greatly increased under high NaCl conditions. Conversely Cowley et al 1990 reported that ALR2 mRNA and sorbitol content increase during dehydration or antidiuresis and that acute decreases in extracellular sodium increases leakage of sorbitol from the renal medulla cells into the extracellular environment. Several other studies carried out by several different groups also reported similar findings. Kaneko et al 1990 using Kidney mesangial cells, Hohman et al 1990 using glomerular endothelial cells, Bekhor et al 1989 using lens epithelial cells and Ferraretto et al 1993 using human embryonic epithelial cells and Petrash et al 1992 studied human renal proximal tubule cells.

Osmotic Response Element (ORE) / Tonicity Enhancer Element (TonE)

Hyperosmolarity responsive expression of the genes involved occurs by stimulation of transcription requiring formation of a DNA-protein complex between a tonicity-responsive enhancer (TonE/ORE) and the respective binding proteins, TonE binding protein (TonEBP) or nuclear factor of activated T cells 5 (NFAT5). Osmotic response element binding protein (OREBP), the tonicity enhancer element binding protein (TonEBP) and nuclear factor of activated T cells 5 (NFAT5) mRNA are ubiquitously expressed and are detectable in the kidney, brain, liver, spleen, gonads and skeletal muscle (Lopez-Rodriguez et al 1999). The tonicity responsive enhancer (TonE) has the putative consensus sequence TGGAAANN(C/T)N(C/T) (Rim et al 1998) which has been found to regulate genes for SMIT (Rim et al 1998), BGT1 (Miyakawa 1998), and aldose reductase (Ferraris et al

1996; Ko *et al* 1997). Miyakawa *et al* 1999 reported the cloning and characterisation of the first animal transcription factor responsible for regulating osmolyte transporter genes during osmotic stress. They named this transcription factor TonE binding protein (TonEBP) because it specifically binds to, and activates the tonicity-responsive enhancer element of osmoprotective genes. The TonE mediates increased transcription of genes involved in the accumulation of compatible osmolytes by renal cells in response to hypertonicity. They described a consensus sequence for TonE and the trans-activating factor for the TonE cis-element as well as the TonEBP. The TonE/TonEBP pathway is known to mediate the tonicity responsive regulation of UT-A expression (Nakayama *et al* 2000; Rim *et al* 1998), the glycine betaine transporter (BGT1), and the myo-inositol transporter (SMIT). Glycine betaine (betaine) is taken up via a specialised transporter (BGT1), hypertonicity raises the number of transporters by increasing their transcription. Yamauchi *et al* 1992 reported the cloning of a Na(+)- and Cl(-)-dependent betaine transporter that is regulated by hypertonicity. The sequence of a putative osmotic response element of the canine betaine transporter gene (TonE) was reported by Takenaka *et al* 1994. The 5' region of the betaine gene contains an ORE that increases its transcription in response to hypertonicity. The sodium/myo-inositol cotransporter (SMIT)1 is a plasma membrane protein catalysing concentrative uptake of myo-inositol (MI) using the electrochemical gradient of sodium across the cell membrane (Kwon *et al* 1992). When cells in the kidney and brain are exposed to hyperosmolar salt concentrations (hypertonicity) they survive by raising the cellular concentration of myo-inositol. Transcription of the Sodium/myo-inositol cotransporter gene is markedly stimulated in response to hypertonicity leading to an increase in the activity of the cotransporter, which in turn drives the osmoprotective accumulation of myo-inositol. Rim *et al* 1998 identified five tonicity responsive enhancers in a 50 kilobase region upstream of the gene, TonEA, TonEB2, TonEC1, TonEC2 and Ton Ep. These five TonEs are reported to provide enough additive enhancer activity to account for greater than 10-fold stimulation of transcription in

response to hypertonicity. Sorbitol is produced via the reduction of D-glucose by the enzyme aldose reductase. Of the organic osmolytes, sorbitol has received special attention since it is one of the principle organic osmolyte accumulated in kidney renal medulla cells in response to hyperosmotic stress (Bagnasco *et al* 1986; Gullans *et al* 1988). Sorbitol therefore plays a beneficial role during anti-diuresis however it also appears to be detrimental in diabetes through its involvement in diabetic microvascular complications (Lee *et al* 1995; Kador *et al* 1985; Greene *et al* 1985). Aldose reductase can therefore be considered as an osmoregulatory protein through its involvement in sorbitol production under hyperosmotic stress. ALR2 is present in a variety of tissues including kidney, liver, ocular lens and retina and in erythrocytes. The induction of ALR2 or ALR2 mRNA by hyperosmolality has been demonstrated in a variety of cells, which include cell lines derived from animal and humans.

Transcription elements involved in osmotic regulation of ALR2

Studies investigating the molecular mechanisms involved in the osmotic regulation of the ALR2 gene have involved cloning of the rabbit AR gene (rAR) and the promoter region and characterising its structure. These studies have demonstrated that the 5' regions' flanking the aldose reductase gene (-3429 to base pair -192) contains osmotic response elements that increase transcription in response to hypertonicity (Ferraris *et al* 1994). The osmotic induction ratio is reduced from 9.4 to 2.0 when the sequence is reduced from 3221 to 11bp, suggesting that there are other *cis*-elements that may potentate the osmotic response. Daoudal *et al* 1996 cloned the AR cDNA from mouse kidney and reported the isolation of the mouse AR gene promoter. They identified a sequence within the promoter region that is required for enhanced activity in hypertonic conditions. The sequence is similar to the tonicity responsive element. A later report by the same group (Daoudal *et al* 1997) reported the isolation of the mouse AR promoter. Using chloramphenicol acetyltransferase reporter constructs containing various 5' flanking regions of the mouse

AR gene in CV1 cells, they identified a sequence spanning base pairs −1053 to −1040, required for an enhancer activity in hypertonic compared with isotonic cell culture conditions. Ferraris *et al* 1996 reported an 11bp osmotic response element essential for osmoregulation of the rabbit AR gene located 1105bp upstream of the transcription start site. This element shares sequence homology with TonE of the canine betaine transporter gene. Aida *et al* 1999 isolated the rat aldose reductase gene and examined the 5' flanking sequence for the presence of transcription regulatory element responsive to hyperosmolarity. Deletion of the promoter region of the aldose reductase gene up to − 1047bp abolished the transcriptional activation in response to osmotic stimuli in transient transfection experiments. In this study glucose was shown to be more effective than NaCl in induction of aldose reductase indicating the possibility of a glucose specific response mechanism. Studies have found the ORE sequence to be in the regulatory region of the vasopressin gene and in the 5'flanking sequences of cyclooxygenase-2 gene (COX-2) indicating that OREBP is the key protein responsible for activating genes involved in protecting cells from hyperosmotic stress.

Ko *et al* 2000 reported the purification and cloning of an osmotic response element binding protein. The protein contains a Rel-like DNA-binding domain and a glutamine rich transactivation domain, suggesting that it is a transcription factor. They demonstrated that OREBP interacts with the ORE and mediates the hyperosmotic expression of aldose reductase in vivo. Two positively charged arginine residues in the DNA binding domain of the OREBP interacting with guanine contact points may also be involved. Possibly two protein species interact with ORE's. Ko *et al* 1997 demonstrated that when the glutamine rich carboxyl end was deleted the hyperosmotic induction of the aldose reductase gene is suppressed, indicating that this portion of the protein is involved in the hyperosmotic transcriptional activation of ALR2 gene. Further confirmation that OREBP is the hyperosmotic transcription factor came from the translocation of cloned OREBP in

transfected cells from the cytoplasm to the nucleus upon hyperosmotic induction. This translocation occurs within 1 hour indicating that the signal of hyperosmolality is quickly relayed to the transcription factor. However, the induction of the ALR2 gene does not occur until 8-12 hours (Ko *et al* 2000). This is a considerable lag time between the arrival of the transcription factor into the nucleus and gene induction. There is an increase in OREBP mRNA after hyperosmotic challenge therefore it is possible that an increase in the amount of OREBP is required through transcription and translation before hyperosmotic gene induction can be mediated. A signal transduction study has also shown that the p38 and the c-Jun N-terminal kinase (SAPK/JNK) are not necessary for the transcriptional regulation of the ALR2 promoter through ORE (Kultz *et al* 1997).

Identification of transcription elements within 5'ALR2 region

Several groups have identified the osmotic response elements (OREs) of ALR2 and its promoter region (Daoudal *et al* 1997; Ferraris *et al* 1996; Ko *et al* 1997; Ferraris *et al* 1994). AR transcription is regulated by a promoter that contains diverse regulatory elements. Recent reports describe the involvement of two ALR2 promoter *cis*-elements, the osmotic response element (ORE) (Iwata et al 1996; Ferraris *et al* 1996; Daoudal *et al* 1997; Ko *et al* 1997), and the aldose reductase enhancer element (AEE) in regulating osmotic response (Iwata *et al* 1997). The ORE and TonE share a putative consensus sequence NGGAAAWDHMC(N) and are responsible for mediating expression of these genes (Ferraris *et al* 1999). Ruepp *et al* 1996 reported the identification of a putative functional ORE located 3.7kb upstream of the transcription start site of the human aldose reductase gene. Specifically, they reported two putative osmotic response elements, pseudo ORE*d* present at position −3834 to −3819 and ORE*p* present at position −3669 to −3654. However, the same group later confirmed that the results came from a cloning artefact and the site could not be confirmed (Ko *et al* 1997). It was also reported that an osmotic response region of the human ALR2 gene mediates its induction during hypertonic stress

(Ko *et al* 1997; Wang *et al* 1997). This was found this to be within a 132bp region located 1325 bp upstream of the transcription start site of the human ALR2 gene. They have shown that hyperosmotic induction of human AR gene is mediated by three sequences homologous to TonE of the canine betaine transporter gene and to the ORE of the rabbit AR gene. These osmotic response elements located within this 132 bp region situated in the 5' flanking sequence of the gene were named;- OreA, OreB and OreC, (figure 13). The 132bp region was found to contain three sequences that resemble part of TonE (**TGGAAAAGTCCA**) of the canine betaine transporter gene (Takenaka *et al* 1994) and the ORE (**CGGAAAATCAC**) of the rabbit AR gene (Ferraris *et al* 1996). ORE-A (-1230 to -1220) and ORE-C (-1157 to -1148) are in the same orientation as TonE, ORE B (-1188 to −1198) is in the opposite direction.

	ORE-A			ORE-B
TTACATGGAA	AAATATCTGG	GCTAGTCTGT	TCTGTATAAA	TTTTTCCAGG

			ORE-C	
AGGGAGCACT	TTTAAAGAAA	GCACCAAATG	GAAAATCACC	GGCATGGAGT

		AP1-D	
TTAGAGAGAC	CTGGTGCTTG	AGTCACTACC	AG

TonE	(TGGAAAAGTCCA)
ORE	(CGGAAAATCAC)
ORE-A (-1230 to -1220)	(TGGAAAAATAT)
ORE-B (-1188 to -1198)	(TGGAAAAATTT)
ORE-C (-1157 to -1148)	(TGGAAAATCA)
AP1 (-1111 to -1117)	(TGAGTCA)

Figure 13. Nucleotide sequence of the 132-bp fragment containing three Ton-E like sequences. **OreA, OreB** and **OreC** are highlighted in red and are indicated by A, B and C respectively. OREA and OREC are in the same orientation as TonE, and OREB is in the opposite orientation. The region indicated by D is an Ap-1 consensus sequence (adapted from Ko *et al* 1997).

The ALR2 ORE is most similar to TonE and to the rabbit AR ORE and it contributes most to the osmotic induction of transcription. The TonE element is functional in both orientations. These ORE's interact with a putative transcription factor under hyperosmotic induction and osmotic response function is reduced when any one of these three sequences

is mutated, suggesting that these sequences have to work in concert to provide maximum transcription induction. The ORE sequence differs from the NFκB binding sequence by one base pair. Iwata *et al* 1999 reported a significant induction of ALR2 by TNF-α, which is mediated by NFκB binding to ORE. Deletion or mutation of this element showed a significant effect on the response of the ALR2 promoter to TNF-α and hyperosmotic stress.

Polymorphisms within AR promoter region-OREs

To date no polymorphisms have been identified within the ORE gene sequences located in the 5'ALR2 promoter region near to the aldose reductase (CA)n microsatellite repeat region. A study carried out by Heesom *et al* 1997 was designed to determine whether polymorphisms within the ORE's may play a role in the development of diabetic complications. A total of 12 patients with T1DM were selected and the ORE's region was sequenced for each patient. No polymorphisms were found in any of the patients studied indicating that the genetic lesion lies within the coding region of the ALR2 gene.
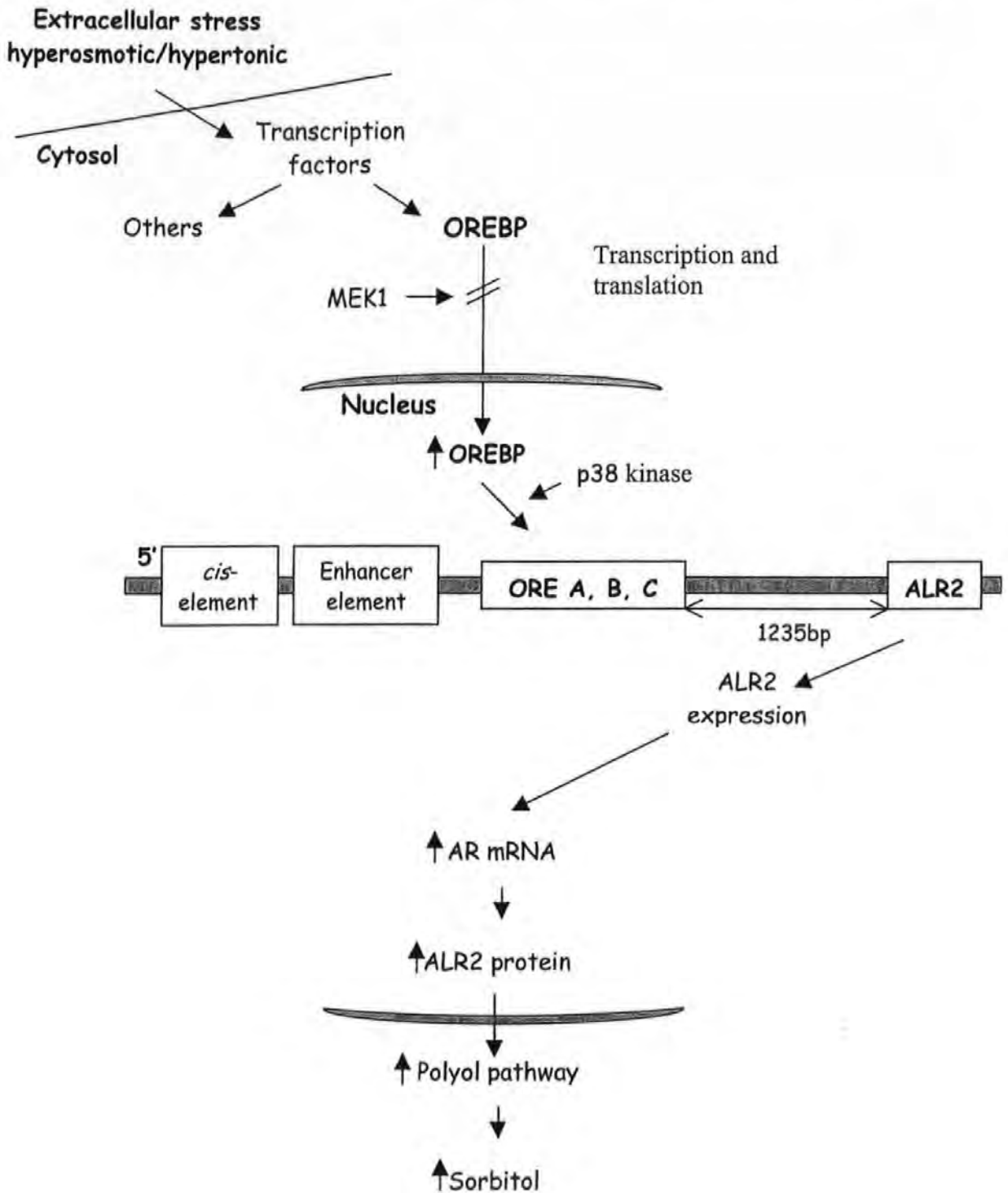
Figure 14. Sequence of intracellular events following extracellular stress (hypertonicity/hyperosmotic) leading to OREBP/ORE binding in the 5'ALR2 promoter region and the resultant induction of ALR2 mRNA synthesis and polyol pathway activation.

Glucose-specific regulation of aldose reductase

Pathophysiological pathways resulting from hyperglycaemia include the activation of the polyol pathway, non-enzymatic glycation of proteins and oxidative stress which probably synergise through their ability to activate the MAPK cascade leading to phosphorylation of transcription factors. This glucose induced transduction pathways lead to alterations of gene expression resulting in altered cellular phenotype, cell division, or increased production of extracellular material (Tomlinson 1999). Henry *et al* 1993 demonstrated human retinal pigment epithelial cells having accelerated and exaggerated production of sorbitol and depletion of myo-inositol upon exposure to 20mM glucose. Aida *et al* 1999 suggested the presence of a glucose specific mechanism of induction in addition to that by NaCl. They showed that glucose was more effective than NaCl in induction of aldose reductase. Portois *et al* 1999 identified a glucose response element in the promoter region of the rat glucagon receptor gene, which contained two 'E-boxes' CACGTG AND CAGCTG. This domain exhibited threshold like activity, with low activity below 5 mM glucose and maximal activation as of 10 mM glucose. More recently, Henry *et al* 2000 tested the hypothesis that pathophysiological levels of glucose regulate aldose reductase (ALR2) gene expression, protein production and activity in human retinal pigment epithelial (RPE) cells in vitro. They determined that elevations in ambient glucose result in greater metabolism of glucose through glycolysis and polyol metabolism. Hyperosmolar stress itself was not a necessary determinant of ALR2 mRNA, ALR2 protein, or ALR2 protein activity in cells that form the outer blood-retinal barrier. They note that increased facilitative glucose transport or increased glucose metabolism is required for glucose specific and nonosmotic regulation of ALR2 in the RPE cell in vitro. To date however, no such specific glucose response element has been found in the region near to the ALR2 gene.

Glucose specific ALR2 gene expression in T1DM subjects

Elevated levels of ALR2 protein have been shown to occur in neutrophils (Dent *et al* 1994), erythrocytes (Hamada *et al* 1991[a and b]; Nishimura *et al* 1994), and mononuclear cells (Ratliff *et al* 1996) isolated from patients with diabetic complications. Studies have shown that the mRNA levels of ALR2 in peripheral blood mononuclear cells (PBMC's) were increased in patients with T1DM and diabetic kidney disease than in T1DM patients without nephropathy, indicating that the degree of ALR2 gene expression modulates the risk of developing nephropathy (Shah *et al* 1997). Patients with diabetic microvascular complications have also been shown to have increased enzymatic activity of ALR2 as well as increased amounts of the protein compared with T1DM subjects with no complications (Hamada *et al* 1993; Maeda *et al* 1999). Hamada *et al* 1993 reported that diabetics who developed severe complications in less than 20 years had the highest red blood cell (RBC) ALR2 activity. In contrast, long standing diabetics, with no complications, had the lowest RBC ALR2 activity. It has also been demonstrated that ALR2 mRNA levels are induced under hyperglycaemia and hypertonicity. Hodgkinson *et al* 2001 reported the quantitation of ALR2 and SORD mRNA in cultured neutrophils from T1DM patients and normal controls using the ribonuclease protection assay (RPA). The study found significantly increased levels of ALR2 mRNA with increasing D-glucose concentrations in T1DM subjects with nephropathy compared to T1DM patients without nephropathy and normal controls. The mRNA expression of SORD was also significantly increased in T1DM nephropathy patients exposed to hyperglycaemic levels of D-glucose compared to uncomplicated T1DM patients and controls. Hamaoka *et al* 1999 used AR-transfected HIT (hamster derived B-cell line) cells, which showed enhanced metabolic activity in the polyol pathway. In this study when ALR2 was over-expressed these cells exhibited typical features of apoptosis, including a decrease in intracellular NADPH and defective activation of NFkB.

Aims of thesis

To investigate the genetic susceptibility to microvascular disease in subjects with DM. This will be achieved by studying polymorphisms (genetic variants) in candidate genes in discrete sporadic population of T1DM patients (n~300) with or without microvascular complications, as well as United Kingdom family trios (Diabetes UK- Warren 3 collection), and a collection of T2DM subjects of Southern Indian origin.

- In the first instance, polymorphisms in the aldose reductase (ALR2) gene and the 5' flanking regions will be studied in these populations. This will include an intragenic polymorphism at Intron 8 A(+11842)C of the ALR2 gene, a C(-106)T polymorphism in the promoter region of the aldose reductase gene, and the 5'ALR2 (CA)n microsatellite polymorphism. The Transmission disequilibrium test (TDT) will be used to measure the transmission of the 5'ALR2 microsatellite from parents to affected probands with diabetic nephropathy.

- Genetic markers for hypertension at position D17S934 proximal to the ACE gene will be studied to determine any association with diabetic nephropathy, retinopathy or neuropathy.

- A study of the mitochondrial polymorphism Mt5178A/C will also be carried out in the British Caucasoid population.

- The study also aims to test the hypothesis that pathophysiological levels of glucose specifically regulate the osmotic response elements situated in the 5'upstream region of the ALR2 gene in T1DM patients with microvascular complications compared to T1DM controls and normal healthy controls.

This investigation aims to be the first comprehensive study of the polymorphisms in the ALR2 gene and its promoter region in case-control, transracial and family studies. It also aims to apply functional protein studies of ORE to determine any associations with diabetic microvascular disease.

Chapter 3

Subjects, materials and methods

Ethical Approval

For the study of T1DM subjects of British Caucasoid origin, local research ethics committee approval had been obtained from the South and West Devon Health Authority, Devon, U.K. Written informed consent was also obtained from all subjects at the time of blood collection prior to inclusion into the study. Ethical approval for research studies using the T2DM subjects of Southern Indian origin was carried out independently by Dr Vijay Viswanathan, Madras, India. The British Diabetic Association (BDA) Warren repository has previously obtained ethical approval from all participating centre's and was also reviewed by a multi-centre Research Ethics Committee.

Subjects

During the course of this thesis several subject groups were investigated, the profiles of which are reviewed here. A large collection of T1DM subjects of British Caucasoid origin, a family trio collection where the affected proband had T1DM and diabetic nephropathy, and a collection of T2DM subjects of Southern Indian/Dravidian origin are included.

British Caucasoid T1DM collection

DNA samples were collected from over 600 unrelated British Caucasoid patients with grandparents born in the UK, diagnosed as having T1DM as defined by the National Diabetes Data Group 1979, who had attended the diabetic clinic of Consultant Diabetologist; Dr B. A. Millward. Venous blood samples were collected from the Diabetic Clinic's at Derriford Hospital in Plymouth, Kings College Hospital and Guys University College in London and the Renal Unit at Dulwich Hospital, London. Basic patient information such as date of birth and ethnic origin was obtained from patients' medical notes and by patients answering a short questionnaire. The progress of patients with regard to their development of diabetic complications was monitored during the progression of their disease. Patients were monitored when they arrived to clinics by Dr Millward and

diabetic research nurses, Caroline Dunster and Karen Holdsworth-Cannon (see acknowledgements) who updated the patient proforma's accordingly. At the diabetes clinic both physical and biochemical parameters were stringently recorded, enabling patients to be classified confidently in accordance to their progression of diabetic complications. Fundoscopy was performed by both a diabetologist and ophthalmologist. This was carried out routinely and according to the criteria defined by the National Diabetes Data Group 1979).

Classification criteria of T1DM subjects according to microvascular disease

Dr Millward also carried out the classification of patients for this study according to their diabetic microvascular complications. The patients were classified at the time of blood sampling in accordance to their onset and progression of microvascular complications as follows:

T1DM uncomplicated control subjects (n=66)

Patients were diagnosed as having T1DM before the age of 40 years and a sufficient duration of disease of at least 20 years. Patients remain free of retinopathy (fewer than 5 dots or blots per fundus), and had normal levels of albumin excretion rate (AER) defined as <20μg/min in an overnight sample on at least two consecutive assessments or urine Albustix® (Bayer, West Haven, CT) negative on three consecutive occasions over 12 months. Patients also showed no signs of overt or autonomic neuropathy. There were 66 uncomplicated diabetic controls included in this study and basic demographic characteristics of this patient group are shown in table 6.

T1DM retinopathy subjects (n=44)

Patients with T1DM and have retinopathy defined as more than 5 dots or blots per fundus, hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or

previous laser treatment for preproliferative retinopathy, and maculopathy or vitreous haemorrhage. Fundoscopy was performed by both a diabetologist and ophthalmologist, and the tests for presence of neuropathy was also performed and patients showed no signs of neuropathy. None of these patients had proteinuria (urine Albustix® negative on 3 consecutive occasions over 12 months). A total of 44 subjects with diabetic retinopathy were included in this study for which basic demographic characteristics are shown in table 6.

T1DM neuropathy subjects (n= 18)

Patients with T1DM and classified as having neuropathy if there was any clinical evidence of overt peripheral or autonomic neuropathy. Symptoms of peripheral neuropathy included the following features; a) symptoms of pain, numbness or parasthesia in the feet and/or hand, b) sensory signs such as loss of vibration sense (assessed using a tuning fork), light touch or pin-prick, and temperature (using warm and cold stimuli), c) loss of flexes in the legs, d) evidence of past or present neuropathic foot ulceration. Autonomic neuropathy was defined if there was evidence of postural hypotension, gustatory sweating, a previously documented Charcot joint, or loss of beat to beat variation on an electrocardiogram (ECG). None of these patients had other causes for neuropathy. A total of 18 subjects with diabetic neuropathy were included in this study for which basic demographic characteristics are shown in table 6.

T1DM nephropathy and retinopathy subjects (n=30)

Patients have had T1DM for at least 10 years and have persistent proteinuria (urine Albustix® positive on at least three consecutive occasions over 12 months or three successive total urinary protein excretion rates >0.5g/24hrs) in the absence of haematuria or infection. In all cases diabetic nephropathy was associated with diabetic retinopathy. A

total of 30 diabetic subjects with nephropathy and retinopathy were included in this study and basic demographic characteristics are shown in table 6.

T1DM retinopathy and neuropathy subjects (n=24)

Patients with T1DM and have retinopathy defined as more than 5 dots or blots per fundus, hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative retinopathy, and maculopathy or vitreous haemorrhage. Patients also showed a positive result for presence of neuropathy (see under neuropathy subgroup). A total of 44 subjects with diabetic retinopathy and diabetic neuropathy were included in this study, basic demographic characteristics are shown in table 6.

T1DM full house complication subjects (n=62)

Patients with T1DM and persistent proteinuria (urine Albustix® positive on at least three consecutive occasions over 12 months or three successive total urinary protein excretion rates >0.5g/24hrs) in the absence of haematuria or infection. In association patients have retinopathy as defined as more than 5 dots or blots per fundus, hard or soft exudates, new vessels or fluorescein angiographic evidence of maculopathy or previous laser treatment for preproliferative retinopathy, and maculopathy or vitreous haemorrhage. Patients also have overt peripheral or autonomic neuropathy (see neuropathy subgroup). A total of 62 T1DM subjects with full house complications were included in this study and basic demographic characteristics are shown in table 6.

Healthy adult control subjects (n=13)

This control group consisted of fresh venous blood taken from normal healthy adult British Caucasoid subjects, with no history of T1DM or T2DM or other autoimmune or renal diseases. This control group was used only for the protein experiments requiring fresh

whole blood samples. A total of 13 healthy adult subjects were included in this study and basic demographic characteristics are shown in table 6.

Cord blood control subjects (n=120)

The normal control group for the genetic studies were taken at random from a bank of cord bloods collected at the time of normal healthy obstetric delivery from the labour ward, Derriford Hospital, Plymouth. A total of 120 cord blood controls were used, and as the only data available at the time was the subjects gender, limited distribution demographics could be established as shown in table 6.

Subjects excluded from study

From the large collection of over 600 T1DM subjects collected, 244 fitted the criteria for the study with confidence and were included in the investigation. Many subjects had to be excluded from the study for reasons as described here. Patients who were diagnosed as having T1DM but pre-insulin treatment were not included in this study, as well as patients who have had diabetes for less than 20 years and showed no signs of complications. This was because there is a possibility that some will develop complications, but as yet we do not know which. Patients were also excluded from the study if they did not completely satisfy the stringent criteria for patient groups or if insufficient data was available or inadequate to confidently classify them. Subjects were excluded from the study if urinary tract infection, cardiac failure or renal vasculitis were present. Subjects were also excluded from the study if they were receiving anti-hypertensive treatment. If DNA quality after extraction was poor, an attempt was made to re-bleed patients, however in some cases patients had to be excluded as re-bleeding was not possible.

Clinical characteristics of T1DM and normal control subjects

Comparison of sex distribution using Chi-squared test showed no significant difference in sex distribution between cord blood controls and T1DM subject groups, or healthy adult controls and T1DM subject groups. The Chi-squared test was also applied to compare whether there is a statistically significant difference in the distribution of sex between study groups, there were no significant differences found.

Subject groups	n	M:F	Age at time of study	Age at diabetes onset (years)	Duration of diabetes (years)
T1DM subjects	244	115:129		NA	NA
• Uncomplicated	66	26:40	46.33 ±15.59 (20-79)	16.94 ± 10.03 (1-42)	31.35 ± 9.04 (20-55)
• Retinopathy	44	20:24	48.16 ±14.16 (24-78)	20.07 ± 10.01 (1-45)	30.0 ± 11.1 (13-57)
• Neuropathy	18	10:8	50.89 ±13.79 (24-68)	25.4 ± 11.79 (1-48)	27.3 ± 9.96 (10-44)
• Nephropathy and Retinopathy	30	13:17	46.57 ±17.38 (19-87)	16.5 ± 11.8 (1-56)	32.03 ± 12.92 (13-61)
• Retinopathy and Neuropathy	24	15:9	51.63 ±13.26 (29-79)	22.0 ± 10.6 (5-38)	31.63 ± 8.84 (19-53)
• Full House	62	31:31	49.18 ±13.59 (19-77)	18.24 ± 10.8 (1-54)	31.92 ± 9.91 (7-52)
Non-diabetics					
• Healthy adult	13	7:6	NA	NA	NA
• Cord bloods	120	53:67	NA	NA[1]	NA[1]

Table 6. Demographic characteristics of British Caucasoid subjects with T1DM and normal healthy control subjects, classified in accordance to onset of diabetic microvascular complications. The results are expressed as mean values +/- standard deviation together with the range in parentheses. All diabetic subjects had T1DM as defined by the National Diabetes Data Group 1997 as previously described.

[1] Not applicable to controls as taken from a collection of cord blood samples.

+/-   Plus or minus standard deviation.
M:F   Male to female ratio.
NA   Not applicable.

British Caucasoid T1DM family trio collection

A United Kingdom resource of DNA was established by the British Diabetic Association (BDA), (now Diabetes U.K.), in order to facilitate the investigation of the genetic susceptibility to diabetic nephropathy in T1DM. The project involved the participation of seven research establishments throughout the United Kingdom. Participating establishments included Belfast, Edinburgh, Newcastle, Manchester, London, Plymouth and Birmingham.

This study used blood collected from 172 British Caucasoids with T1DM and diabetic nephropathy. Blood was also collected from the parents of these individuals creating a trio pedigree of two parents and an affected proband. Where both parents were not available, blood was collected from siblings of the proband so as to facilitate the generation of parental haplotypes. In total 516 subjects (172 probands and 344 non-diabetic first-degree relatives) were included in this diabetic nephropathy family trio collection.

The subject's included into the study were British Caucasoid with grandparents born in the UK, having T1DM diagnosed under the age of 31 years and requiring insulin from diagnosis. Subjects showed no history of recurrent urinary tract infection, cardiac failure or renal vasculitis. Patients in whom renal imaging (ultrasonography or intravenous urography) showed any abnormality were excluded from the study. The diagnosis of diabetic nephropathy was made at the time of venesection, on the following basis;

- Development of persistent[a] proteinuria[b] > 10 years after diagnosis of diabetes.
- Presence of hypertension[c] at or after the onset of persistent proteinuria.
- Presence of diabetic retinopathy[d] at the time of persistent proteinuria.

[a] Persistent proteinuria is defined as proteinuria present continuously for >6 months.

[b] Proteinuria can be defined in various ways including: >0.5g protein/24 hours; >300mg albumin / 24 hours; >200 micrograms albumin / min on timed overnight collection; urinary Albumin/ Creatinine Ratio >35mg/ml; dipstick positive clinic urine testing.

[c] Hypertension is defined as blood pressure >135/85 (JNC VI guidelines) and/or treatment with antihypertensive agents.

[d] Presence of diabetic retinopathy includes any degree of disease (background, pre-proliferative & proliferative) on routine clinical examination.

Clinical characteristics of family trio collection

Subject information was not available and consequently subject demographic characteristics cannot be calculated for the Diabetes UK-Warren repository T1DM family trio collection.

Southern Indian T2DM collection

Blood samples were collected from a population of 62 Indian patients with T2DM (non-insulin dependent diabetes mellitus). The blood was collected and the DNA extracted by Dr Vijay Viswanathan and co-workers at the Diabetes Research Centre, Chennai, India. The samples were then delivered on dry ice in 1.5ml Eppendorf tubes to our laboratory along with comprehensive clinical data. The recorded clinical data included age, sex, evidence of family history of diabetes and subjects diabetic complications, which are summarised in table 7.

Classification criteria of T2DM subjects according to microvascular disease

Dr Vijay Viswanathan carried out classification of patients for this study according to their diabetic microvascular complications. The patients were classified according to their microvascular complications at the time of blood sampling as follows:

T2DM uncomplicated controls (n= 28)

Patients were diagnosed as having T2DM and were normoalbuminuric without retinopathy. Out of the 28 subjects included in this group 17 had a positive family history of diabetes and 10 had no family history of diabetes and 1 subject did not know. A total of 28 uncomplicated diabetic subjects were included in this study and basic demographic characteristics are shown in table 7.

T2DM nephropathy and retinopathy subjects (n= 32)

Patients were diagnosed as having T2DM and subjects tested positive for proteinuria and fundoscopy also revealed retinopathy. Out of the 32 subjects included in this group 18 had a positive family history of diabetes and 16 had no family history of diabetes. A total of 32 nephropathy and retinopathy diabetic subjects were included in this study and basic demographic characteristics are shown in table 7.

Healthy adult control subjects (n=43)

This control group consisted of normal healthy adult Southern Indian subjects of whom 9 had a positive family history of diabetes and 34 had no known family history of diabetes. A total of 43 healthy adult subjects were included in this study but no record had been taken of subject gender, therefore no subject demographic analysis could be made. A total of 43 healthy adult control subjects were included in this study.

Clinical characteristics of T2DM and normal control subjects

Comparison of sex distribution using chi-squared test showed no significant difference in sex distribution between T2DM subject groups ($\chi^2 = 0.2$, p= 0.65). As there was no record of control subjects gender comparisons could not be made between this group and T2DM subjects.

| | T2DM subjects | | Non-diabetics |
	Uncomplicated	Nephropathy and retinopathy	Healthy adult
n	28	32	43
M:F	15:13	19:13	[1]DNA
Age at time of study	55 ±10.11 (27-76)	53.8 ±8.94 (37-68)	38 +6.3 (30-58)
Age at diabetes onset (years)	42.07 ± 11.26 (15-60)	42.34 ± 8.46 (26-57)	[2]NA
Duration of diabetes (years)	13.11 ± 7.45 (2-29)	11.81 ± 5.38 (3-25)	[2]NA

Table 7. Demographic characteristics of Southern Indian subjects with T2DM and normal healthy control subgroups classified in accordance to onset of diabetic microvascular complications. The results are expressed as mean values +/- standard deviation together with the range in brackets. All patients had T2DM as defined by the National Diabetes Data Group 1997 as previously described.

+/-   Plus or minus standard deviation.
M:F   Male to female ratio.
NA   Not applicable.
DNA   Data not available

[1] Data regarding subjects gender was not recorded.
[2] Not applicable to controls as taken from a collection of healthy adults.

Blood sampling

Trained phlebotomists and research nurses (see acknowledgements) carried out all blood sample collection. Venupuncture was performed using a BD-Vacutainer™ blood collection system (Becton Dickinson Vacutainer Systems, Oxford, U.K.). For DNA extraction 5-10 ml of peripheral venous blood was collected into 6.0-ml BD Vacutainer Plus™ whole blood tubes containing potassium ethylene diamine tetra-acetic acid (K_2EDTA, spray dry, 10.8mg). The blood was subsequently transferred to BD Falcon™ sterile polypropylene 50-ml conical centrifuge tubes (Becton Dickinson, Plymouth, UK) and stored at -20°C, prior to subsequent DNA extraction. For cell culture 20-ml of peripheral venous blood was collected into 6.0-ml BD Vacutainer Plus™ Sodium Heparin whole blood tubes. These samples were then directly transferred to sterile 50-ml BD Falcon™ tubes and processed immediately. Cord blood samples were collected into heparin syringes and transferred to sterile 50-ml BD Falcon™ tubes and stored at −20°C, prior to DNA extraction.

Materials

Water

Purified tap water was obtained using an Elix Water Purification System unit and Polyethylene Reservoir (Millipore Ltd, Watford, UK) and was used to make up all stock, general purpose and specialist solutions. Sterile water (Baxter Healthcare, Thetford, UK) was used to make amplimer dilutions, restriction digest reactions, and used for PCR applications.

Reagents

All general-purpose reagents used were analytical grade or equivalent and were obtained either directly from the manufacturers or through a local supplier listed in table 8. Super *Taq* polymerase and 10 x PCR buffer was purchased from HT Biotechnology Ltd (Cambridge, UK). Ultrapure dNTP set 2-Deoxynucleoside 5'-triphosphates (dNTP's), radioactivity (γ^{32P} ATP), and Ready-To-Go™ T4 Polynucleotide kinase were purchased from Amersham Pharmacia Biotech LTD (Buckinghamshire, UK). Quik-Precip™ was purchased from Edge BioSystems Inc. (Oxon., UK). Nucleon® BACC2 DNA extraction kits for whole blood were purchased from Nucleon Biosciences (Lanarkshire, Scotland, UK), and Wizard® PCR Preps DNA Purification System was purchased from Promega (Southampton, UK). Restriction endonucleases and incubation buffers were purchased from New England BioLabs Inc. (Herts, UK), and Roche Diagnostics LTD (East Sussex, UK) respectively. Perbio Science LTD (Chester, UK) supplied Coomassie® Plus Protein Assay Reagent Kit. Reagents used for the Electrophoretic Mobility Shift Assay which include T4 polynucleotide kinase 10X buffer and T4 polynucleotide kinase were supplied by Promega (Southampton, UK). Custom oligonucleotide synthesis was carried out by MWG-Biotech UK Ltd (Milton Keynes, UK).

Reagents/ chemical/ solution	Supplier
Redivue™ [Gamma 32P]-ATP (3000Ci/mmol), Stop solution, 1.25ml.	Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK.
Kodak X-Omat™ Scientific Imaging Film, X-ray developer, Rapid fixer	Anachem LTD, Beds, UK.
Sterile water	Baxter Healthcare, Thetford, UK.
2% Bisacrylamide, 40% Acrylamide	Bio-Rad Laboratories, Hemel Hempstead, UK.
Acetic acid, ethylene diamine tetra-acetic acid (EDTA), Glycerol, Hydrochloric acid, Magnesium Chloride, Maleic acid, Orthoboric acid, Sodium Chloride, Sodium citrate, Sodium dodecyl sulphate, Sodium Hydroxide, Sucrose, Tris (hydroxymethyl) aminomethane	BDH Lab Supplies, Merck Limited, Poole, UK.
Orange G	Fisher Scientific, Loughborough, UK
Lymphoprep™, Fetal Calf Serum (FCS), Dulbecco's Modified Eagles Medium (DMEM), L-glutamine, Penicillin/Streptomycin, Phosphate Buffered Saline (PBS), Roswell Park Memorial Institute 1640 (RPMI)	Life Technologies Ltd, Paisley, UK.
Sequagel concentrate, Sequagel diluent and Sequagel buffer	National Diagnostics, Flowgen Instruments, Staffordshire, UK.
Chloroform, Ethanol, Methanol, Industrial Methylated Spirit	Rathburns Limited, Walkerburn, UK.
DNA molecular weight marker XIV (100bp ladder), Agarose MP (multi purpose agarose)	Roche Diagnostics Ltd, East Sussex, UK
Nucleon® DNA extraction kits	Scotlab, Lanarkshire, Scotland, U.K.
Ammonium persulphate (APS), TEMED, Formamide, 3% Acetic Acid stop bath, Trypan blue, Xylene cyanol, Ethidium bromide	Sigma Chemicals, Poole, UK.

Table 8. Specialist reagents listed in accordance to the manufacturer from which items were purchased.

General purpose, glass and plastic ware

Soda-glass test tubes, Falcon™ 250-ml polystyrene tissue culture flasks, Falcon™ polystyrene round bottom tubes, Falcon™ 50-ml polypropylene conical tubes and serological pipettes were all supplied by Becton-Dickinson (Oxford, UK). Disposable polystyrene cuvettes were purchased from Sigma Chemical LTD (Poole, UK), and filter paper used for polyacrylamide gel support and drying was purchased from Heto Laboratory Equipment (Surrey, UK). Gilson liquid handling pipettes and tips were purchased from Anachem (Beds. U.K).

Specialist laboratory equipment

This study employed the use of a range of specialist laboratory apparatus which included the MJ Research PTC-200 DNA Engine (MJ Research, Massachusetts, USA), Fluor-S multi-imaging systems and Image analysis software (Bio-Rad, UK), Ultra-Violet transilluminator and UVP-LabWorks™ Image acquisition and analysis software (Ultra-Violet Products, Cambridge, UK), and γ scintillation counter 5010 Cobra 10 (Packard Biosciences, UK). Also employed were a range of general-purpose laboratory apparatus which included a CK40-F Olympus Inverted Microscope (Jencons Scientific LTD, Bedfordshire, UK), Priorclave PS/LAC/EV100 (PriorClave LTD, London, UK), UV1101 Biotech Photometer (Cecil, UK), Cecil 5500 spectrophotometer (Cecil, UK) and a Heraeus Sepatech Biofuge 15/15 / 13/13 (Heraeus Instruments GmbH, Germany). For tissue culture work a Microflow Biological Safety Cabinet (Steriliser and Airflow Service Care, Hampshire, UK) and a Nuaire™ Water-Jacketed TS autoflow CO_2/O_2 incubator (Indis Scientific, Glasgow, UK) were used. For protein mobility shift assays the Sequigen® GT Nucleic Acid Electrophoresis Cell (Bio-Rad, UK), and Protean® II XI Cell (Bio-Rad, UK) were used with a PowerPac 3000 (Bio-Rad, UK).

Methods

Autoclaving

All solutions, and all glassware and plasticware used in the techniques of DNA and protein analysis were autoclaved at a temperature of 121°C, and pressure of 15 p.s.i for 30 minutes in a steam autoclave (PriorClave Ltd, London, UK).

DNA extraction

Extraction of high molecular weight genomic and mitochondrial DNA from peripheral blood leukocytes, was carried out employing the Nucleon® DNA extraction method and also a salting out method as described below.

DNA extraction using Nucleon® BACC2 method

The principle method utilised for DNA extraction employed the Nucleon® BACC2 (whole blood and cell culture) genomic DNA extraction kit (Scotlab Ltd, Lanarkshire, UK) according to manufacturers protocol (figure 15). The kit extracted both genomic and mitochondrial DNA in the same process. The kit provided 'Nucleon® Reagent B' (400mM Tris-HCL pH 8.0, 60mM EDTA, 150mM NaCl, 1% SDS), sodium perchlorate and Nucleon® silica resin. In addition a 'Nucleon® Reagent A' (10mM Tris-HCL, 320mM sucrose, 5mM $MgCl_2$, 1% Triton X-100,- adjusted to pH 8.0 using 40% NaOH and autoclaved) stock solution was used which was made up in house.

Stored frozen peripheral venous blood samples were thawed at room temperature, and an aliquot of 7.5 ml was transferred to a 50-ml Falcon® tube (Becton Dickinson, Oxford, UK). To the sample a four-times volume of 'Nucleon® Reagent A' was added and mixed either by hand or on a Luckham R100/TW Rotatest shaker for 4 minutes. The blood/'Nucleon® Reagent A' solution was then centrifuged at 1300 x rpm for 4 minutes

using a MSE Mistral 1000 centrifuge and the supernatant containing lysed red cells was separated from the pellet and discarded. The PBMC pellet was then re-suspended in 2ml of 'Nucleon® Reagent B' (400mM Tris-HCL pH 8.0, 60mM EDTA 150mM NaCl, 1% SDS) as supplied, and incubated at 37°C for 10-15 minutes, enabling membrane disruption to occur. The suspension then was transferred to a 15ml Falcon® tube and 500µl of sodium perchlorate was added and mixed by gentle inversion. To extract the DNA, 2ml of ice cold chloroform (-20°C) was added and mixed to emulsify the two phases, which were then separated by centrifugation at 1300 x rpm for 3 minutes. After centrifugation, 300µl of Nucleon® silica suspension was added to the interface of the two layers by careful pipetting prior to a further centrifugation at 1300 x rpm for 3 minutes. The upper aqueous phase containing the DNA was then carefully transferred to a fresh tube, ensuring the interphase with silica resin and the underlying organic phase were not disturbed. Precipitation of DNA then carried out by inverting gently with 2-x volume of 100% ice-cold (-20°C) ethanol (Rathburns Ltd, U.K.). A sterilised glass pipette with a sealed tip was used to hook out the precipitated DNA and transfer it to a sterile Eppendorf tube. The DNA was then washed in 70% ethanol prior to dilution in 500µl of TE buffer (10mM Tris pH 7.6, 0.1 mM EDTA pH 8.0) over 24 hours.

7.5ml Blood + K2 EDTA

Add 40ml
Reagent A

Mix for 4 min at room temperature

Centrifuge at 1300 x rpm for 4 min.
Discard supernatant

Pellet

Add 2ml **Reagent B** to pellet and vortex to re-suspend

Incubate at 37°C for 15 minutes.
Transfer to 15ml falcon

Add 500µl **Na Perclorate** and mix at room temperature for 15 min.

Add 2mls ice cold **Chloroform** & shake for 10 min.

Centrifuge at 1300 x rpm for 1 min.

Aqueous phase

Organic phase

Add 300µl **Nucleon Silica Suspension**.

Centrifuge at 1300 x rpm for 3 min.

DNA containing upper phase.

Nucleon Silica Stratum

Organic phase

Remove upper layer and transfer to fresh tube.

Precipitate the DNA using 100% ice cold **ethanol**, wash in **70% ethanol** and re-suspend in **TE Buffer**

Figure 15. Flow Diagram of Nucleon DNA extraction using BACC2 extraction kit Scotlab. Adapted with modification from Scotlab Nucleon II protocol II(a) flow diagram (Scotlab, UK).

DNA extraction using 'salting out' method

A second method employed to extract genomic DNA from peripheral blood leukocytes was the salting out method as described by Miller *et al* 1988. Frozen whole blood samples were taken from the freezer and thawed at room temperature, 5-10ml was then transferred to a sterile 50-ml Falcon[®] tube. Next, 25ml of 'Red Cell Lysis Buffer' (RCLB; 0.144 M NH_4Cl, 0.001 M $NaHCO_3$) was added and incubated at room temperature for 10 minutes. The sample was then centrifuged for 10 minutes at 1300 x rpm and the haemolysate removed and discarded. This step was repeated to ensure the removal of all residual red blood cells. The white cell pellet was re-suspended in 3ml 'Nuclei Lysis Buffer' (NLB: 10mM Tris-HCL pH 8.0, 400mM NaCl and 2mM Na_2/EDTA), 300µl 10% SDS and 600µl Proteinase K (2mg/ml) (Sigma Chemicals UK). This was incubated overnight in a 37°C water-bath. The following day 1ml of saturated sodium chloride solution (6M) was added, shaken vigorously for 15 seconds to precipitate proteins, and centrifuged at room temperature for 15 minutes at 1300 x rpm. The supernatant was transferred to 2 x volume of 100% ice cold (-20°C) absolute ethanol and gently inverted to precipitate the DNA. The DNA was hooked out using a sterile glass Pasteur pipette and dissolved in 500µl of TE buffer pH 8 at 4°C for 72 hours and then stored at –20°C.

Quantification of DNA

To ensure a homogeneous solution, the DNA samples were stored at 4°C for at least three days before taking optical density readings. Next, 1μl of stock DNA was combined with 99μl of TE buffer in a 1.5-ml micro-centrifuge tube. Optical density readings were taken at 260nm and 280nm and compared with a TE buffer reference sample using optically matched quartz cuvettes employing a Cecil 5500 spectrophotometer (Cecil, UK). The concentration (μg/μl) of the DNA in the stock solution was determined by multiplying the absorbency reading at 260nm by a factor of 100, and based upon a reading of 1-optical density (OD) being equivalent to 50mg/ml of double stranded DNA. The purity of DNA was assessed using the ratio of λ260nm/λ280nm which should be between 1.7-2.0. Suitable DNA yields ranged from between 200μg and 250μg per 10 ml of blood. The DNA samples were then stored at –20°C prior to use. Dilutions of stock DNA samples were made for every day use, either in sterile water (Baxter, UK) or TE buffer to give a working concentration of 50ng/μl for use in PCR amplifications. These dilutions were stored at 4°C to minimise the number of degradative freeze-thaw processes.

DNA clean-up process

DNA samples which did not respond well to PCR, were 'cleaned-up' by re-precipitating the DNA and re-suspending in sterile water to remove any salts or inhibitors. This was achieved by adding 1/10 volume 5M NaCl and 2-x volume of absolute ethanol to the diluted DNA. This was then mixed and 'snap frozen' in liquid nitrogen for 2 minutes, and centrifuged at 15110 x rpm for 15 minutes. The supernatant was removed and samples were allowed to air dry, then the pellet was re-suspended in double distilled water.

Polymorphism detection

All assays used for the detection of polymorphisms at gene loci were PCR based. Specific assays for the different polymorphism's studied will be described in the following sections.

Amplification of DNA using the Polymerase Chain Reaction (PCR)

The technique of Polymerase Chain Reaction (PCR) was first introduced by Saiki *et al* 1985; and Mullis and Faloona 1987 and has since become a fundamental tool in molecular biology. The principle technique of PCR involves combining a DNA sample with oligonucleotides (amplimers) complementary to sequences on either side of the sequence targeted for amplification, deoxynucleotide triphosphates and the thermostable *Taq* DNA polymerase and a suitable buffer. The mixture is then repeatedly heated and cooled to enable the specific amplification of the region of interest. New DNA synthesis is limited to the region of DNA bounded by the two amplimers. By repeating this cycle, a single copy of target DNA can be exponentially amplified.

Amplimer design and production

Pairs of amplimers were designed from sequences known to flank the specific locus of interest. These amplimers permit PCR amplification of the DNA region of interest, which may contain a known polymorphism. Design was enabled by reference to sequences deposited in the publicly available Internet website 'Genbank' (www.ncbi.nlm.nih.gov.genebank). For all sequences of interest amplimers were designed complementary to the 5'-3' strand (sense) and complimentary to the 3'-5' strand (antisense) either side of the sequence of interest. All amplimers were designed to be between 18-30 bases in length with a random base distribution, and an approximate guanosine and cytidine (G+C) ratio of approximately 50% or similar to that of the fragment being amplified. Sequences were checked on DNA Star Software (Lasergene, USA) to check the amplimers against each other for complementarity to prevent the

possibility of creating 'primer dimers' or 'hairpin loops', where the amplimers bind to each other and may result in the formation of anomalies or artefacts. Amplimers containing stretches of polypurines, polypyrimidines or other unusual sequences were avoided. All amplimers were commercially synthesised on an oligonucleotide synthesiser, 'Pharmacia Gene Assembler' (MWG Biotech, Milton Keynes, UK) at a scale of 0.05-0.2 µmoles, and diluted for use to concentrations of ~10pmol µl^{-1}.

Standard reaction mixture

The PCR conditions required achieving optimum efficacy of DNA amplification varied depending upon the application for which it was applied. All PCR mixtures were prepared on ice in 0.2ml thin walled PCR strips (Advanced Biotechnology, Epsom, UK). Most reaction mixtures required a 50µl volume containing; 100-500ng of genomic DNA, 5µl of 10 x PCR buffer (250mM KCL, 50 mM Tris.HCL pH 8.4 at room temperature, 1% Triton X-100, 0.1% gelatin), 1.5-2.5 mM $MgCl_2$, 10-20 pmoles of forward and reverse primers, 200 µM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 2.5 units of *Taq* DNA polymerase. Sterile water was added to a total volume of 50µl and a few drops of mineral oil were added to each reaction to seal it and prevent evaporation and condensation. The samples were then briefly centrifuged and the PCR reaction carried out automatically using a PTC-200 'Thermal Cycler' (MJ Research, Essex, U.K.) or a 'Cyclogene Thermocycler' (Techne, Cambridge, UK).

PCR cycling strategies

Amplification was performed by incubating the samples at three temperatures corresponding to the three steps in a cycle of amplification-denaturation, annealing and extension. Typically the double stranded DNA is denatured by briefly heating the samples to 90-95°C, and then cooled to 40-60°C to allow the primers to anneal to their

complementary sequences. This was followed by heating the reaction to 70-75°C for approximately 1-minute per kilobase of sequence, to extend the annealed primers with the *Taq* DNA polymerase. Calculation and optimisation of the required annealing temperature was derived from the melting temperature (Tm) values of the primer-template pairs, where $Tm = 4(G+C) + 2(A+T)$. This annealing temperature was determined by applying the following formula;

$$\text{Annealing temp. (°C)} = \frac{\text{Tm Primer 1 + Tm Primer 2}}{2} - 3$$

Modifications to the standard PCR protocol were made for each of the PCR amplifications carried out in order to enhance specificity of PCR products. These strategies involved carrying out a $MgCl_2$ titration (1.5-6.5mM) to determine the optimal concentration required, increasing or decreasing the annealing temperature, decrease in primer concentration and decreasing the dNTP concentration. These strategies were applied when PCR products showed spurious bands upon agarose gel electrophoresis alongside a molecular weight marker (Sambrook, Fritsch and Maniatis 1989).

Determination of PCR efficacy by agarose gel electrophoresis

Amplification efficacy and the product size was checked by running out an aliquot of the PCR product on a 1-3.5% multi-purpose agarose gel containing 0.01% ethidium bromide. A 1% agarose/ethidium bromide gel was prepared by heating 1g of ultra-pure multi-purpose agarose (Roche Diagnostics Ltd, Sussex, U.K.) and 100ml of 0.5 Tris Borate EDTA (TBE) buffer. This was carried out using a 400ml-heat-proof glass bottle in a microwave on low power for 3 minutes or until all the agarose had dissolved. The agarose solution was then allowed to cool at room temperature to approximately 65°C. Next, 10μl of ethidium bromide solution (10mg/ml) (Sigma-Aldrich, Dorset, U.K) was added to create

a 0.01w/v concentration, and gently mixed. The agarose/ethidium bromide mixture was carefully poured into a 20x24 cm ultraviolet light transparent Perspex casting tray (Gibco BRL, UK), that was sealed at both ends with masking tape. Two 28 tooth vinyl combs were inserted into the gel to generate the sample loading wells. The gel was allowed to set at room temperature and was placed at 4°C to harden. The masking tape was then removed from the gel tray and the gel was placed into 0.5% TBE buffer in a 'Flowgen Submarine Electrophoresis Tank' (Flowgen, UK) and the combs were carefully removed.

A 10μl aliquot of the PCR product was mixed with 1.5μl of xylene cyanol track dye (15% Ficoll-type 400, 0.25% w/v Xylene Cyanol FF, 0.25% Bromophenol Blue, in 10 X TBE), or Orange G loading buffer (0.25% orange G, 10% v/v glycerol in 10 X TBE). The PCR product/loading buffer mixture was than loaded into the wells of the gel guided by a Gilson pipette tip. A 100bp molecular weight marker (Life Technologies, Paisley, U.K) was also loaded into an adjacent well, along with appropriate positive and negative controls. The gels were electrophoresed for 30-45 minutes at a constant voltage of 100-150V (constant voltage) using a BioRad 3000 power pack, (BioRad, Herts, U.K.). Gels were then viewed and photographed under UV illumination using an Ultra-Violet transilluminator linked to UVP-LabWorks™ Image acquisition and analysis software (Ultra-Violet Products, Cambridge, UK), and DPII + Dpsoft digital camera and software.

Dinucleotide repeat analysis

Short tandem repeat polymorphisms (STRP's) otherwise termed microsatellite polymorphisms, are polymorphic genetic markers consisting of short nucleotide sequences which are sequentially repeated. Typically the repeated sequences are 1-4bp in length and are tandemly repeated 10 to 60 times. They provide highly informative markers for human genetic linkage studies. Microsatellite sequences are randomly distributed in the genome, and occur on average every 6 kilobases (kb) (Weber and May 1989). Dinucleotide repeat polymorphisms were typed using the PCR (as previously described) but incorporated the use of Redivue [γ^{32}P] dATP radiolabelled amplimers designed to flank the region of interest. High-resolution acrylamide electrophoresis was carried out to resolve the allelic fragments that differ in length by as little as 2 nucleotides.

Two microsatellite markers were studied within this thesis, firstly the (CA)n dinucleotide repeat marker of the hypertension-linked region on chromosome 17q (D17S934), residing 18cM to the ACE locus, and the second a (CA)n dinucleotide repeat marker on chromosome 7q35, situated 2.1kb upstream of the aldose reductase (ALR2) start site. For the D17S934 locus associated to hypertension, amplimers were designed from sequences known to flank the (CA)n repeat region to create a 185bp fragment illustrated in figure 16. Each amplimer was composed of 14 (G+C) and 11 (A+T) bases, the sequences of which are outlined in table 9. The amplimers were chosen to share the optimum annealing temperature of ≈68°C. Template sequences for H. sapiens D17S934, accession number Z23831, were obtained from the GenBank Internet website- www.ncbi.nlm.nih.gov (Gyapay *et al* 1994). For the 5'ALR2 locus pairs of amplimers were designed from sequences known to flank the (CA)n repeat sequence region to create a 138bp fragment illustrated in figure 16 (Heesom *et al* 1994, Shah *et al* 1998). The sense amplimer was composed of 9 (G+C) and 13 (A+T) bases, and the antisense primer was composed of 12 (G+C) and 7 (A+T) bases the sequences of which are also outlined in table 9. As before amplimers were

chosen to share the optimum annealing temperature of ≈68°C. Template sequences for H. sapiens, aldose reductase gene promoter region; accession number U72619 were also obtained from GenBank Internet website- www.ncbi.nlm.nih.gov (Ko and Chung 1997).

The amplification optimisation procedure as previously described in this section was carried out for both microsatellite regions studied in order to achieve maximum efficacy of the reaction. Final PCR reaction mixture and cycling conditions for both markers are outlined in table 10 and table 11 respectively. All PCR products were checked against a molecular weight marker for size using agarose gel electrophoresis as described.

D17S934 (H.sapiens, accession number Z23831):

> agctctgaat ggcccttggt ccatgcctct ctccctctct cctccctctt cctccacttcc
> tgacttcagg acctgttccc tcctgctccc tacaagcatg cat*acacaca* cat*acacaca*
> ***cacacacaca cacacacaca cacacacaca c***gcactcata ccccacctca gataggaaac
> aggacttgcc ctcccaggac ctatattaag acctatgggg agtggctggg atctgggccc
> ccagtgggtc tgtgagcatg aggtggtgag tgtgcaaaag tgtgagccta tgagagtgag

5'ALR2 (H.sapiens, accession number U72619):

> cctcatttgt cttaccttgg tcccagccca gccctatacc ta*gtgtgtgt gtgtgtgtgt*
> *gtgtgtgt gtgtgtgt gtgtgtgtg* tttcctttta aattatttcc ttaggaaaaa
> ttcccatgat gggagattac tggttcagag catgttaaga ttccaattac tagagtgttt

Figure 16. Nucleotide sequences for D17S934 and 5'ALR2 microsatellite regions under investigation, obtained through the GenBank Internet website- www.ncbi.nlm.nih.gov. Dinucleotide repeat regions are italicised, and amplimer sequences are highlighted.

Microsatellite	Direction	Sequence
D17S934	Sense	5'-AGCTCTGAATGGCCCTTGGTCCATG-3'
	Antisense	5'-GTCCTGTTTCCTATCTGAGGTGGGG-3'
5'ALR	Sense	5'-GAATCTTAACATGCTCTGAACC-3'
	Antisense	5'-GCCCCAGCCCTATACCTAG-3'

Table 9. Amplimer pair sequences designed to amplify microsatellite polymorphisms at regions 5'ALR2 and D17S934.

Master Mix	5'ALR2	D17S934
DNA	300ng	300ng
10 x PCR buffer	5µl	5µl
Sense primer	15pmol	10pmol
Antisense primer	10pmol	10pmol
MgCl₂	1.5mM	1.5mM
dNTP mix	0.2mM	0.2mM
Taq DNA Polymerase	0.5 units	0.5 units
Sterile H₂O	make up to 50µl	make up to 50µl

Table 10. Optimised PCR reaction mixture for 5'ALR2 and D17S934 microsatellite regions.

Cycle	D17S934	5'ALR
Denaturation then 30 cycles of:	94°C for 3 minutes	94°C for 1 minute
Denaturation	94° C for 30 seconds	94°C for 30 seconds
Annealing	56°C for 2 minutes	61°C for 2 minutes
Extension	72°C for 2 minutes	72°C for 2 minutes
Samples were cooled at 4°C until further use.		

Table 11. Optimised PCR reaction times and temperatures for 5'ALR2 and D17S934 microsatellite regions.

Incorporation of radio-labelled amplimers into PCR

Once the PCR conditions were optimised for the microsatellites studied, radiolabelling of the 5' flanking amplimer with Redivue [γ^{32}P] dATP was carried out. This radiolabelled amplimer was then incorporated into a new PCR reaction.

5'End labelling of amplimers using T4 polynucleotide kinase (T4-PNK)

To facilitate detection a radioactive nucleotide precursor was incorporated into the PCR reaction mix. Amplimers were labelled with Redivue [γ^{32}P] dATP (Amersham U.K) using Ready-to-Go™ T4-polynucleotide kinase (T4-PNK) (Pharmacia Biotech). The lyophilised T4-PNK was re-suspended in 25µl of sterile water and incubated at room temperature for 2-5 minutes. The solution was then mixed by gently pipetting up and down using a Gilson pipette tip. 5-10 pmoles of sense amplimer was added along with sufficient water to bring the reaction volume up to 49µl. 1µl of [γ^{32}P ATP (10mCi/ml) radioactive isotope (Amersham U.K), was added and briefly centrifuged for 30 seconds at 1300 x rpm (Biofuge 13 microcentrifuge, Heraeus Sepatech, Germany). Samples were then incubated at 37°C in a water-bath for 30 minutes.

Precipitation of labelled amplimer

A 5.5µl volume of 5M NaCl, and 2µl QuickPrecip® (Edge Biosystems, Oxon, UK) was added along with approximately three volumes of ice-cold 100% ethanol (-20°C) to the reaction tube to precipitate the DNA. After vortexing, the samples were centrifuged for 3 minutes. Each sample was then briefly vortexed and re-centrifuged for 30 sec's. All the supernatant fluid was decanted off and discarded and the precipitate was rinsed in both 100% and 70% ethanol. 50µl of sterile water was used to resuspend the precipitated DNA. Incorporation of γ^{32}P was then measured using a Beckman scintillation counter (Packard Biosciences, UK).

Assessment of efficacy of amplimer labelling

1µl of the labelled amplimer was counted for radioactivity using a Beckman scintillation counter. This gave a count per minute (cpm) reading. Determination of the cpm for 1µl of the sample allowed calculation of the volume of labelled amplimer required for each reaction when the desired amount of radioactivity for the PCR is 25,000cpm.

PCR was carried out as previously described, on average 25000cpm of the labelled amplimer was added to the reaction mixture. All PCR products were checked by running a 10-15µl aliquot of the sample and 1-1.5µl of loading buffer against a 100bp molecular weight marker on a 1% agarose/ethidium bromide gel as previously described. The gel was visualised under UV light and a photograph was taken. If the PCR was successful the PCR products were then electrophoresed on a vertical polyacrylamide gel.

Polyacrylamide Gel Electrophoresis (PAGE)

For microsatellite analysis the PCR products were then size fractionated by electrophoresis on a vertical polyacrylamide gel. A BioRad Sequi-Gen GT electrophoresis cell (30 x 50cm) (BioRad Laboratories, Hemel Hempstead, UK), was used with a 49 well vinyl sharks-tooth comb and 0.4 mm spacers. All components of the gel rig were thoroughly cleaned in warm water, followed by 70% Industrial Methylated Spirit (IMS) solution (Rathburns) and allowed to air dry. The inner surfaces of the gel plates were then siliconised with a thin layer of Repelcote® (BDH, UK) to prevent the gel from adhering to the glass plates. A small amount of petroleum jelly was run down the edges of the base plate to grip the vinyl 0.4-mm gel spacers that were positioned at the edges. The glass top plate was then positioned onto the base plate and sandwiched together using two arm clamps. The casting tray was then attached to the assembled plate and levelled horizontally using a spirit level.

A 5% polyacrylamide (formamide/urea) gel was prepared using a series of solutions (National Diagnostics U.S). 30mls Sequagel concentrate (237.5g acrylamide, 12.5g methylene bisacrylamide and 500g urea), 15 ml Sequagel buffer (50% 8.3M urea in 1M Tris-Borate-20mM EDTA buffer pH 8.3), 99ml Sequagel diluent (500g 8.3M urea), 12ml formamide (Sigma chemicals) were mixed together in a 250 ml conical flask. 70μl TEMED and 1500μl 10% ammonium persulphate (APS) were used to polymerise the gel (Sigma, UK).

The gel solution was cast into the horizontally positioned cell using a 200 ml syringe with a delivery tube, according to the manufacturer instructions. The flat edge of the sharks-tooth comb was inserted into the gel in an inverted position at the top of the gel plates, and the gel was allowed to polymerise over 2 hours at room temperature. The gel rig was then assembled vertically into the base and equilibrated overnight in 1 x TBE buffer. The system was connected to a power-pack (BioRad PC3000) and pre-warmed at 1800V for 30 minutes or until the temperature indicator read 40°C. The sharks-tooth comb was then removed and reinserted in the correct orientation to form the sample wells.

PCR amplification products (6μl) were mixed with 3μl of 10 x formamide stop solution (Gibco BRL, UK) and loaded into the wells on the gel using a 10μl Drummond® sequencing pipette (Drummond Laboratories, USA). The electrophoresis was carried out for 2.5 to 3 hours at 1500–2000V or maintained at a constant temperature of 50°C. The marker dye indicated how far the PCR products had migrated down the gel, this acted as an indicator to detect when the separation of bands was reached. The gel was removed from the plate and fixed in 10% Methanol/Acetic acid, transferred from the cell plate to filter paper and dried on a vacuum/heated gel drying system (Heto, UK) at 80°C for approximately 1-2 hours.

Autoradiography of polyacrylamide gel

The gel was then exposed to Kodak X-OMAT films between Cronex intensifying screens for 8 hours at -80°C. The film was developed in Kodak developing and fixing agents (according to manufacturer's instructions). Alternatively gels were exposed onto plates using a BioRad Fluor S analyser, and printed onto photographic paper (BioRad Phosphoimager).

Scoring of microsatellite alleles

Allele scoring was carried out by two independent observers. Description of how alleles were assigned for each microsatellite is given in the result's section. Analysis of bands was carried out either by eye or using BioRad analysis software.

Single Nucleotide Polymorphism (SNP) detection

Three single base polymorphisms were studied by restriction site enzyme digestion, firstly a C to T (*Bfa*1 forming) polymorphism situated −106bp upstream of the aldose reductase start site, secondly an A to C polymorphism situated at nucleotide +11842 of intron 8 within the aldose reductase gene itself, and thirdly an A to C polymorphism at nucleotide position 5178 within the mitochondrial genome.

Restriction site polymorphisms

Restriction site polymorphisms are single base polymorphic sites, which result in the formation or alteration of a restriction enzyme cut site. Restriction enzyme digestion of a PCR product containing the known polymorphic site enables the genotype to be determined. Amplimers can be designed to flank the known polymorphic region such that restriction enzyme cutting results in the production of different sized fragments, which can be determined by agarose gel electrophoresis.

C(-106)T polymorphism detection by restriction enzyme digestion.

The amplimers for the basal promoter region of the ALR2 gene were used to amplify the 263 base pair fragment that included the novel C(-106)T polymorphic site. The amplimer sequences were previously designed by Heesom *et al* 1994 and Patel *et al* 1996 from gene sequences obtained through the GenBank Internet website- www.ncbi.nlm.nih.gov (figure 17). The upstream amplimer consists of 24 nucleotides spanning the −222 to −199 region, and the downstream amplimer consists of 21 nucleotides which extended from nucleotide +21 to the translation start ATG start codon (table 12). The polymorphism creates a new *Bfa*1 restriction site and digestion produced fragments detectable by size fractionation (table 13). DNA was amplified by PCR as previously described using a reaction mixture outlined in table 14, and cycling strategy as outlined in table 15. PCR products were checked for efficacy on an agarose/ethidium bromide gel as previously described. It proved

to be very difficult to genotype all of the samples for the C-106T polymorphism as many failed to respond to the restriction enzyme digestion stage of the assay. Failure primarily lay with poor or incomplete restriction enzyme digestion. This probably resulted from an inhibiting factor preventing the enzyme from working. Despite stringent purification protocols being applied some samples still failed to respond. This may have been due to different batches of DNA used and differences in the DNA extraction protocols applied.

Direct Purification of PCR products

The restriction enzyme *Bfa*1 is an isoschizomer of *Mae* 1 and *Rma* 1, and according to the manufacturers protocol *Bfa*1 has shown minimal cleavage of unpurified PCR products. Therefore, PCR products required purification before *Bfa*1 digestion. PCR products were purified using the Wizard® PCR PrepDNA purification system (Promega, Madison, USA) and a Vac-Man® laboratory vacuum manifold. PCR products were transferred to a sterile microcentrifuge tube and 100μl of direct purification buffer (KCl 50mM, 10mM Tris-HCl (pH 8.8 at 25°C), 1.5mM $MgCl_2$ and 0.1% Triton X®-100) was added. After vortexing, 1ml of Wizard® PCR prep DNA purification resin was added and the mixture was briefly vortexed 3 times over a 1-minute period.

For each PCR product one Wizard® mini-column was prepared by attaching a syringe barrel to the Luer-Lok® extension of the mini-column according to manufacturers instructions. This mini-column/syringe barrel assembly was inserted into the vacuum manifold (Promega). The resin/DNA mix was pipetted into the syringe barrel and a vacuum was applied to draw the mixture through the mini-column. The mini-column was washed with 2 ml of 80% isopropanol (Sigma chemicals). The resin was dried by centrifuging the mini-column at 800 x rpm in a micro-centrifuge for 2 minutes. The mini-column was transferred to a new micro-centrifuge tube and 50μl of sterile water was added

for 1 minute. The mini-column was re-centrifuged for 20 seconds at 800 x rpm to elute the DNA fragment, which was collected in a sterile 1.5ml microcentrifuge tube.

*Bfa*1 restriction endonuclease digestion

The C(-106)T substitution polymorphism creates a new *Bfa*1 restriction endonuclease site. To detect the C(-106)T polymorphism 20 µl of purified amplification product was digested using 8 units of *Bfa*1 (New England Biolabs, UK) and incubated in 1 x NE buffer 4 (50mM potassium acetate, 20mM tris acetate, 10mM magnesium acetate, 1mM dithiolthreitol, pH 7.9) (New England Biolabs, UK) at 37°C for 3 ½ hours. The allelic variant C enabled cleavage at one site only producing two fragments 206bp and 57bp in size. The allelic variant T enabled Bfa1 cleavage at two sites, producing 147bp, 59bp and 57bp fragments. A heterozygous CT genotype therefore produced restriction fragments 206bp, 146bp, 59bp and 57bp (table 13). Digestion fragments were separated using 3.5% agarose/ethidium bromide gel electrophoresis against a 100bp molecular weight marker (as previously described). Timed digestion was checked by running a 3µl aliquot with 1.5µl of loading buffer and if digestion was incomplete a further 5-10 units of restriction enzyme was added in 3µl of 10 x buffer and incubated for a further hour. The enzyme concentration in solution was kept below 10% to minimise non-specific cleavage of DNA due to the presence of glycerol. Digestion fragments were viewed using a UV transilluminator and genotyping was carried out by eye as illustrated in figure 25.

5'ALR2 (H. sapiens, accession number U72619):
-273

tattttcgct aaagcattcg ctttcccacc agatacagca gctgaggaac tcctttctgc

cacgcggggc gcgggcgagc gttgggggcg gaaagaatcc gctgcca*cta g*gaccaggcg

gaagaagcat ccccgccgac ccttggggaa ggccgccgcg gcaccc*ccag* cgcaaccaat

cagaaggctc cttcgcgcag cggcgcgcca accgcaggcg ccctttctgc cgacctcacg

ggctatttaa aggtacgcgc cgcggccaag gccgcaccgt tactgggcggg ggtctggggа

gcgcagcagc catggcaagc cgtctcctgc tcaacaacgg cgccaagatg cccatcctgg

+

Figure 17. Nucleotide sequence for 5'ALR2 C(-106)T polymorphic region under investigation, obtained through the GenBank Internet website- www.ncbi.nlm.nih.gov. *Bfa*1 restriction sites are italicised and amplimer sequences are highlighted.

Polymorphism	Direction	Sequence
C(-106)T	Sense	5' CCT TTC TGC CAC GCG GGG CGC GGG 3'
	Antisense	5' CAT GGC TGC TGC GCT CCC CAG 3'

Table 12. Amplimer pair sequences designed to amplify the 5'ALR2 C(-106)T polymorphic region.

C(-106)T	Restriction endonuclease	Allele fragment sizes
	*Bfa*I	C: 57bp, 206bp
	5'...C▾T AG...3'	T: 57bp, 59bp, 147bp.
	3'...G A▴TC...5'	

Table 13. *Bfa*1 restriction enzyme allele fragment sizes for 5'ALR2 C(-106)T polymorphism.

Master Mix	C(-106)T
DNA	300ng
10 x PCR buffer	5µl
Sense primer	10pmol
Antisense primer	10pmol
MgCl$_2$	1.5mM
dNTP mix	0.2mM
Taq DNA Polymerase	1.0 units
Sterile H$_2$O	make up to 50µl

Table 14. Optimised PCR reaction mixture for 5'ALR2 C(-106)T polymorphic region.

Cycle	C(-106)T
Denaturation then 32 cycles of:	96°C for 3 minutes
Denaturation	94° C for 30 seconds
Annealing	70°C for 2 minutes
Extension	72°C for 1 minutes
Samples were cooled at 4°C until further use.	

Table 15. Optimised PCR reaction times for 5'ALR2 C(-106)T polymorphic region.

(a) Allelic variant C

Bfa 1 cut   no cut

C(-106)T-Forward amplimer   C(-106)T-Reverse amplimer
ctttctgccacgcgggggc gcgggᴡᴡᴡᴡᴡ▶ ctag ᴡᴡᴡᴡᴡ▶ ccag ᴡᴡᴡᴡᴡ▶ctgggggagcgcagcagccatg
gatc   ggtc

57bp fragment   206bp fragment

(b) Allelic variant T

Bfa 1 cut   Bfa 1 cut

ctttctgccacgcgggggc gcgggᴡᴡᴡᴡᴡ▶ ctag ᴡᴡᴡᴡᴡ▶ ctag ᴡᴡᴡᴡᴡ▶tgggggagcgcagcagccatg
gatc   gatc

57bp fragment   59bp fragment   147bp fragment

57/59 bp
147 bp
206 bp
263 bp

Genotype   TT   CC   CT   Control

Figure 18. Illustration of C(-106)T restriction site polymorphism (RSP's). This figure illustrates the C(-106)T restriction site polymorphism where the 263bp PCR product is cut by the restriction enzyme *Bfa* I which detects and cuts at the C▾TAG sequence. Alleleic variant C is cut into two restriction fragments 57bp and 206bp, and an allele which contains the C to T polymorphism is further cut into three fragments 57bp, 59bp and 147bp. Subjects heterozygous for C(-106)T polymorphism showed restriction fragments 57bp, 59bp, 147bp and 206bp in size. A control was always included where no restriction enzyme was added, producing a 263 bp uncut band. Alternative primer design would have resolved the 57bp and 59bp fragments.

ALR2 A(+11842)C polymorphism detection by restriction endonuclease digestion

Amplimers previously designed by Kao *et al* 1999 for the aldose reductase intragenic region containing the novel A(+11842)C polymorphism were used to amplify the 252bp fragment of interest. The amplimer sequences were checked from gene sequences obtained through GenBank Internet website- http://www.nlm.nih.gov (figure 19). The upstream amplimer consists of 21 nucleotides spanning the +11763 to +11783 region, and the downstream amplimer consists of 21 nucleotides which span from +11993 to +12012 nucleotides upstream of the aldose reductase start site (table 16). The polymorphism abolishes a *Bam*H1 restriction enzyme site and digestion produced fragments detectable by size fractionation (table 17). DNA was amplified by PCR as previously described using a reaction mixture outlined in table 18, and cycling strategy as outlined in table 19.

_Bam_H1 restriction endonuclease digestion

The A(+11842)C substitution polymorphism abolishes a *Bam*H1 restriction endonuclease site. To detect the A(+11842)C polymorphism 5µl of amplification product was then digested using 10 units of *Bam*H1 (New England Biolabs, UK) and incubated at 37°C for 45 minutes. The allelic variant C showed no cleavage by *Bam*H1 digestion and the 252bp PCR product remained intact. The allelic variant A enabled *Bam*H1 cleavage at one site, producing two restriction fragments, 174bp and 78bp in length, detectable by agarose gel electrophoresis. A heterozygous AC genotype therefore produced all three fragments, 252bp, 174bp, and 78bp. To ensure completed restriction enzyme digestion an internal control was always incorporated alongside. The internal control consisted of patient DNA for which the correct genotype had previously been confirmed by direct sequencing methods (MWG). Digestion fragments were separated using 2.5% agarose/ethidium bromide gel electrophoresis alongside a 100bp molecular weight marker (as previously described). Timed digestion was checked by running

a 3μl aliquot of digestion mixture with 1.5μl of loading buffer and if digestion was incomplete a further 5-10 units of restriction enzyme was added and incubated for a further 30 minutes. Digestion fragments were viewed using a UV transilluminator and genotyping was carried out by eye.

ALR2 (H. sapiens, accession number AF032455):

tcttggctgg tcaggcctgg ccctcctcca tggagtgggg gatgggggag gcctctcatc
ctgtctctgg agtgtcatct gtg*ggatcc*c caccatcctc tcttctgagg ccagggagct
gtggcgagca agccaagact gagactgaca cctcaccagt ggagccgtgt gccaggggca
ggccttgggt ccagggccgt gctgtggcaa tacacctaca cctttgctca ggcccttcag
cacaccgaga ggttacccgg ggagaatctc gctcttgagc ttcactgcct ggacctgccc

Figure 19. Nucleotide sequence for ALR2 A(+11842)C polymorphic region within intron 8 of the aldose reductase gene, obtained through the GenBank Internet website-http://www.ncbi.nlm.nih.gov. *Bam*HI restriction site containing the A(+11842)C polymorphism is italicised and amplimer sequences are highlighted.

Polymorphism	Direction	Sequence
A(+11842)C	Sense	5' CTG GTC AGG CCT GGC CCT CCT 3'
	Antisense	5' GTA ACC TCT CGG TGT GCT GAA 3'

Table 16. Amplimer pair sequences designed to amplify the ALR2 A(+11842)C polymorphic region.

A(+11842)C	Restriction endonuclease	Allele fragment sizes
	*Bam*H1	C: 252bp
	5'...G▾GATC C...3'	A: 174bp, 78bp
	3'...C CTAG▴G...5'	

Table 17. Restriction enzyme allele fragment sizes for ALR2 A(+11842)C polymorphism.

Master Mix	A(+11842)C
DNA	300ng
10 x PCR buffer	5µl
Sense primer	10pmol
Antisense primer	10pmol
$MgCl_2$	1.5mM
dNTP mix	0.2mM
Taq DNA Polymerase	1.0 units
Sterile H_2O	make up to 50µl

Table 18. Optimised PCR reaction mixture for ALR2 A(+11842)C polymorphic region, detected using *Bam*H1 restriction enzyme digestion.

Cycle	A(+11842)C
Denaturation then 32 cycles of:	94°C for 2 minutes
Denaturation	95° C for 1 minute
Annealing	66°C for 1 minute
Extension	72°C for 1 minutes
Samples were cooled at 4°C until further use.	

Table 19. Optimised PCR reaction times for ALR2 A(+11842)C polymorphic region.

Mitochondrial C(5178)A polymorphism detection by restriction endonuclease digestion

Amplimers previously designed by Gong *et al* 1998 for the mitochondrial gene C(5178)A polymorphism were used to amplify the 417bp fragment of interest. The amplimer sequences were checked from gene sequences obtained through GenBank Internet website-http://www.nlm.nih.gov (figure 20). The upstream amplimer consists of 24 nucleotides spanning the Mt4949 to Mt4972 region, and the downstream amplimer consists of 24 nucleotides which span from Mt5342 to Mt5365 (table 20). The polymorphism abolishes a *Alu*1 restriction enzyme site and digestion produced fragments detectable by size fractionation (table 21). DNA was amplified by PCR as previously described using a restriction mixture outlined in table 22, and cycling strategy as outlined in table 23.

AluI restriction endonuclease digestion

The C(5178)A substitution polymorphism abolishes a *Bam*H1 restriction endonuclease site. To detect the C(5178)A polymorphism 10μl of amplification product was then digested using 10 units of *Alu*1 (New England Biolabs, UK) with NE buffer 2 (50 mM NaCl,10 mM Tris-HCL, 10 mM MgCl, 1 mM dithiothreitol) and incubated at 37°C for 4 hours. The allelic variant A showed *Alu*1 cleavage at one site, producing two digestion fragments, 43bp and 374bp. The allelic variant C enabled *Alu*1 cleavage at two sites, producing three restriction fragments 43bp, 188bp and 186bp in size. To ensure completed restriction enzyme digestion an internal control was always incorporated alongside. The internal control consisted of patients DNA for which the genotype had previously been confirmed by direct sequencing. Digestion fragments were separated using a 3% agarose/ethidium bromide gel electrophoresis alongside a 100bp molecular weight marker (as previously described). Timed digestion was checked by running a 3μl aliquot of digestion mixture with 1.5μl of loading buffer and if digestion was incomplete a further 5-10 units of restriction enzyme was added and incubated

for a further 30 minutes. Digestion fragments were viewed using a UV tranilluminator and genotyping was carried out by eye.

(H. sapiens, accession number;NC_001807):

tcacatgaca aaaactagcc cccatctcaa tcatatacca aatctctccc tcactaaacg
taagccttct cctcactctc tcaatcttat ccatcatagc aggcagttga ggtggattaa
accaaaccc*a gct*acgcaaa atcttagcat actcctcaat tacccacata ggatgaataa
tagcagttct accgtacaac cctaacataa ccattcttaa tttaactatt tatattatcc
taactactac cgcattccta ctactcaact taaactccag caccacgacc ctactactat
ctcgcacctg aaaca*agct*a acatgactaa cacccttaat tccatccacc ctcctctccc
taggaggcct gcccccgcta accggctttt tgcccaaatg ggccattatc gaagaattca
caaaaaacaa tagcctcatc atccccacca tcatagccac catcaccctc cttaacctct
acttctacct acgcctaatc tactccacct caatcacact actccccata tctaacaacg
taaaaataaa atgacagttt gaacatacaa aacccacccc attcctcccc acactcatc

Figure 20. Nucleotide sequence for Mt C(5178)A polymorphic region within the mitochondrial genome, obtained through the GenBank Internet website-http://www.ncbi.nlm.nih.gov. The *Alu*1 restriction site containing the C(5178)A polymorphism is italicised and amplimer sequences are highlighted.

Polymorphism	Direction	Sequence
Mt C(5178)A	Sense	5' ATC CAT CAT AGC AGG CAG TTG AGG 3'
	Antisense	5' GAG TAG ATT AGG CGT AGG TAG AAG 3'

Table 20. Amplimer pair sequences designed to amplify the mitochondrial C(5178)A polymorphic region.

Mt C(5178)A	Restriction endonuclease	Allele fragment sizes
	*Alu*1	A: 43 bp, 374bp
	5'...AG▾CT...3'	C: 43bp, 188bp, 186bp
	3'...TC▴GA...5'	

Table 21. Restriction enzyme allele fragment sizes for mitochondrial C(5178)A polymorphism.

Master Mix	Mt C(5178)A
DNA	300ng
10 x PCR buffer	5µl
Sense primer	10pmol
Antisense primer	10pmol
$MgCl_2$	1.5mM
dNTP mix	0.2mM
Taq DNA Polymerase	1.0 units
Sterile H_2O	make up to 50µl

Table 22. Optimised PCR reaction mixture for mitochondrial C(5178)A polymorphic region, detected using *Alu*1 restriction enzyme digestion.

Cycle	Mt C(5178)A
Denaturation then 32 cycles of:	94°C for 4 minutes
Denaturation	94° C for 30 seconds
Annealing	49°C for 2 minutes
Extension	72°C for 2 minutes
Samples were cooled at 4°C until further use.	

Table 23. Optimised PCR reaction times for mitochondrial C(5178)A polymorphic region, detected using *Alu*1 restriction enzyme digestion.

Electrophoretic mobility shift assay for 5'ALR2 ORE's

The electrophoretic mobility shift assay (EMSA) was used to quantitatively detect the interaction between the sequence specific osmotic response element (ORE) binding proteins and the ORE gene sequences in the 5' promoter region of the aldose reductase gene (ALR2). The assay was performed by incubating a known quantity of purified nuclear protein extracts with a ^{32}P end-labelled DNA fragments containing the putative protein binding sites, which in this study were the ORE's of 5'ALR2. The assay incorporates the use of a non-denaturing polyacrylamide gel to separate protein/DNA complexes from free DNA, which can then be analysed quantitatively. This study was carried out using a Gel Shift Assay System (Promega Life Sciences, Southampton, UK; http://www.promega.com). The study used the established cell lines Jurkat E6.1 as well as PBMC's extracted from fresh whole blood samples.

Established cell lines

Jurkat E6.1 Human Leukaemic T cell lymphoblast- ECACC No. 88042803

This cell line was obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK), and are an IL-2 producing cell line derived by incubating the cells at 41°C for 48h followed by limited dilution cloning over macrophages. Morphologically these cells grew as a suspension. Cells were initially decanted into a centrifuge tube and centrifuged at 70-100g for 5 minutes. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 + 2mM Glutamine + 10% Foetal Bovine Serum (FBS) at 37°C in a 5% CO^2 incubator. Cells were grown and were sub-cultured during their log phase of growth 2-3 days following seeding determined by haemocytometer counting. Cells were maintained at between 3-9 x 100,000 cells/ml.

Extraction of Peripheral blood mononuclear cells (PBMC's) from whole blood

Peripheral blood mononuclear cells (PBMC's) were extracted from 20 ml fresh whole blood/ Heparin samples. Briefly the blood was diluted in equal volume of Dulbecco's Phosphate Buffered Saline (PBS) (Life Technologies, Paisley, UK) pre-warmed to 37°C in a water-bath (Grant, UK). 7 ml of Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) was added to each of 6 x 15 ml Falcon® centrifuge tubes and 7 ml of the blood/PBS solution was carefully added using a sterile Pasteur pipette without disturbing the Lymphoprep™ surface. Lymphoprep™ has a density of 1.077 $\pm$ 0.001 g/ml, and osmolality of 280 + 15 mosm to facilitate the isolation of PBMC's by density gradient centrifugation. The samples were centrifuged at 1600 x rpm with no break setting for 30 min at room temperature. The lymphocytes at the interface layer were then removed using a sterile Pasteur pipette and transferred into a clean 15-ml Falcon® centrifuge tube. Cells were washed twice, using 4 x volume RPMI 1640 and centrifuging at 1400 x rpm for 10 minutes in a bench centrifuge. Cells were then re-suspended at a concentration of 0.5 x 10^6 /ml in RPMI 1640 (11mM D-glucose) supplemented with 5% L-Glutamine, 10% Foetal calf serum (FCS) and 5% Penicillin/Streptomycin. The cell suspension was divided into two 250 ml cell culture flasks (Becton-Dickinson). The cells were stimulated by the addition of the mitogen stimulant phytohaemagglutinin (PHA) (1μg/ml) and incubated in a Nuaire Flow incubator (Indis Scientific, Glasgow, UK) at 37°C with 5% CO_2 until cell replication was achieved.

Cryopreservation

For future work cultured cells were stored by cryopreservation. Actively growing cells, free from contamination were suspended in the cryoprotectant dimethylsulphoxide (DMSO) complete medium 10% (v/v) and FBS to give 1 x 10^6-1 x 10^7 cells/ml in medium. Cell number was counted using trypan blue solution (Sigma Chemicals, Poole, UK) and a Neubauer

haemocytometer. Cells were cooled at ~1°C/min and frozen at a continuous rate to -196°C and transferred to gaseous phase liquid nitrogen. When required the ampoules of frozen cell lines were resuscitated by removing from liquid nitrogen pod, and held at room temperature for approximately 1 minute and then transferred to a 37°C water bath for 1-2 minutes until fully thawed. To reduce risk of contamination, ampoules were wiped with tissue soaked in 70% alcohol prior to opening. The contents of the ampoule were then pipetted directly into a flask of media pre-warmed to 37°C.

Establishment of stress conditions

For the purposes of studying ORE binding protein activity, cells were incubated under hyperosmotic and hypertonic conditions to simulate the diabetic hyperglycaemic state. PBMC's from T1DM patients and normal controls, as well as the cell lines were incubated at 37°C for 24 hours before the addition of the stress factor. For the hyperglycaemia experiments cells were cultured in either normoglycaemic conditions (11 mmol/l D-glucose) or supplemented with 17 mmol/l D-glucose to create a 28mM/l environment (hyperglycaemia). Optimal glucose dosage was obtained by carrying out the assay using 14 mmol and 28 mmol D-glucose. The best results were obtained from the 28 mmol assay, and this was therefore used for the experiments. Cells were then incubated for 5 days at 37°C in a Nuaire flow incubator. Cells remained viable for 5 days and this was confirmed by examination under the microscope and by Trypan Blue staining techniques.

Extraction of nuclear protein from whole cells

Nuclear protein extracts were prepared using methods previously described by Dignam *et al* 1983 [a and b]. Non-adherent cells were re-suspended in the culture flask by gently pipetting the culture medium up and down using a Pasteur pipette, and transferred to a Falcon®

centrifuge tube. Adherent cells were detached from the cell culture flask using trypsin/EDTA solution and also transferred to Falcon® centrifuge tubes. Cells were then pelleted by centrifugation at 800 x rpm for 4 minutes. The supernatant was removed and cells were washed twice with PBS pre-warmed to 37°C in a water-bath and centrifuged at 600 x rpm for 30 sec's. The supernatant was discarded and the pelleted cells were re-suspended in 100μl of buffer A (10mM HEPES, pH 7.9, 1.5 mM MgCl$_2$, 10mM KCl, 0.5mM DTT, 0.2% NP-40, 100mM AEBSF, 18.4mg/ml sodium orthovanadate, 42mg/ml sodium flouride and 2.2mg/ml aprotonin) and held on ice for 15 minutes. The resulting cell lysate was then centrifuged at 1300 x rpm for 10 minutes. The supernatant containing cytoplasmic proteins was removed and the nuclear pellet was re-suspended in 50μl of buffer C (20mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5mM MgCl$_2$, 0.5mM DTT, 0.2 EDTA, 100 mM AEBSF, 18.4 mg/ml sodium orthovanadate, 42 mg/ml sodium fluoride, 2.2 mg/ml aprotonin), and incubated on ice for 10 minutes. After centrifugation at 1300 x rpm for 10 minutes the supernatant containing the nuclear protein was extracted and stored in a fresh tube at -70°C until use.

Determination of protein concentration for nuclear fraction

The concentration of protein in the samples was determined using a Coomassie® Plus Protein Assay reagent kit (Peribo Science Ltd., Chester, UK). Serial dilutions of a 2.0mg/ml bovine serum albumin (BSA) protein standard were made, ranging from 0 to 2.0 mg/ml (0, 0.2, 0.4, 0.8, 1.6, 2.0) in clean dry glass test tubes. 1 ml of Coomassie® Plus Protein dye reagent was added to 50μl of each protein dilution, gently vortexed and left to stand for 5min-1 hour. The OD$_{595}$ versus reagent blank was measured using a WPA UV1101 Biotech Photometer (Biotech, UK), and a standard curve was plotted. Sample protein concentrations were established by reading from the standard curve, and accounting for appropriate dilution factors.

Oligonucleotide probe design and labelling

Oligonucleotide sequences were designed with the aid of sequences downloaded from GenBank database (www.ncbi.nlm.nih.gov). Three oligonucleotides were designed, each to incorporate one ORE sequence within the promoter region of the ALR2 gene (figure 21, table 24). All oligonucleotides were commercially synthesised by MWG Biotech (Milton Keynes, UK) at a scale of 0.2μmol. Oligonucleotide probes were re-suspended in sterile water to create a working dilution of 1.75 pmoles/ul. Phosphorylation was carried out by incubating 1.75 pmol of oligonucleotide for 10 minutes at 37°C in a reaction mixture containing; T4 polynucleotide kinase 10 X buffer [100mM Tris-HCl (pH 7.6), 100 mM MgCl$_2$, 50mM DTT], 1μl λ-^{32}P-ATP, nuclease free water and 5-10 units T4 polynucleotide kinase (Promega Life Sciences, Southampton, UK). The reaction was stopped by adding 1μl of 0.5M EDTA and diluted in 89μl of TE buffer (10mM Tris-HCl pH 8.0, 1 mM EDTA).

5'ALR2 (H. sapiens, accession number U72619):

caaattctat   tacttgggga   caaatgaatt   tgcccaatag   caacaatttt   tacaaaag*tt*
*acatggaaaa   atatctggg*c   tagtctgtt*c   tgtataaatt   tttccaggag   gg*agcacttt
taaagaaag*c   accaaatgga   aaatcaccgg   catgg*agttt   agagagacct   ggtgcttgag

Figure 21. Nucleotide sequences for 5'ALR2 promoter region containing osmotic response elements. Oligonucleotide sequences are highlighted in blue (OREA), green (ORE B), and yellow (ORE C).

ORE	Sequence
ORE A sequence	5' TGG AAA AAT AT 3'
• **ORE A probe**	5' TTA CAT GGA AAA ATA TCT GGG
ORE B sequence	5' AAA TTT TTC CA 3'
• **ORE B probe**	5' CTG TAT AAA TTT TTC CAG GAG GG 3'
ORE C sequence	5' GAA AAT CA 3'
• **ORE C probe**	5' ACC AAA TGG AAA ATC ACC GGC ATG G 3'

Table 24. Oligonucleotide sequences designed to incorporate the osmotic response element of interest.

Preparation of non-denaturing 4% acrylamide gel

Gel plates were wiped clean using 70% IMS solution and rinsed using warm purified water to remove any ionic contaminants. The glass plates were assembled using 0.75mm spacers and held together using clamps and a casting tray. The rig was loaded with distilled water to ensure a good seal and no signs of leakage. The water was poured away and well combs were inserted between the glass plates. The gel solution was prepared by mixing 5 ml 10 X TBE, 2.5 ml 2% bisacrylamide (BioRad), 10 ml 40% acrylamide, 3.1 ml 80% glycerol, 79.5ml sterile water glass wide necked conical flask. To polymerise the gel 70µl of TEMED and 750µl of 10%APS. The gel solution was drawn up into a syringe and the gel was poured into the gel casting system, care being taken not to form air bubbles, and left for 30 minutes to polymerise. Prior to running the samples the gel was positioned in a vertical position in a running tank and equilibrated in a 0.5% TBE running buffer at 100V for 30 minutes. The well comb was removed and the wells were washed out with 0.5 TBE using a 21-gauge needle and syringe.

Control and competition assay

The specificity of the DNA binding protein for the putative binding sites was established by competition experiments using an AP1 consensus oligonucleotide as a probe which contains other unrelated DNA sequences (figure 22).

SP-1 5′ GAT CGA ACT GAC CGC CCG CGG CCC GT 3′

AP-1 3′ ATT CGA TCG GGG CGG GGC GAG C 5′

Figure 22. Nucleotide sequence of SP-1 and AP1 consensus oligonucleotides used in competition experiments.

The AP1 (1.75 *p*mol) was labelled using [γ-^{32}P] ATP using a phosphorylation reaction as previously described. Four reaction conditions were set up; a negative control, a positive control and two competition assays to demonstrate binding specificity. The reactions were set up in sterile microcentrifuge tubes in accordance to table 25. These were incubated for 10 minutes at room temperature and then 1µl of [γ-^{32}P] ATP labelled ORE oligonucleotide was added to each reaction. This was then incubated at room temperature for a further 20 minutes, following which 1µl of gel loading 10 x buffer (250mM Tris-HCl, 0.2% bromophenol blue, 40% glycerol) was added to each reaction. The samples were then loaded into the wells of the 4% acrylamide gel and electrophoresed at 100V for 3-4 hours. The gel was then transferred to Whatman 3M paper (Whatman, Maidstone, UK) and overlaid with Saran Wrap™. The gel was then exposed to X-Omat photographic film (Kodak, UK) between intensifying screen for 48 hours at -80°C. The film was then developed using developer and fixing solutions (Kodak, UK). The bands were analysed and quantified using a phosphoimager (BioRad) with multi analyst software.

Negative control	Positive control
7µl Nuclease free water	5µl Nuclease free water
2µl Gel shift binding 5X buffer	2µl Gel shift binding 5X buffer
0µl Nuclear extract	2µl Nuclear extract
Specific competitor	**Non-specific competitor**
4µl Nuclease free water	4µl Nuclease free water
2µl Gel shift binding 5X buffer	2µl Gel shift binding 5X buffer
2µl Nuclear extract	2µl Nuclear extract
1µl Unlabelled competitor oligo (SP1)	1µl Unlabelled noncompetitor oligo (AP1)

Table 25. Reaction conditions for control and competition experiments for ORE A, B and C, using SP-1 as the competitive probe, (Promega Gel Shift Assay Systems Technical Bulletin No.110.

Protein reaction for subject gel shift assay

The labelled ORE probe along with a gel shift binding 5 x buffer (20% glycerol, 5mM MgCl2, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 50mM Tris-HCl, 0.25 mg/ml poly (d1-dc)• poly (d1-dc)) was incubated with 5-10μg of nuclear protein (determined by the protein assay described previously) at room temperature for 20 minutes. 5μl of gel loading dye (40% glycerol, 0.2% bromophenol blue, 250mM tris-HCl) was added and samples were loaded into the wells of the gel and electrophoresed as before.

Approaches to the detection of susceptibility loci for diabetic complications

There are two strategies that may be applied to identify susceptibility genes for diabetic complications, namely linkage analysis using affected pedigrees, and population association case-control studies (Chowdhury *et al* 1999). Linkage studies involve using affected sib pairs or discordant sib pairs and require a large number of families with two or more affected offspring. It is recognised that due to the complex inheritance of T1DM and T2DM large affected pedigrees of this sort are rare and somewhat difficult to collect. Association studies, which involve comparing allele and genotype frequencies of candidate genes, in individuals with microvascular disease and control subjects, are therefore an alternative strategy. There are two main approaches to performing association based studies which include case-control analysis and transmission disequilibrium analysis. Since there is no superior approach to identifying genetic determinants of diabetic microvascular complications more than one approach is needed for candidate gene analysis. The candidate gene approach has the advantage that the availability of a polymorphism close to the gene makes it possible to report clear positive or negative results because recombinants between the marker and the gene are unlikely. However, most of the methods have limited power in assessing minor rather than major gene effects. The approaches that were employed in this study were as follows;

Population based case-control association analysis

Population association studies require the use of large numbers (at least 200/group), of similar age distributions obtained from the same geographical population to try to exclude ethnic admixture. Confirmation of a positive or negative result is also required in a separate population, through multi-centre collections. However, despite stringent controls to match cases and controls, there still remains the possibility that significant associations are due to hidden population stratification and are therefore spurious. A population of British Caucasoid diabetic subjects with microvascular disease, diabetic subjects with no evidence of

microvascular disease and non-diabetic controls were studied, as well as a population of Southern Indian Dravidian diabetic subjects and controls in the course of this thesis.

Family based association analysis

Family based association analysis using a collection of trios has been regarded as the gold standard test for genetic association studies. Trios can be used to confirm and ultimately fine map linkages from linkage studies, facilitate candidate gene studies and to examine genetic associations. The transmission/disequilibrium test (TDT) is used in the study and involves the analysis of the frequency of transmission of designated alleles from heterozygous parents to affected offspring. A large collection of British Caucasoid nephropathy trios was collected through a multi-centre research programme from seven locations nation-wide and used in this thesis. Significant variance from the expected Mendelian transmission of 50:50 would indicate that the allele has a role in the susceptibility to microvascular complications of diabetes.

Statistical analysis of data

All the data collected was entered into a Microsoft Excel spreadsheet (Microsoft, UK) and statistical analysis was performed through this program, and also by the use of Epi-Info 6, (Centres for Disease Control and Prevention, Atlanta, Georgia, USA) program.

Allele and genotype frequencies

The number of alleles detected was obtained using gene counting. Allele frequencies were expressed as decimals and calculated from the number of copies of an allele divided by the total number of chromosomes tested in the population. A homozygous genotype was counted as two copies of the allele. Genotype frequencies were determined as the percentage of subjects positive for each genotype. The frequency of the alleles and genotypes between the patient subgroups and normal control groups were compared using Chi-squared distribution

test and 2 x 2 contingency tables to test significance by degrees of freedom (df). This was performed on the 'Stat-Calc' program (Epi Info 6). The P values were corrected for the number of comparisons made ($p= 0.05/n$, where n is the number of independent associations detected), and Pc-values of <0.05 were considered to be significant (Bland 1995). Where the observed number in each category is <5 Fishers exact test was applied.

Hardy-Weinberg equilibrium

The Hardy-Weinberg principle was used in order to determine whether a bi-allelic polymorphism is conforming to normal distribution within a population. The underlying assumption is that the frequency of alleles remains constant from generation to generation. The expected gene frequencies are calculated by the formula; $1 = p^2 + 2pq + q^2$ where p and q are the frequencies of the two alleles within the population under investigation. The observed gene frequencies were then compared with the expected frequencies using 2 x n tables and the Chi-squared test. Hardy-Weinberg was established if the expected frequency did not differ significantly (<= 0.05) from the observed (Strachan and Read 1996).

Transmission Disequilibrium Test (TDT)

The TDT was used in the analysis of the family data (Spielman *et al* 1993; Todd 1996; Schaid and Sommer 1994). The test evaluates the frequency with which a suspected susceptibility allele is transmitted from heterozygous parents to the affected offspring. TDT assesses deviation from 50% transmission. The association of an allele is determined by the use of the χ^2 test and 2x2 contingency tables. A Pc value of <0.05 was considered to be significant. In order to obtain an unbiased multihaplotype TDT analysis all transmissions were counted and a significance level was estimated empirically (Dudbridge *et al* 2000).

Chapter Four

Results

Results

Profile of T1DM Caucasoid patient groups

Table 6 shows the clinical features of the patient sub-groups with the mean, standard deviation and the range for subjects of British Caucasoid origin with T1DM. The total number of T1DM subjects studied was 244 consisting of 155 males and 129 females. The T1DM subjects were subdivided according to their diabetic complication phenotype. These groups include uncomplicated diabetes (n=66), diabetic retinopathy (n=44), diabetic neuropathy (n=18), diabetic nephropathy with diabetic retinopathy (n=30), diabetic retinopathy with neuropathy (n=24), and full house patients (n=62). The mean average age at the time of the study in all of the groups was between 46.3 ($\pm$15.6) and 51.6 ($\pm$13.3). The distribution of the mean average age at onset of diabetes in all groups was between 16.5 ($\pm$ 11.8) and 25.4 ($\pm$ 11.8) years of age and the range was similar in all of the groups. The mean average duration of diabetes in all groups was between 27.3 ($\pm$9.9) and 32.0 ($\pm$ 12.9). The range of this variable was also similar in all of the groups. There is a general elevation in the onset of diabetes between the ages of 11 and 13, and a sharp increase in incidence in the early twenties. Following this the incidence drops around mid twenties with a relatively low incidence from the late thirties and older.

There is no clinical data for the normal control population as they were taken from the cord blood.

Profile of T2DM Southern Indian population

Table 7 shows the clinical features of the patient sub-groups with the mean, standard deviation and the range for subjects of Southern Indian/Dravidian origin diagnosed with T2DM. The total number of T2DM subjects was 60, consisting of 34 males and 26 females. The T2DM subjects were sub-divided according to their diabetic complication phenotype. These consisted of two groups which were uncomplicated/normoalbuminuric diabetics (n=28), and diabetics with proteinuria (n=32). The mean average age at the time of the study in both groups was 55 ($\pm$10.1) and 53.8 ($\pm$8.9) respectively, and the range was similar in both groups. The mean average age at onset of diabetes in both groups was 42.1 ($\pm$11.3) and 42.3 ($\pm$8.5) respectively, and the range was similar in both groups. The mean average duration of diabetes in both groups was between 13.1 ($\pm$7.5) and 11.8 ($\pm$5.4) and the range of this variable in both groups was also similar. It is important to note that the T2DM normoalbuminuria patient subgroup had a duration of diabetes ranging from 2-29 years (mean 13.11 $\pm$ 7.5). It is possible that some of these subjects will develop microvascular disease later during the course of their disease. The peak age at onset of diabetes was between 40 and 50 years of age with a relatively low incidence before and after this age group.

The normal control population had a mean age of 38 $\pm$6.3 with a range from 30 to 58, which was a slightly larger age range than the diabetic subjects included in the study.

Aldose Reductase Gene Study

Associations of polymorphism's within the ALR2 gene and promoter region and the microvascular complications of T1DM and T2DM

Polymorphisms within the ALR2 gene and its promoter region

Polymorphisms within the ALR2 gene and its promoter region were investigated. The study incorporated three subject groups of which two were case control studies and one was a family trio based study. The study used a population of T1DM and normal control subjects of British Caucasoid origin, a population of T2DM and normal control subjects of Southern Indian origin, and the 'Diabetes UK- Warren nephropathy family trio' collection of T1DM diabetic subjects of British Caucasoid origin where the proband had diabetic nephropathy.

Three polymorphisms of the ALR2 gene region were studied, a (CA)n repeat microsatellite polymorphic marker located 2.1kb upstream of the aldose reductase gene start site, a C to T single base substitution polymorphism located −106bp upstream of the ALR2 start site, and an A to C polymorphism located within intron 8 (+11842) of the ALR2 gene itself (figure 23). The results of these studies are presented in tabular form in the following section.

For the case-control studies the data was analysed for allelic and genotypic frequencies between subjects with diabetes and subjects without diabetes, and then with regard to subjects onset of diabetic microvascular disease. For the family based study the TDT was applied to determine transmission frequency of alleles from parents to affected offspring.

Not all of the populations studied were investigated for all of the polymorphisms, but where possible the whole population of each group was analysed for the polymorphism studied for ease of comparability.
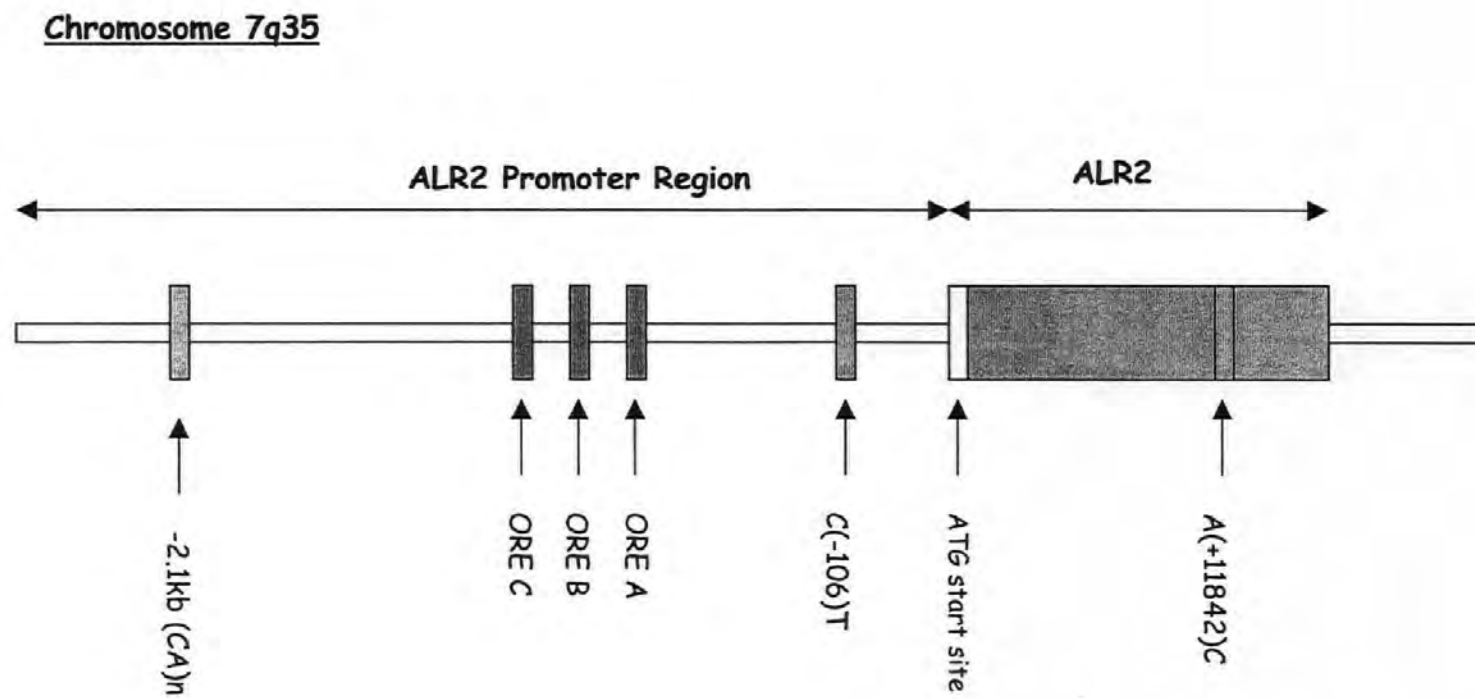
Figure 23. Diagrammatic representation of the polymorphic region studied within the ALR2 gene and promoter region located on chromosome 7q35.

5'ALR2-(CA)n microsatellite polymorphic marker

The (CA)n dinucleotide repeat microsatellite situated 2.1kb upstream of the aldose reductase transcription start sequence was investigated. Polymerase Chain Reaction was carried out to amplify the region upstream of the aldose reductase gene containing the (CA)n repeat microsatellite as described in chapter 3. Amplification products (6µl) mixed with stop solution (Amersham, UK) were separated on a 6% polyacrylamide gel at 1900V for 3 hours, and alleles were revealed by autoradiography [figure 24] and typed using the Fluor-s multi-imaging system (BioRad). The size of the bands was checked by running a [32P] ATP radiolabeled molecular weight marker alongside the subjects. The (CA)n microsatellite was investigated in T1DM subjects and normal controls of British Caucasoid origin, T2DM subjects and non-diabetic adult controls of Southern Indian/Dravidian origin and the 'Diabetes UK- Warren family trios' collection of British Caucasoid origin, where the proband had diabetic nephropathy. In these studies we were able to find 12 alleles which differed by the number of integral repeats. Subjects were assigned their allele and genotype according to the number of CA repeats which were identified (table 26), where Z is the most common allele and consists of 24 CA repeats. Allele and genotypes were assigned by eye and where stutter bands were present the PCR conditions were altered by increasing the annealing temperature and re-running the gel.
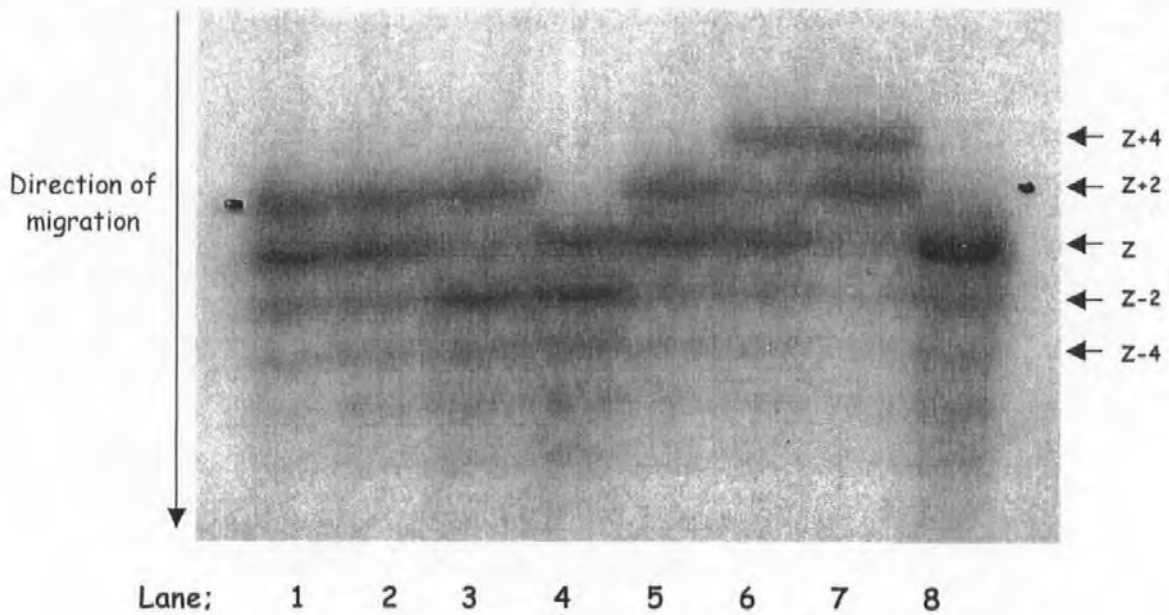
Figure 24. 5'ALR2 microsatellite autoradiograph. Bands were assigned by eye, and compared alongside alleles of known genotypes. Assignment was carried out by two independent observers. Where stutter peaks were observed, the PCR was altered and optimised in order to remove the shadow bands.

5'ALR2 microsatellite autoradiograph. Seven alleles are identified in this autoradiograph and allocated genotypes for each of the subjects shown in lanes 1-8 are as follows:
Lane 1: Z/Z+2
Lane 2: Z/Z+2
Lane 3: Z+2/Z-2
Lane 4: Z/Z-2
Lane 5: Z/Z+2
Lane 6: Z/Z+4
Lane 7: Z+2/Z+4
Lane 8: Z/Z

Allele	PCR product size (bp)	CA repeats
Z+10	148	29
Z+8	146	28
Z+6	144	27
Z+4	142	26
Z+2	140	25
Z	138	24
Z-2	136	23
Z-4	134	22
Z-6	132	21
Z-8	130	20
Z-10	128	19
Z-16	122	16

Table 26. Allele sizes in base pairs for the 12 different CA repeat polymorphisms identified. This table shows the size of the PCR amplified region corresponding to each allele, along with the number of dinucleotide (CA)n repeats.

5'ALR2- (CA)n microsatellite marker in British Caucasoid T1DM subjects and normal controls

The (CA)n microsatellite marker in the 5' region of the ALR2 gene was investigated using 244 subjects with T1DM of British Caucasoid origin as well as a collection of 120 British Caucasoid normal controls (clinical demographics shown in table 6). Within the T1DM subject population studied, there were 66 diabetic controls, 62 full house patients, 18 with diabetic neuropathy, 24 with diabetic retinopathy and neuropathy, 30 with diabetic nephropathy and retinopathy and 44 with diabetic retinopathy alone.

The frequency of the (CA)n alleles in both T1DM and normal control populations is shown in table 27. There were 9 (CA)n alleles detected at the 5'ALR2 locus in the British Caucasoid T1DM and control population; Z+8, Z+6, Z+4, Z+2, Z, Z-2, Z-4, Z-6 and Z-16 where Z is the most common allele and consists of 24 CA repeats. Nine alleles gave rise to 54 possible genotypes, of which 23 were identified here. In the analysis of allelic frequencies homozygotes were counted as 2 alleles, and alleles and genotypes that are not detected are not shown. Tables show the percentage frequency of detected alleles and genotypes with actual numbers detected shown in parenthesis. Possible associations were investigated with respect to allelic frequency and genotype and comparisons were made between groups using the χ^2 test and 2x2 contingency tables. Where the observed frequencies were <5 a two tailed Fishers exact test was applied.

This data set includes and extends results previously obtained and published by Angela Heesom (refer to acknowledgements), all of which have been re-analysed here as confirmation of the earlier findings. The tables include a further 76 T1DM subjects and a further 18 control subjects, that have been genotyped since earlier publications (Heesom *et al* 1997).

5'ALR2- (CA)n allelic frequencies in British Caucasoid T1DM and normal control subjects

The frequency of the 5'ALR2 alleles in T1DM Caucasoid subjects is shown in tables 27 to 33. Table 27 shows the percentage frequency of the detected 5'ALR2 (CA)n alleles in the total T1DM population studied compared to the normal control population studied. With the exception of the Z+6 allele there were no significant differences found in the allelic frequencies between the two groups. In the case of the Z+6 allele the incidence was significantly higher in the normal controls compared to the T1DM population (2.9 vs. 0.6%, $\chi^2 = 6.3$, p= 0.01 (1df), Fishers exact correction; 0.02), this however, involved small numbers.

The T1DM subjects with diabetes of more than 20 years duration of diabetes with insulin dependence, who had not progressed to complications, were then separated. This group of patients has a lower risk of developing complications after this time and therefore serve as an important diabetic control population. The allelic frequencies for these groups are shown in table 28. When comparing the T1DM subjects with one or more microvascular complications to the T1DM control subjects significant differences in the frequency of detected alleles were found. The Z-2 allele was higher in the complicated group compared to the uncomplicated controls (28.1 vs. 15.9%, $\chi^2 = 7.7$, p= 0.006 (1df), Pc = 0.05). The Z+2 allele was significantly higher in the T1DM control population compared to T1DM subjects with one or more microvascular complication (16.9 vs. 34.9%, $\chi^2 = 18.3$, p= 0.00002 (1df), Pc = 0.0002). Of interest, the Z-4 allele was also increased in the T1DM subjects with one or more microvascular complications compared to the uncomplicated, and the Z+4 allele was slightly increased in the uncomplicated versus the complicated group. Comparison of the T1DM control allelic frequencies with the normal control allelic frequencies showed no significant difference between the frequencies of the Z-2 allele (15.9 vs. 21.3%, although there was a slight reduction in percentage frequency in the

diabetic controls. A difference was also found in the frequency of the Z+2 allele (34.9 vs. 21.7%, χ^2 = 7.6, p= 0.006 (1df), Pc = 0.05).

Table 29 shows the allelic frequencies for T1DM subjects according to the precise phenotype of the T1DM subject's microvascular complications. The frequency of the Z allele remains constant between all groups. All five T1DM complication subgroups show an increase in the percentage frequency of the Z-2 allele compared to both the uncomplicated and normal controls. Conversely, the Z+2 allele percentage frequency is decreased in all five T1DM complication subgroups compared to the uncomplicated diabetic controls. The groups for which these trends reach statistical significance for Z+2 are the diabetic nephropathy and retinopathy subgroup (9.9 vs. 34.9%, χ^2 = 12.9, p= 0.0003 (1df), Pc = 0.003), and the full house patients subgroup (14.5 vs. 34.9%, χ^2 = 14.1, p= 0.0002 (1df), Pc = 0.002).

Table 30 shows the percentage frequency of the 5'ALR2 alleles in the amalgamated subgroups according to specific microvascular complications. The frequency of the Z allele remains consistent in all groups. All three of the complication subgroups show decreased frequency of the Z+2 allele compared to the uncomplicated. Firstly, the diabetic nephropathy subgroup (13.04 vs. 34.9%, χ^2 = 21.2, p= 0.0000004 (1df), Pc = 0.000004). Secondly, the diabetic retinopathy subgroup (16.9 vs. 34.9%, χ^2 = 17.5, p= 0.00003 (1df), Pc = 0.0003). Thirdly, the diabetic neuropathy subgroup (16.4 vs. 34.9%, χ^2 = 15.4, p= 0.00009 (1df), Pc = 0.0008). The Z-2 allele was increased in all three of the complicated sub-groups. In the case of diabetic nephropathy, (29.9 vs. 15.9%, χ^2 = 8.2, p= 0.004 (1df), Pc = 0.04). For diabetic retinopathy, (28.4 vs. 15.9%, χ^2 = 7.9, p= 0.005 (1df), Pc = 0.05). Finally, in the case of diabetic neuropathy, (29.3 vs. 15.9%, χ^2 = 7.9, p= 0.005 (1df), Pc = 0.05).

Alleles	T1DM subjects	Normal Controls (NC)
n	488 (244)	240 (120)
Z-16	0.2 (1)	- (0)
Z-6	1.0 (5)	1.3 (3)
Z-4	4.9 (24)	4.2 (10)
Z-2	24.8 (121)	21.3 (51)
Z	41.6 (203)	45.3 (110)
Z+2	21.7 (106)	21.7 (52)
Z+4	4.5 (22)	2.9 (7)
Z+6	0.6 (3)	2.9 (7)
Z+8	0.6 (3)	- (0)

Table 27. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite marker alleles in British Caucasoid T1DM subjects compared to normal controls

This table shows the frequency of detected alleles for T1DM subjects and normal control subjects. Frequencies are expressed as the percentage incidence out of the total number of alleles detected. Comparisons were made between the allelic frequencies for control and T1DM subjects using the χ^2 test and 2 x 2 contingency tables. For comparisons of less than 5 samples Fishers exact test was applied.

n = number of alleles detected, number of subjects studied is shown in parentheses.

T1DM vs. NC;

$$Z+6; \chi^2 = 6.3, p= 0.01 \text{ (1df)}, \text{Fishers Exact} = 0.02$$

No other significant differences were found in the allelic frequencies between T1DM patients and the normal control groups.

	T1DM Nephropathy and/or Retinopathy and/or Neuropathy (DR/N/Nu)	Uncomplicated (DC)	Normal Controls (NC)
n	356 (178)	132 (66)	240 (120)
Z-16	0.3 (1)	- (0)	- (0)
Z-6	1.4 (5)	- (0)	1.3 (3)
Z-4	5.9 (21)	2.3 (3)	4.2 (10)
Z-2	28.1 (100)	15.9 (21)	21.3 (51)
Z	42.4 (151)	39.4 (52)	45.8 (110)
Z+2	16.9 (60)	34.9 (46)	21.7 (52)
Z+4	4.2 (15)	5.3 (7)	2.9 (7)
Z+6	0.3 (1)	1.5 (2)	2.9 (7)
Z+8	0.6 (2)	0.8 (1)	- (0)

Table 28. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite marker alleles in British Caucasoid T1DM subjects with diabetic nephropathy and/or retinopathy and/or neuropathy (DR/N/Nu) compared to T1DM of short duration (SD), uncomplicated (DC) and normal controls (NC).

This table shows the percentage frequency of detected alleles for T1DM subjects with microvascular disease (nephropathy, retinopathy, neuropathy) compared to the uncomplicated and normal controls. The percentage frequency is shown and the actual number of each of the alleles detected for each group is shown in parenthesis. n = number of alleles detected, the number of subjects is shown in parenthesis. Only significant differences by χ^2 and correction by multiplying by the number of observed alleles, are shown.

DR/N/Nu vs. Uncomplicated diabetic controls;
　　　;Z-2 ; χ^2 = 7.7, p= 0.006 (1df), Pc = 0.05
　　　;Z+2 ; χ^2 = 18.3, p= 0.00002 (1df), Pc = 0.0002

NC vs. Uncomplicated diabetic controls;
　　　;Z+2 ; χ^2 = 7.6, p= 0.006 (1df), Pc = 0.05

	[1]Diabetic Retinopathy	[2]Diabetic Nephropathy and Retinopathy	[3]Diabetic Retinopathy and Neuropathy	[4]Diabetic Neuropathy	[5]Full House Patients	Uncomplicated (DC)	Normal Controls (NC)
n	88 (44)	60 (30)	48 (24)	36 (18)	124 (62)	132 (66)	240 (120)
Z-16	- (0)	- (0)	- (0)	- (0)	0.8 (1)	- (0)	- (0)
Z-6	2.3 (2)	1.7 (1)	- (0)	- (0)	1.6 (2)	- (0)	1.3 (3)
Z-4	3.4 (3)	6.7 (4)	4.2 (2)	5.6 (2)	8.1 (10)	2.3 (3)	4.2 (10)
Z-2	22.7 (20)	31.7 (19)	33.3 (16)	24.9 (9)	29.0 (36)	15.9 (21)	21.3 (51)
Z	38.6 (34)	46.7 (28)	35.4 (17)	47.2 (17)	44.4 (55)	39.4 (52)	45.8 (110)
Z+2	22.7 (20)	9.9 (6)	20.8 (10)	16.7 (6)	14.5 (18)	34.9 (46)	21.7 (52)
Z+4	9.1 (8)	1.7 (1)	6.3 (3)	2.8 (1)	1.6 (2)	5.3 (7)	2.9 (7)
Z+6	- (0)	1.7 (1)	- (0)	- (0)	- (0)	1.5 (2)	2.9 (7)
Z+8	1.1 (1)	- (0)	- (0)	2.8 (1)	- (0)	0.8 (1)	- (0)

Table 29. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite marker alleles in British Caucasoid T1DM subjects categorised in accordance to the onset of diabetic complications and normal control subjects.

This table shows the percentage frequency of detected alleles for T1DM subjects with retinopathy (DR), nephropathy and retinopathy (DN/DR), retinopathy and neuropathy (DR/DNu), neuropathy (DNu), full house complications patients (FHC) and uncomplicated diabetics controls (DC). Also shown here is the allelic frequency of normal healthy controls. n = number of alleles detected, the number of subjects is shown in parenthesis.

[1] vs. Uncomplicated diabetic controls; Z+2; $\chi^2 = 3.7$, p= ns

[2] vs. Uncomplicated diabetic controls; Z-2; $\chi^2 = 6.2$, p= 0.01 (1df), Pc = ns
Z+2; $\chi^2 = 12.9$, p= 0.0003 (1df), Pc = 0.003

[3] vs. Uncomplicated diabetic controls; Z-2; $\chi^2 = 6.5$, p= 0.01 (1df), Pc = ns

[4] vs. Uncomplicated diabetic controls; Z+2; $\chi^2 = 4.4$, p= 0.04 (1df), Pc = ns

[5] vs. Uncomplicated diabetic controls; Z-2; $\chi^2 = 6.4$, p= 0.01 (1df), Pc = ns
Z+2; $\chi^2 = 14.1$, p= 0.0002 (1df), Pc = 0.002

	[1]Diabetic Nephropathy (DN)	[2]Diabetic Retinopathy (DR)	[3]Diabetic Neuropathy (DNu)	Uncomplicated (DC)	Normal Controls (NC)
n	184 (92)	320 (160)	208 (104)	132 (66)	240 (120)
Z-4	7.6 (14)	5.9 (19)	6.7 (14)	2.3 (3)	4.2 (10)
Z-2	29.9 (55)	28.4 (91)	29.3 (61)	15.9 (21)	21.3 (51)
Z	45.1 (83)	41.9 (134)	42.8 (89)	39.4 (52)	45.8 (110)
Z+2	13.0 (24)	16.9 (54)	16.4 (34)	34.9 (46)	21.7 (52)
Z+4	1.6 (3)	4.4 (14)	2.9 (6)	5.3 (7)	2.9 (7)

Table 30. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite alleles in Caucasoid T1DM subjects categorised in accordance to onset of diabetic nephropathy, retinopathy or neuropathy.

This table presents the allelic frequency of the (CA)n microsatellite in T1DM subjects of British Caucasoid origin. Subjects are classified in accordance to onset of retinopathy, nephropathy or neuropathy. The nephropathy group (n=92) is an amalgamation of the nephropathy and retinopathy (n=30) and full house subjects (n=62) groups previously identified. The retinopathy group (n=160) is an amalgamation of the retinopathy (n=44), nephropathy with retinopathy (n=30), retinopathy and neuropathy (n=24) and full house patients (n=62) groups previously identified. The neuropathy group (n=104) consists of subjects diagnosed as having neuropathy in the absence of any other microvascular complication (n=18), the diabetic retinopathy with neuropathy (n=24) and full house patients (n=62) groups. Also presented is the allelic frequency in the uncomplicated and normal controls. Only the Z-2, Z+2, Z, Z-4 and Z+4 alleles are shown. n = number of alleles detected, the number of subjects is shown in parenthesis.

[1] **vs. Uncomplicated diabetic controls;**

$$Z-2; \chi^2 = 8.2, p= 0.004 \ (1df), Pc = 0.04$$
$$Z+2; \chi^2 = 21.2, p= 0.0000004 \ (1df), Pc = 0.000004$$

[2] **vs. Uncomplicated diabetic controls;**

$$Z-2; \chi^2 = 7.9, p= 0.005 \ (1df), Pc = 0.05$$
$$Z+2; \chi^2 = 17.5, p= 0.00003 \ (1df), Pc = 0.0003$$

[3] **vs. Uncomplicated diabetic controls;**

$$Z-2; \chi^2 = 7.9, p= 0.005 \ (1df), Pc = 0.05$$
$$Z+2; \chi^2 = 15.4, p= 0.00009 \ (1df), Pc = 0.0008$$

5'ALR2- (CA)n genotype frequencies in British Caucasoid T1DM and normal control subjects

The frequencies of the 5'ALR2 genotypes in Caucasoid T1DM subjects and normal controls are shown in tables 31 to 35. Table 31 shows the detected frequency of the 5'ALR2 genotypes in all T1DM subjects studies compared to the normal controls. No differences were detected between the two groups. Table 32 shows the frequency of the detected genotypes in the sub-groups of the T1DM population studied, diabetic complications, uncomplicated diabetic controls and normal controls subgroups. The low frequencies of many of the genotypes detected resulted in no significant differences being identified. However, the genotypes, which occurred more frequently in the population, were analysed for differences in percentage frequency. There was a significant decrease in the frequency of the Z/Z+2 genotype in those with complications (nephropathy, retinopathy, neuropathy) compared with the uncomplicated sub-group (15.7 vs. 37.9%, χ^2 = 13.9, p= 0.0002 (1df), Pc = 0.009). There was an increase in the frequency of the Z/Z-2 genotype in the diabetic complication group compared with the uncomplicated group (31.5 vs. 12.1%, χ^2 = 9.3, p= 0.002 (1df), Pc = 0.05). The Z-2/Z-2 genotype however did not show a significant increase between the two groups.

Table 33 shows the frequency of each of the genotypes detected in all of the patient subgroups according to the precise phenotype of the diabetic microvascular complications. Comparisons were made between each of the complication subgroups and the diabetic uncomplicated controls. With regard to the diabetic retinopathy subgroup there was an increase in the Z/Z-2 genotype compared to the diabetic controls, (29.6 vs. 12.1%, χ^2 = 5.2, p= 0.02 (1df), Pc = ns). The Z/Z+2 genotype was decreased in the diabetic retinopathy group compared to the uncomplicated group, (15.9 vs. 37.9%, χ^2 = 6.01, p= 0.01 (1df), Pc = ns). The Z/Z-2 genotype was increased in the nephropathy and retinopathy group compared to diabetic controls (36.7 vs. 12.1%, χ^2 = 4.0, p= 0.05 (1df), Pc = ns). The

Z/Z+2 genotype was decreased in the nephropathy and retinopathy group compared to the diabetic controls (9.9 vs. 37.9%, χ^2 = 7.8, p= 0.005 (1df), Pc = 0.1). The diabetic retinopathy with neuropathy, and the diabetic neuropathy only, subgroups followed a similar trend whereby the Z/Z-2 genotype was increased and the Z/Z+2 genotype was decreased in the complicated groups compared to the uncomplicated controls. The strongest evidence for a correlation came from the full house patient subgroup compared to the diabetic controls. The Z/Z-2 genotype was increased in the full house patient group compared to the diabetic control group (32.3 vs. 12.1%, χ^2 = 7.6, p= 0.006 (1df), Pc = ns). The Z/Z+2 genotype was decreased in the full house patient groups compared to uncomplicated controls (14.5 vs. 37.9%, χ^2 = 8.9, p= 0.003 (1df), Pc = 0.07). No other differences were found in the frequency of any of the other genotypes detected.

Table 34 shows the frequency of the detected genotypes in the T1DM patient groups amalgamated according to particular complication present. The diabetic nephropathy group showed significant correlation's whereby a reduction in the Z/Z+2 genotype and an increase in the Z/Z-2 genotype were both significant by the χ^2 test (Z/Z-2; 33.7% vs. 12.1%, χ^2 = 9.6, p= 0.002 (1df), Pc = 0.04, Z/Z+2; 13.04% vs. 37.9%, χ^2 = 13.2, p= 0.0003 (1df), Pc = 0.007. The diabetic retinopathy group also showed significance with the Z/Z+2 allele whereby there was a reduction compared to the uncomplicated group, (17.7 vs. 37.9%, χ^2 = 14.4, p= 0.0002 (1df), Pc = 0.005). The Z/Z-2 genotype was increased in the retinopathy group compared to the uncomplicated (31.3 vs. 12.1%, χ^2 = 8.9, p= 0.003 (1df), Pc = 0.07). In the case of the diabetic neuropathy group the Z/Z-2 genotype was increased in the neuropathy group compared to the uncomplicated (30.8 vs. 12.1%, χ^2 = 7.8, p= 0.005 (1df), Pc = ns). In the case of Z/Z+2, there was a significant increase in the uncomplicated compared to the neuropaths (17.3 vs. 37.9%, χ^2 = 9.04, p= 0.003 (1df), Pc = 0.07).

Of the 92 patients with nephropathy, 45.7% had the Z-2/X genotype (where X is not Z+2), compared with only 24.2% in the uncomplicated diabetes group (χ^2 = 7.6, p= 0.006 (1df), Pc = 0.02). In contrast, the Z+2/Y genotype (where Y is not Z-2) was found in only 17.4% of the patients with nephropathy compared to 51.5% in the uncomplicated (χ^2 = 20.7, p= 0.000005 (1df), Pc = 0.00002) (table 35). Similarly, in the case of the 160 patients with retinopathy, 41.25% had the Z-2/X genotype compared with only 24.2% in the uncomplicated (χ^2 = 5.9, p= 0.02 (1df), Pc = ns). In contrast, the Z+2/Y genotype was found in only 23.1% of patients with retinopathy, compared to 51.5% in the uncomplicated (χ^2 = 17.5, p= 0.00003 (1df), Pc = 0.0001). Also, in the case of the 104 patients with neuropathy, 42.3% had the Z-2/X genotype compared with only 24.2% in the uncomplicated (χ^2 = 5.8, p= 0.02 (1df), Pc = 0.08). In contrast, the Z+2/Y genotype was found in only 23.08% of patients with neuropathy, compared to 51.5% in the uncomplicated (χ^2 = 14.5, p= 0.001 (1df), Pc = 0.0004).

Genotype	T1DM subjects	Normal Controls (NC)
n	244	120
Z/Z	12.3 (30)	20.8 (25)
Z/Z-2	26.3 (64)	21.7 (26)
Z/Z-4	4.9 (12)	2.5 (3)
Z/Z-6	1.2 (3)	0.8 (1)
Z/Z-16	0.4 (1)	- (0)
Z/Z+2	21.7 (53)	20.8 (25)
Z/Z+4	3.3 (8)	2.5 (3)
Z/Z+6	0.4 (1)	1.7 (2)
Z/Z+8	0.4 (1)	- (0)
Z-2/Z-2	6.9 (17)	6.7 (8)
Z-2/Z-4	2.5 (6)	3.3 (4)
Z-2/Z+2	6.1 (15)	4.2 (5)
Z-2/Z+4	1.2 (3)	- (0)
Z-4/Z-4	0.4 (1)	- (0)
Z-4/Z-6	0.8 (2)	1.7 (2)
Z-4/Z+2	0.4 (1)	0.8 (1)
Z-4/Z+8	0.4 (1)	- (0)
Z+2/Z+2	4.9 (12)	8.3 (10)
Z+2/Z+4	4.1 (10)	- (0)
Z+2/Z+6	- (0)	0.8 (1)
Z+2/Z+8	0.4 (1)	- (0)
Z+4/Z+4	0.4 (1)	1.7 (2)
Z+6/Z+6	0.4 (1)	1.7 (2)

Table 31. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite marker genotypes in British Caucasoid T1DM subjects compared to normal controls
This table shows the frequency of detected genotypes for T1DM subjects and normal control subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. n = number of genotypes detected. Comparisons were made between the allelic frequencies for normal controls and T1DM subjects using the Chi-squared test and 2 x 2 contingency tables. No significant differences were found in the genotype frequencies between T1DM patients and the normal control groups.

T1DM vs. NC;   Z/Z; χ^2 = 5.9, p= 0.02 (1df), Pc = ns

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Genotypes	T1DM Nephropathy and/or Retinopathy and/or Neuropathy (DR/N/Nu)	Uncomplicated (DC)	Normal Controls (NC)
n	178	66	120
Z/Z	12.4	12.1	20.8
	(22)	(8)	(25)
Z/Z-2	31.5	12.1	21.7
	(56)	(8)	(26)
Z/Z-4	6.2	1.5	2.5
	(11)	(1)	(3)
Z/Z-6	1.7	-	0.8
	(3)	(0)	(1)
Z/Z-16	0.6	-	-
	(1)	(0)	(0)
Z/Z+2	15.7	37.9	20.8
	(28)	(25)	(25)
Z/Z+4	3.9	1.5	2.5
	(7)	(1)	(3)
Z/Z+6	0.6	-	1.7
	(1)	(0)	(2)
Z/Z+8	-	1.5	-
	(0)	(1)	(0)
Z-2/Z-2	7.9	4.6	6.7
	(14)	(3)	(8)
Z-2/Z-4	2.3	3.03	3.3
	(4)	(2)	(4)
Z-2/Z+2	6.7	4.6	4.2
	(12)	(3)	(5)
Z-2/Z+4	-	4.6	-
	(0)	(3)	(0)
Z-4/Z-4	0.6	-	-
	(1)	(0)	(0)
Z-4/Z-6	1.1	-	1.7
	(2)	(0)	(2)
Z-4/Z+2	0.6	-	0.8
	(1)	(0)	(1)
Z-4/Z+8	0.6	-	-
	(1)	(0)	(0)
Z+2/Z+2	2.8	10.6	8.3
	(5)	(7)	(10)
Z+2/Z+4	4.5	3.03	-
	(8)	(2)	(0)
Z+2/Z+6	-	-	0.8
	(0)	(0)	(1)
Z+2/Z+8	0.6	-	-
	(1)	(0)	(0)
Z+4/Z+4	-	1.5	1.7
	(0)	(1)	(2)
Z+6/Z+6	-	1.5	1.7
	(0)	(1)	(2)

Table 32. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite marker genotypes in British Caucasoid T1DM subjects with nephropathy and/or retinopathy and/or neuropathy (DR/N/Nu) compared to T1DM uncomplicated (DC) and normal controls (NC). This table shows the percentage frequency of detected genotypes for T1DM subjects with microvascular disease (nephropathy, retinopathy, neuropathy) compared to the uncomplicated and normal controls. The percentage frequency is shown and the actual number of genotypes detected for each group is shown in parenthesis. n = number of genotypes detected.

DN/R/Nu vs. uncomplicated diabetic controls;

Z/Z-2; χ^2 = 9.3, p= 0.002 (1df), Pc = 0.05

Z/Z+2; χ^2 = 13.9, p= 0.0002 (1df), Pc = 0.005

Genotypes	[1] Diabetic Retinopathy	[2] Diabetic Nephropathy and Retinopathy	[3] Diabetic Retinopathy and Neuropathy	[4] Diabetic Neuropathy	[5] Full House Patients	Uncomplicated (DC)	Normal Controls (NC)
n	44	30	24	18	62	66	120
Z/Z	11.4	13.3	4.2	16.7	14.5	12.1	20.8
	(5)	(4)	(1)	(3)	(9)	(8)	(25)
Z/Z-2	29.6	36.7	24.9	33.3	32.3	12.1	21.7
	(13)	(11)	(6)	(6)	(20)	(8)	(26)
Z/Z-4	2.3	9.9	8.3	5.6	6.5	1.5	2.5
	(1)	(3)	(2)	(1)	(4)	(1)	(3)
Z/Z-6	2.3	3.3	0	-	1.6	-	0.8
	(1)	(1)	(0)	(0)	(1)	(0)	(1)
Z/Z-16	-	-	-	-	1.6	-	-
	(0)	(0)	(0)	(0)	(1)	(0)	(0)
Z/Z+2	15.9	9.9	20.8	22.2	14.5	37.9	20.8
	(7)	(3)	(5)	(4)	(9)	(25)	(25)
Z/Z+4	4.6	3.3	8.3	-	3.2	1.5	2.5
	(2)	(1)	(2)	(0)	(2)	(1)	(3)
Z/Z+6	-	3.3	-	-	-	-	1.7
	(0)	(1)	(0)	(0)	(0)	(0)	(2)
Z/Z+8	-	-	-	-	-	1.5	-
	(0)	(0)	(0)	(0)	(0)	(1)	(0)
Z-2/Z-2	4.6	9.9	12.5	5.6	8.1	4.6	6.7
	(2)	(3)	(3)	(1)	(5)	(3)	(8)
Z-2/Z-4	-	3.3	-	5.6	3.2	3.0	3.3
	(0)	(1)	(0)	(1)	(2)	(2)	(4)
Z-2/Z+2	6.8	3.3	16.7	-	6.5	4.6	4.2
	(3)	(1)	(4)	(0)	(4)	(3)	(5)
Z-2/Z+4	-	-	-	-	-	4.6	-
	(0)	(0)	(0)	(0)	(0)	(3)	(0)
Z-4/Z-4	-	-	-	-	1.6	-	-
	(0)	(0)	(0)	(0)	(1)	(0)	(0)
Z-4/Z-6	2.3	-	-	-	1.6	-	1.7
	(1)	(0)	(0)	(0)	(1)	(0)	(2)
Z-4/Z+2	-	-	-	-	1.6	-	0.8
	(0)	(0)	(0)	(0)	(1)	(0)	(1)
Z-4/Z+8	2.3	-	-	-	-	-	-
	(1)	(0)	(0)	(0)	(0)	(0)	(0)
Z+2/Z+2	4.6	3.3	-	-	3.2	10.6	8.3
	(2)	(1)	(0)	(0)	(2)	(7)	(10)
Z+2/Z+4	13.6	-	4.2	5.6	-	3.03	-
	(6)	(0)	(1)	(1)	(0)	(2)	(0)
Z+2/Z+6	-	-	-	-	-	-	0.8
	(0)	(0)	(0)	(0)	(0)	(0)	(1)
Z+2/Z+8	-	-	-	5.6	-	-	-
	(0)	(0)	(0)	(1)	(0)	(0)	(0)
Z+4/Z+4	-	-	-	-	-	1.5	1.7
	(0)	(0)	(0)	(0)	(0)	(1)	(2)
Z+6/Z+6	-	-	-	-	-	1.5	1.7
	(0)	(0)	(0)	(0)	(0)	(1)	(2)

Table 33. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite marker genotypes in British Caucasoid T1DM subjects categorised in accordance to the onset of diabetic complications and uncomplicated (DC) and normal controls (NC)

This table shows the percentage frequency of detected genotypes for T1DM subjects with respect to onset of microvascular complications, and normal control subjects. The percentage frequency is shown and the actual number of genotypes detected for each group is shown in parenthesis. n = number of subjects studied and genotypes detected.

[1] **vs. uncomplicated diabetic controls;**

Z/Z-2; $\chi^2 = 5.2$, p= 0.02 (1df), Pc = ns

Z/Z+2; $\chi^2 = 6.01$, p= 0.01 (1df), Pc = ns

[2] **vs. uncomplicated diabetic controls;**

Z/Z-2; $\chi^2 = 4.0$, p= 0.05 (1df), Pc = ns

Z/Z+2; $\chi^2 = 7.8$, p= 0.005 (1df), Pc = ns

[4] **vs. Uncomplicated diabetic controls;**

Z/Z-2; $\chi^2 = 4.6$, p= 0.03(1df), Pc = ns

[5] **vs. Uncomplicated diabetic controls;**

Z/Z-2; $\chi^2 = 7.6$, p= 0.006 (1df), Pc = ns

Z/Z+2; $\chi^2 = 8.9$, p= 0.003 (1df), Pc = ns

Genotypes	[1] Diabetic Nephropathy	[2] Diabetic Retinopathy	[3] Diabetic Neuropathy	Uncomplicated (DC)	Normal controls (NC)
n	92	160	104	66	120
Z/Z	16.3	11.9	12.5	12.1	20.8
	(15)	(19)	(13)	(8)	(25)
Z/Z-2	33.7	31.3	30.8	12.1	21.7
	(31)	(50)	(32)	(8)	(26)
Z/Z-4	7.6	6.3	6.7	1.5	2.5
	(7)	(10)	(7)	(1)	(3)
Z/Z+2	13.0	15.0	17.3	37.9	20.8
	(12)	(24)	(18)	(25)	(25)
Z/Z+4	3.3	4.4	3.9	1.5	2.5
	(3)	(7)	(4)	(1)	(3)
Z-2/Z-2	8.7	8.1	8.7	4.6	6.7
	(8)	(13)	(9)	(3)	(8)
Z-2/Z-4	3.3	1.9	2.9	3.0	3.3
	(3)	(3)	(3)	(2)	(4)
Z-2/Z+2	5.4	7.5	7.7	4.6	4.2
	(5)	(12)	(8)	(3)	(5)
Z-2/Z+4	-	-	-	4.6	-
	(0)	(0)	(0)	(3)	(0)
Z-4/Z-4	1.1	0.6	0.9	-	-
	(1)	(1)	(1)	(0)	(0)
Z-4/Z+2	1.1	0.6	0.9	-	0.8
	(1)	(1)	(1)	(0)	(1)
Z+2/Z+2	3.3	3.1	1.9	10.7	8.3
	(3)	(5)	(2)	(7)	(10)
Z+2/Z+4	-	4.4	1.9	3.0	-
	(0)	(7)	(2)	(2)	(0)
Z+4/Z+4	-	-	-	1.5	1.7
	(0)	(0)	(0)	(1)	(2)

Table 34. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite marker genotypes in British Caucasoid T1DM subjects categorised in accordance to onset of diabetic nephropathy, retinopathy or neuropathy

This table presents the genotype frequency of the (CA)n microsatellite in T1DM subjects of British Caucasoid origin. Subjects are classified in accordance to onset of retinopathy, nephropathy or neuropathy. The nephropathy group (n=92) is an amalgamation of the nephropathy and retinopathy (n=30) and full house subjects (n=62) groups previously identified. The retinopathy group (n=160) is an amalgamation of the retinopathy (n=44), nephropathy with retinopathy (n=30), retinopathy and neuropathy (n=24) and full house patients (n=62) groups previously identified. The neuropathy group (n=104) consists of subjects diagnosed as having neuropathy in the absence of any other microvascular complication (n=18), the diabetic retinopathy with neuropathy (n=24) and full house patients (n=62) groups. Also presented is the genotype frequency in uncomplicated and normal controls. Only the Z-2, Z+2, Z, Z-4 and Z+4 genotypes are shown. n = number of subjects analysed and genotypes detected. The percentage frequency is shown and the actual number of each genotype detected for each subjects group is shown in parenthesis.

[1] vs. uncomplicated diabetic controls;

Z/Z-2; χ^2 = 9.6, p= 0.002 (1df), Pc = 0.0

Z/Z+2; χ^2 = 13.2, p= 0.0003 (1df), Pc = 0.007

[2] vs. uncomplicated diabetic controls;

Z/Z-2; $\chi^2 = 8.9$, p= 0.003 (1df), Pc = 0.07

Z/Z+2; $\chi^2 = 14.4$, p= 0.0002 (1df), Pc = 0.005

Z+2/Z+2; $\chi^2 = 5.2$, p= 0.02 (1df), Pc = ns

[3] vs. uncomplicated diabetic controls;

Z/Z-2; $\chi^2 = 7.8$, p= 0.005 (1df), Pc = ns

Z/Z+2; $\chi^2 = 9.04$, p= 0.003 (1df), Pc = 0.07

Z+2/Z+2; $\chi^2 = 6.07$, p= 0.01 (1df), Pc = ns

	1 Diabetic Nephropathy	[2] Diabetic Retinopathy	[3] Diabetic Neuropathy	Uncomplicated (DC)	Normal Controls (NC)
n	92	160	104	66	120
Z-2/X	45.7 (42)	41.3 (66)	42.3 (44)	24.2 (16)	31.7 (38)
Z+2/Y	17.4 (16)	23.1 (37)	23.08 (24)	51.5 (34)	30.8 (37)
Z-2/Z+2	5.4 (5)	7.5 (12)	7.7 (8)	4.5 (3)	4.2 (5)
X/Y	31.5 (29)	28.1 (45)	26.9 (28)	19.7 (13)	34.2 (41)

Table 35. Frequency of the Z-2 and Z+2 5'ALR2 genotypes in patients with or without diabetic microvascular disease. Figures in parenthesis indicate the number of subjects. X allele is any allele other than Z+2 and Y is any allele other than Z-2. P-values have been corrected by multiplying by the number of comparisons made.

[1] **frequency vs. Uncomplicated diabetic controls;**
    Z-2/X; $\chi^2 = 7.6$, p= 0.006 (1df), Pc = 0.02
    Z+2/Y; $\chi^2 = 20.7$, p= 0.000005 (1df), Pc = 0.00002

[2] **frequency vs. Uncomplicated diabetic controls;**
    Z-2/X; $\chi^2 = 5.9$, p= 0.02 (1df), Pc = ns
    Z+2/Y; $\chi^2 = 17.5$, p= 0.00003 (1df), Pc = 0.0001

[3] **frequency vs. Uncomplicated diabetic controls;**
    Z-2/X; $\chi^2 = 5.8$, p= 0.02 (1df), Pc = 0.03
    Z+2/Y; $\chi^2 = 14.5$, p= 0.0001 (1df), Pc = 0.0004

5'ALR2- (CA)n microsatellite marker in Southern Indian T2DM subjects and non-diabetic controls

The (CA)n microsatellite marker in the 5' region of the ALR2 gene was investigated using 60 subjects with T2DM of Southern Indian/Dravidian origin as well as a collection of 43 Southern Indian/Dravidian non-diabetic controls (clinical demographics shown in table 7). Within the T2DM subject population studied, there were 28 subjects that were normoalbuminuric without retinopathy and 32 subjects with proteinuria. The frequency of the (CA)n alleles in both patient and control populations is shown in table 36. There were 10 (CA)n alleles detected at the 5'ALR2 locus in the Southern Indian T2DM and non-diabetic control population; Z+10, Z+8, Z+6, Z+4, Z+2, Z, Z-2, Z-4, Z-6 and Z-10 where Z is the most common allele and consists of 24 CA repeats. Ten alleles gave rise to 65 possible genotypes, of which 27 were identified here. In the analysis of allelic frequencies homozygotes were counted as 2 alleles, and alleles and genotypes that were not detected are not shown. Tables show the percentage frequency of detected alleles and genotypes, with actual numbers detected shown in parenthesis. Possible associations were investigated with respect to allelic frequency and genotype and comparisons were made between groups using the χ^2 test and 2x2 contingency tables. Where comparisons of <5 samples were made the Fishers exact test was applied. All of the data obtained from the 5'ALR2 study of the Southern Indian population is presented in the following tables. Possible associations with the 5'ALR2 (CA)n allelic frequencies and genotypes and the onset of proteinuria were investigated.

5'ALR2 (CA)n allele frequencies in Southern Indian T2DM and non-diabetic subjects

Table 36 presents the frequency of the detected alleles in the T2DM Southern Indian subjects investigated. All T2DM subjects are compared with the non-diabetic control subjects. No significant differences were found in the frequency of the alleles between the two groups suggesting that there is no involvement of these alleles with the onset of T2DM. The T2DM group was then separated into the two groups representative of their clinical diabetic complication phenotype, T2DM with proteinuria and T2DM with normoalbuminuria, which is shown in table 37. There were no significant difference in the frequencies of the respective alleles identified between these two groups, although upon observation there was a small increase in the Z-2 allele in subjects with proteinuria compared to those without (15.6% vs. 10.7%). Conversely, there was also a decrease in the frequency of the Z+2 allele in the subjects with proteinuria compared to the subjects without (12.5 vs. 23.2%). The lack of any significant correlation may be due to the small number of patients studied. It is important to note that the normoalbuminuria patients had a diabetes duration of between 2 and 29 years. It is possible that some of these patients may progress to developing proteinuria later on in the course of their disease. This may be masking possible associations.

Alleles	T2DM subjects	Non-diabetic Controls (NDC)
n	120 (60)	86 (43)
Z-10	- (0)	1.2 (1)
Z-6	1.7 (2)	- (0)
Z-4	3.3 (4)	2.3 (2)
Z-2	13.3 (16)	15.1 (13)
Z	27.5 (33)	44.2 (38)
Z+2	17.5 (21)	19.8 (17)
Z+4	15.0 (18)	10.5 (9)
Z+6	10.0 (12)	5.8 (5)
Z+8	10.0 (12)	1.2 (1)
Z+10	1.7 (2)	- (0)

Table 36. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite marker alleles in Southern Indian/Dravidian T2DM subjects compared to non-diabetic controls.

This table shows the frequency of detected alleles for T2DM subjects and non-diabetic control subjects. Frequencies are expressed as the percentage incidence out of the total number of alleles detected. Actual numbers of alleles detected is shown in parenthesis.
n = number of alleles detected, number of subjects studied is shown in parentheses. Fishers exact test was also applied where values <5 were used.

No significant differences were found between these groups.

	[1]T2DM with Proteinuria	T2DM with Normoalbuminuria	Non-diabetic Controls (NDC)
n	64 (32)	56 (28)	86 (43)
Z-10	- (0)	- (0)	1.2 (1)
Z-6	- (0)	3.6 (2)	- (0)
Z-4	3.1 (2)	3.6 (2)	2.3 (2)
Z-2	15.6 (10)	10.7 (6)	15.1 (13)
Z	29.7 (19)	24.9 (14)	44.2 (38)
Z+2	12.5 (8)	23.2 (13)	19.8 (17)
Z+4	17.2 (11)	12.5 (7)	10.5 (9)
Z+6	9.4 (6)	10.7 (6)	5.8 (5)
Z+8	10.9 (7)	8.9 (5)	1.2 (1)
Z+10	1.6 (1)	1.8 (1)	- (0)

Table 37. Percentage frequency of the detected 5'ALR2 microsatellite marker alleles in Southern Indian/Dravidian T2DM subjects with proteinuria compared to T2DM subjects with normoalbuminuria and non-diabetic control subjects.

This table shows the percentage frequency of the detected alleles for T2DM subjects with normoalbuminuria and T2DM subjects with proteinuria as well as non-diabetic controls. The actual number of alleles within each group is shown in parentheses. n = number of alleles detected, number of subjects studied is shown in parentheses.

[1] **frequency compared with T2DM normoalbuminuria;**
No significant differences in the frequencies of the alleles were found.

5'ALR2 (CA)n genotype frequencies in Southern Indian T2DM and non-diabetic control subjects

Table 38 presents the frequency of the genotypes in the T2DM Southern Indian subjects against the non-diabetic controls, all genotypes detected are presented in the tables. Differences in frequencies of the detected genotypes were found for the Z/Z genotype. There was a higher incidence of the Z/Z genotype in the non-diabetic control population compared to the T2DM subjects (3.3 vs. 18.6%, χ^2 = 6.7, p= 0.01 (1df), Fishers exact = 0.01). Table 39 presents the genotypes detected for the T2DM subjects according to the onset of proteinuria or normoalbuminuria. There were no significant difference found in the frequencies of the detected genotypes in these groups. Through general observation, however, there was an increase in the Z/Z-2 genotype in the T2DM with proteinuria group compared to the normoalbuminuria group (15.6% vs. 7.1%). There was also a decrease in the frequency of the Z/Z+2 genotype in the T2DM proteinuria group compared to the T2DM normalbuminuria group (12.5 vs. 25.00%). Genotypes detected were then scored according to the presence of a Z+2 or Z-2 allele as shown in table 40. There were no other significant differences found.

Genotypes	T2DM subjects	Non-diabetic Controls (NDC)
n	60	43
Z/Z	3.3 (2)	18.6 (8)
Z/Z-2	11.7 (7)	18.6 (8)
Z/Z-4	- (0)	2.3 (1)
Z/Z-10	- (0)	2.3 (1)
Z/Z+2	18.3 (11)	16.3 (7)
Z/Z+4	8.3 (5)	9.3 (4)
Z/Z+6	1.7 (1)	2.3 (1)
Z/Z+8	6.7 (4)	- (0)
Z/Z+10	1.7 (1)	- (0)
Z-2/Z-2	- (0)	2.3 (1)
Z-2/Z-4	5.0 (3)	2.3 (1)
Z-2/Z-6	1.7 (1)	- (0)
Z-2/Z+2	1.7 (1)	2.3 (1)
Z-2/Z+4	3.3 (2)	2.3 (1)
Z-2/Z+6	1.7 (1)	- (0)
Z-2/Z+8	1.7 (1)	- (0)
Z-4/Z+2	1.7 (1)	- (0)
Z+2/Z-6	1.7 (1)	- (0)
Z+2/Z+2	- (0)	7.00 (3)
Z+2/Z+4	3.3 (2)	- (0)
Z+2/Z+6	1.7 (1)	7.00 (3)
Z+2/Z+8	6.7 (4)	- (0)
Z+4/Z+4	- (0)	2.3 (1)
Z+4/Z+6	11.7 (7)	2.3 (1)
Z+4/Z+8	3.3 (2)	2.3 (1)
Z+6/Z+8	1.7 (1)	- (0)
Z+6/Z+10	1.7 (1)	- (0)

Table 38. Percentage frequency of the detected 5'ALR2 (CA)n microsatellite genotypes in T2DM subjects compared to non-diabetic control subjects of Southern Indian/Dravidian origin. This table shows the percentage frequency of detected genotypes for T2DM subjects with respect to onset of diabetes, and non-diabetic control

subjects. n = number of genotypes detected. Only significant differences by χ^2 and correction are shown. Fishers exact test was also applied where values of <5 were used.

[1] **vs. non-diabetic controls;**

Z/Z; χ^2 = 6.7, p= 0.01 (1df), Fishers Exact = 0.01

Genotype	T2DM Proteinuria	T2DM Normoalbuminuria	Non-diabetic Controls (NDC)
n	**32**	**28**	**43**
Z/Z	3.1	3.6	18.6
	(1)	(1)	(8)
Z/Z-2	15.6	7.1	18.6
	(5)	(2)	(8)
Z/Z-4	-	-	2.3
	(0)	(0)	(1)
Z/Z-10	-	-	2.3
	(0)	(0)	(1)
Z/Z+2	12.5	25.0	16.3
	(4)	(7)	(7)
Z/Z+4	12.5	3.6	9.3
	(4)	(1)	(4)
Z/Z+6	3.1	-	2.3
	(1)	(0)	(1)
Z/Z+8	6.3	7.1	-
	(2)	(2)	(0)
Z/Z+10	3.1	-	-
	(1)	(0)	(0)
Z-2/Z-2	-	-	2.3
	(0)	(0)	(1)
Z-2/Z-4	6.3	3.6	2.3
	(2)	(1)	(1)
Z-2/Z-6	-	3.6	-
	(0)	(1)	(0)
Z-2/Z+2	-	3.6	2.3
	(0)	(1)	(1)
Z-2/Z+4	6.3	-	2.3
	(2)	(0)	(1)
Z-2/Z+6	-	3.6	-
	(0)	(1)	(0)
Z-2/Z+8	3.1	-	-
	(1)	(0)	(0)
Z-4/Z+2	-	3.6	-
	(0)	(1)	(0)
Z+2/Z-6	-	3.6	-
	(0)	(1)	(0)
Z+2/Z+2	-	-	6.98
	(0)	(0)	(3)
Z+2/Z+4	3.1	3.6	-
	(1)	(1)	(0)
Z+2/Z+6	3.1	-	6.98
	(1)	(0)	(3)
Z+2/Z+8	6.3	7.1	-
	(2)	(2)	(0)
Z+4/Z+4	-	-	2.3
	(0)	(0)	(1)
Z+4/Z+6	9.4	14.3	2.3
	(3)	(4)	(1)
Z+4/Z+8	3.1	3.6	2.3
	(1)	(1)	(1)
Z+6/Z+8	3.1	-	-
	(1)	(0)	(0)
Z+6/Z+10	-	3.6	-
	(0)	(1)	(0)

Table 39. Percentage frequency of the detected (CA)n 5'ALR2 microsatellite marker genotypes in Southern Indian T2DM subjects classified and compared in accordance to presence of proteinuria or normoalbuminuria against non-diabetic controls. This table shows the percentage frequency of detected genotypes for T2DM subjects with respect to onset of proteinuria, and non-diabetic control subjects. n = number of genotypes detected. No significant differences by χ^2 and correction were found.

	T2DM with Proteinuria	T2DM with Normoalbuminuria	Non-diabetic Controls
n	32	28	43
Z-2/X	34.4 (11)	17.9 (5)	25.6 (11)
Z+2/Y	25.0 (8)	42.9 (12)	30.2 (13)
Z-2/Z+2	- (0)	3.6 (1)	2.3 (1)
X/Y	43.8 (14)	35.7 (10)	41.9 (18)

Table 40. Frequency of Z-2 and Z+2 5'ALR2 genotypes in T2DM patients with and without proteinuria and non-diabetic controls of Southern Indian origin. Figures in parenthesis indicate the number of subjects. X allele is any allele other than Z+2 and Y is any allele other than Z-2. No significant differences by χ^2 and correction were found.

Association of 5'ALR2 and diabetic nephropathy in family based trio studies

The 'Diabetes UK- Warren nephropathy family trio' collection was used in this study of the 5'ALR2 microsatellite polymorphism. The aim of which was to allow the determination of the transmission of alleles from parents to proband to be made, and to identify any alleles showing preferential transmission. There were 9 alleles identified at the 5'ALR2 locus in the family study, which gave rise to 18 genotypes. A total of 172 family trios were analysed for the 5'ALR2 microsatellite polymorphism. Possible associations with the 5'ALR2 genotypes and alleles were investigated. Out of the 172 families genotyped for the 5'ALR2 microsatellite 10 showed non-paternity (5.8%), 2 trios were not fully genotyped due to degradation of DNA, and a further 45 trios were non-informative. This was due to several reasons, firstly if both of the parents within a family trio were heterozygous for the allele marker, transmission could not be determined, and secondly where parental genotype could not be accurately determined from incomplete trios where sibling genotypes were used. This resulted in a considerable reduction in the number of families that could be used to determine any significant associations. The informative 115 family trios were analysed using the transmission disequilibrium test (TDT). The 160 successfully genotyped nephropathy probands were also analysed (where 12 subjects were excluded due to non-paternity family trios and poor genotyping). In addition there were 11 fully informative trios analysed, where there was also a sibling to the proband included and these were also successfully genotyped.

5'ALR2 microsatellite marker in British Caucasoid families where proband has T1DM and diabetic nephropathy

A total of 160 T1DM subjects with diabetic nephropathy were genotyped for the 5'ALR2 polymorphism as part of the Diabetes UK –Warren Nephropathy studies. The frequencies of alleles and genotypes are presented in tables 41 and 42 respectively. The percentage frequency of the Z-2 allele was 25.9% (n=83) and the percentage frequency of the Z+2 allele was 21.3% (n=68). The percentage frequency of the Z/Z-2 genotype was 31.3% (n=50), and the percentage frequency of the Z/Z+2 genotype was 34.4 (n=55).

In table 43 the frequency of transmission of the 5'ALR2 alleles from parents who were heterozygous for the allele to affected offspring is shown. Under the hypothesis that there is no linkage the expected number of transmitted versus non-transmitted alleles should be equal. The difference therefore between the expected number of transmissions and observed transmission of alleles was calculated. The 115 informative family trios investigated showed no significant association for the transmission of the Z+2/Z/Z-2 alleles from parents to proband with T1DM and nephropathy. Table 43 presents the frequency of transmission of the 5'ALR2 Z+2/Z/Z-2 alleles from parents to the proband with T1DM and nephropathy. The Z allele was transmitted 51.5% (n=88) vs. not transmitted 48.5% (n= 83), (χ^2 = 0.07, p= 0.8 (1df), Pc = ns). The Z+2 allele was transmitted 45.4% (n=39) vs. not transmitted 54.7% (n=47), (χ^2 = 0.4, p= 0.5 (1df), Pc = ns). The Z-2 allele was transmitted 56.8% (n=71) vs. not transmitted 43.2% (n=54), (χ^2 = 1.2, p= 0.3 (1df), Pc = ns). Therefore, there were no significant differences between the expected and observed number of transmissions of alleles from parents to proband. Although, upon observation there was a slight preference for the transmission of the Z-2 allele and a slightly reduced preference for the transmission of the Z+2 allele from the expected 50:50 ratio.

	T1DM Nephropathy
n	320 (160)
Z-8	0.3 (1)
Z-6	0.3 (1)
Z-4	3.8 (12)
Z-2	25.9 (83)
Z	41.3 (132)
Z+2	21.3 (68)
Z+4	5.6 (18)
Z+6	1.3 (4)
Z+10	0.3 (1)

Table 41. Percentage frequency of the detected (CA)n 5'ALR2 microsatellite marker alleles in British Caucasoid T1DM subjects with diabetic nephropathy, taken from the 'DUK-Warren nephropathy collection'. This table shows the percentage frequency of detected alleles for T1DM subjects with nephropathy, actual number of alleles detected in the population is shown in brackets. n = number of alleles detected, number of subjects is shown in parenthesis.

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	T1DM Nephropathy
n	160 (320)
Z/Z	4.4 (7)
Z/Z+2	34.4 (55)
Z/Z+4	5.0 (8)
Z/Z+6	1.3 (2)
Z/Z-2	31.3 (50)
Z/Z-4	1.3 (2)
Z/Z-8	0.6 (1)
Z+2/Z+10	0.6 (1)
Z+2/Z+2	0.6 (1)
Z+2/Z+4	2.5 (4)
Z+2/Z+6	0.6 (1)
Z+2/Z-2	3.1 (5)
Z+4/Z+6	0.6 (1)
Z-2/Z+4	3.1 (5)
Z-2/Z-2	4.4 (7)
Z-2/Z-4	5.0 (8)
Z-2/Z-6	0.6 (1)
Z-4/Z-4	0.6 (1)

Table 42. Percentage frequency of the detected (CA)n 5'ALR2 microsatellite marker genotypes in British Caucasoid T1DM subjects with diabetic nephropathy, taken from the 'DUK-Warren nephropathy collection'. This table shows the percentage frequency of detected genotypes for T1DM subjects with nephropathy, actual number of genotypes detected in population is shown in brackets. n = number of genotypes detected, number of alleles is shown in parenthesis.

		Transmitted	Non-transmitted	Total	χ^2	p-value	*Pc*
Z	**Observed**	51.5 (88)	48.5 (83)	(171)	0.07	ns	ns
	Expected	50 (85.5)	50.0 (85.5)	(171)			
Z+2	**Observed**	45.4 (39)	54.7 (47)	(86)	0.4	ns	ns
	Expected	50.0 (43)	50.0 (43)	(86)			
Z-2	**Observed**	56.8 (71)	43.2 (54)	(125)	1.2	ns	ns
	Expected	50.0 (62.5)	50.0 (62.5)	(125)			

Table 43. Frequency of transmission of the 5'ALR2 Z, Z-2 and Z+2 alleles with respect to T1DM and diabetic nephropathy in affected proband family trios.

This table shows the frequency of transmission of the Z, Z-2 and Z+2 alleles from parents to affected offspring with respect to diabetic nephropathy. The frequency of transmission of the alleles from parents who were heterozygous for the 5'ALR2 allele, to affected offspring was determined in a total of 115 families. The number in the transmitted column in the observed row is the actual number of copies of the allele that was transmitted from parents to affected offspring. The non-transmitted value in the observed column is the actual number of times that the allele was not transmitted from parent to affected offspring. The expected number of Z, Z-2 and Z+2 alleles to be transmitted and not transmitted is 50% of the total number of alleles. The deviation from 50% transmission or non-transmission of the alleles from parents to affected offspring was determined using the χ^2 test and 2 x 2 contingency tables. There was no significant association found for the transmission of the Z+2/Z/Z-2 alleles from parents to proband with T1DM and nephropathy in the British Caucasoid family trios studied.

C(-106)T polymorphism marker

The cytosine to thymine single base polymorphism situated -106bp upstream of the ALR2 gene ATG transcription start sequence was investigated. Polymerase Chain Reaction was carried out to amplify the 263bp region upstream of the ALR2 gene containing the C(-106)T polymorphism which creates a new *Bfa I* restriction site as described in chapter 3. Purified amplification products were cut using *Bfa I* restriction enzyme digestion, and separated on a 2.5% agarose/ethidium bromide gel at 100V for 1 hour. The fragment sizes were checked by running a 100bp molecular weight marker alongside. Bands were revealed by UV (320nm) transillumination (figure 25) and subjects were assigned genotypes according to restriction fragments identified. A PCR control band was also included. After *Bfa*1 restriction enzyme digestion the homozygote genotype CC produced 206bp and 57bp fragments, while the homozygote genotype TT produced 174bp, 59bp and 57bp fragments. The heterozygte CT genotype produced all three fragments (174bp, 57bp, 59bp and 206bp). The C(-106)T polymorphism was investigated in T1DM subjects and normal controls of British Caucasoid origin and the 'DUK-Warren diabetic nephropathy family trios' of British Caucasoid origin. The assay however, failed to work using the T2DM Southern Indian subjects and consequently these have not been included here. A possible reason for the assay failing to work in the T2DM population could be that the DNA stock solutions contained an inhibiting factor preventing the BfaI enzyme from cutting the PCR product. Failure primarily lay with poor or incomplete restriction enzyme digestion. It is likely that despite the stringent purification protocols applied some inhibiting factors remained in the samples.
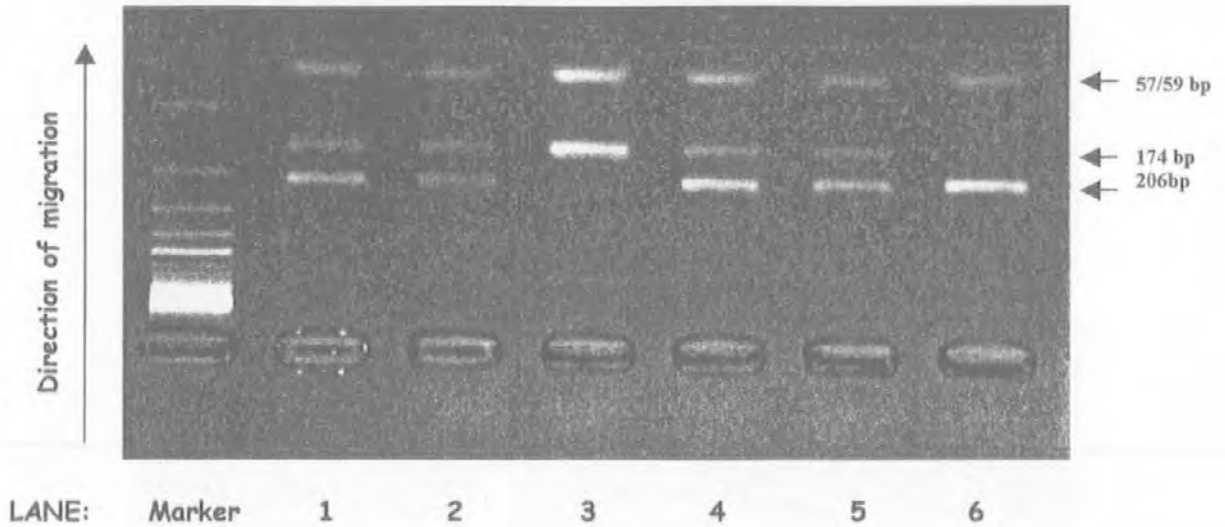
Figure 25. C(-106)T polymorphism by *Bfa 1* restriction digest and fragment separation on 2.5% agarose gel with ethidium bromide staining. Three genotypes were identified; homozygous TT, homozygous CC and heterozygous CT. Allocated genotypes for each of the subjects shown in this figure (lanes 1-6) are as follows:

Lane 1; CT	Lane 4; CT
Lane 2; CT	Lane 5; CT
Lane 3: TT	Lane 6: CC

C(-106)T polymorphism in T1DM subjects and normal controls of British Caucasoid origin

The C(-106)T polymorphism located in the 5' promoter region of the ALR2 gene, -106bp upstream of the ATG start sequence was successfully investigated using 155 T1DM subjects and 120 normal controls of British Caucasoid origin (clinical demographics shown in table 6). The remainder of the available 285 patients failed to respond to the assay and allele and genotypes could not be obtained with confidence. This was primarily due to poor or incomplete restriction enzyme digestion probably due to an inhibiting factor. Within the T1DM subject population studied there were 39 uncomplicated, 39 full house patients, 12 diabetic neuropathy, 16 retinopathy and neuropathy, 26 nephropathy and retinopathy and 23 retinopathy. The frequency of the C(-106)T alleles in the total T1DM population versus the normal populations is shown in table 44. In this study we were able to find subjects who were homozygous CC, homozygous TT and heterozygous CT. In the analysis of allelic frequencies homozygotes were counted as 2 alleles, and alleles and genotypes that are not detected are not shown. Tables show the percentage frequency of detected alleles and genotypes with actual numbers detected shown in parenthesis. Possible associations were investigated with respect to allelic and genotype frequency and comparisons were made between groups using the χ^2 test and 2 x 2 contingency tables. All of the data obtained from this study is presented in the following tables. Possible associations were investigated with respect to allelic and genotypic frequencies and the onset of diabetic complications.

The expected frequencies for C(-106)T genotypes were calculated using 2 x n analysis to determine whether frequencies of heterozygotes and homozygotes in control groups were in Hardy-Weinberg equilibrium (table 45).

Allele and genotype frequencies of C(-106)T polymorphism in British Caucasoid T1DM subjects and normal control subjects

The C(-106)T polymorphism was investigated in 155 T1DM subjects and 120 normal controls of British Caucasoid origin. Table 44 presents the percentage frequency of the detected C(-106)T alleles and genotypes for all T1DM subjects studied compared to the normal controls. There was a small increase in the CC (-106)-ALR2 genotype in the T1DM subjects compared to the normal controls (NC), (40.0% vs. 28.3%, χ^2 = 4.05, p= 0.04 (1df), Pc = ns). There is consequently also a significant increase in the frequency of the C allele and a subsequent decrease in the T allele in T1DM subjects compared to the NC group (χ^2 = 6.8, p= 0.009 (1df), Pc = 0.03). The Hardy Weinberg equilibrium principle was applied to the T1DM group and the NC group respectively. In both groups there were no significant differences between the expected and the observed frequencies according to the χ^2 test for significance, implying compliance with Hardy Weinberg equilibrium (table 45).

Table 46 presents the alleles and genotypes for the T1DM subjects according to the presence or absence of diabetic microvascular disease, where T1DM subjects with DN/DR/DNu are compared with DC and NC groups. A non-significant increase in the CC genotype was seen in the DR/DN/DNu group compared to the DC group (45.7 vs. 23.1%, χ^2 = 6.2, p= 0.01 (1df), Pc = ns). There was also a non-significant difference observed in the frequency of the TT genotype. The frequency was higher in the DC group compared to the DR/DN/DNu (12.8 vs. 3.5%, χ^2 = 4.7, p= 0.03 (1df), Pc = ns). There was a significant increase in the frequency of the C (-106) ALR2 allele in the DN/DR/DNu group compared to the uncomplicated DC group (71.1 vs. 55.1%, χ^2 = 6.8, p= 0.009 (1df), Pc = 0.02). Table 47 presents the frequency of the ALR2 C(-106)T alleles and genotypes where the T1DM group has been subgrouped according to the precise diabetic complications observed. In the case of the FHP vs. uncomplicated DC group the frequency of the CC

genotype was 51.3% vs. 23.1% (χ^2 = 6.6, p= 0.01 (1df), Pc = 0.03), and the C allele was 74.4% vs. 55.1%, χ^2 = 6.3, p= 0.01 (1df), Pc = ns), where significance was lost by correction. No other significant differences were found.

Table 48 presents the C(-106)T ALR2 alleles and genotypes detected in T1DM subjects according to the presence of retinopathy, or nephropathy or neuropathy. In all three groups the frequency of the CC genotype and the C allele was increased in the complications groups compared to the DC group. In the case of the DR vs. uncomplicated DC group the frequency of the CC genotype was 48.1% vs. 23.1% (χ^2 = 7.3, p= 0.007 (1df), Pc = 0.02), and the C allele was 72.6% vs. 55.1%, χ^2 = 7.9, p= 0.005 (1df), Pc = 0.01). In the case of the DN vs. uncomplicated DC group the frequency of the CC genotype was 50.8% vs. 23.1% (χ^2 = 7.8, p= 0.005 (1df), Pc = 0.02), and the C allele was 73.9% vs. 55.1%, χ^2 = 7.7, p= 0.006 (1df), Pc = 0.02). In the case of the DNu vs. DC group the frequency of the CC genotype was 46.3% vs. 23.1% (χ^2 = 5.7, p= 0.02 (1df), Pc = ns), and the C allele was 71.6% vs. 55.1%, χ^2 = 5.95, p= 0.01 (1df), Pc = 0.04). Therefore, with respect to the CC genotype statistical significance was not attained in the DNu vs. DC group.

	T1DM Subjects	Normal Controls (NC)	χ2	p-value	Pc
C(-106)T Genotype	n=155	n=120			
CC	40.0 (62)	28.3 (34)	4.05	0.04	ns
CT	54.2 (84)	55.8 (67)	0.07	ns	ns
TT	5.8 (9)	15.8 (19)	7.4	0.006	0.02
C(-106)T Alleles	n=310	n=240			
C	67.1 (208)	56.3 (135)	6.8	0.009	0.03
T	32.9 (102)	43.8 (105)	6.8	0.009	0.03

Table 44. Percentage frequency of the detected C(-106)T alleles and genotypes in all British Caucasoid T1DM subjects studied, compared to normal control subjects. This table displays C(-106)T allele and genotype frequencies in T1DM subjects and normal control subjects.

	Predicted	Observed	χ^2	p-value	Pc-value
Normal Controls (NC)	(n=120)	(n=120)			
CC	38	34	0.3	ns	ns
CT	59	67	1.1	ns	ns
TT	23	19	0.5	ns	ns
T1DM Patients	(n=155)	(n=155)			
CC	70	62	0.84	ns	ns
CT	68	84	3.3	ns	ns
TT	17	9	2.7	ns	ns

Table 45. Comparison between the expected and observed frequency % of C(-106)T polymorphism genotypes in British Caucasoid T1DM and normal subjects.

This table shows the expected and observed frequencies of C(-106)T genotypes in T1DM subjects and normal control subjects. This demonstrated that the distribution in the normal controls (NC) conforms to Hardy-Weinberg equilibrium principal ($p^2 + 2pq + q^2 = 1$). There were no significant differences between the expected and observed frequencies in the normal controls according to the Chi-squared test for significance. There was also no difference between the expected and observed frequencies in the T1DM population. This indicates that the T1DM population is in Hardy-Weinberg equilibrium for the C-106T polymorphism.

	T1DM Nephropathy and/or retinopathy and/or neuropathy	Uncomplicated (DC)	Normal Controls (NC)
C(-106)T Genotype	116	39	120
CC	45.7 (53)	23.1 (9)	28.3 (34)
CT	50.9 (59)	64.1 (25)	55.8 (67)
TT	3.5 (4)	12.8 (5)	15.8 (19)
C(-106)T Alleles	232	78	240
C	71.1 (165)	55.1 (43)	56.3 (135)
T	28.9 (67)	44.9 (35)	43.8 (105)

Table 46. C(-106)T allele and genotype frequencies in normal controls and diabetic patients according to the presence of microvascular complications of diabetes. This table displays C(-106)T allele frequencies in normal controls (NC), uncomplicated (DC) and T1DM Nephropathy/Retinopathy/Neuropathy. For each allele the percentage frequency and the actual number of alleles (in parenthesis) is given. n represents the number of subjects in each group. Comparisons were made for each allele and genotype between patient and control groups using 2 x 2 contingency tables and the χ^2 test. P values were corrected for the number of comparisons made. Only significant differences by χ^2 and correction by multiplying by the number of comparisons made, are shown.

DR/N/Nu vs. Uncomplicated diabetic controls;

CC; χ^2 = 6.2, p= 0.01 (1df), Pc = 0.03

TT; χ^2 = 4.7, p= 0.03 (1df), Pc = ns

C/T; χ^2 = 6.8, p= 0.009 (1df), Pc = 0.02

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	[1]Diabetic Retinopathy	[2]Diabetic Nephropathy and Retinopathy	[3]Diabetic Retinopathy and Neuropathy	[4]Diabetic Neuropathy	[5]Full House Patients	Uncomplicated (DC)	Normal Controls (NC)
C(-106)T Genotype	23	26	16	12	39	39	120
CC	39.1	50.0	50.0	25.0	51.3	23.1	28.3
	(9)	(13)	(8)	(3)	(20)	(9)	(34)
CT	56.5	46.2	50.0	66.7	46.2	64.1	55.8
	(13)	(12)	(8)	(8)	(18)	(25)	(67)
TT	4.4	3.9	-	8.3	2.6	12.8	15.8
	(1)	(1)	(0)	(1)	(1)	(5)	(19)
C(-106)T Alleles	46	52	32	24	78	78	240
C	67.4	73.1	75.0	58.3	74.4	55.1	56.3
	(31)	(38)	(24)	(14)	(58)	(43)	(135)
T	32.6	26.9	25.0	41.7	25.6	44.9	43.8
	(15)	(14)	(8)	(10)	(20)	(35)	(105)

Table 47. C(-106)T allele and genotype frequencies in normal controls and diabetic patients according to onset of diabetic complications. This table displays C(-106)T allele and genotype frequencies in control and patient subjects according to their diabetic complication phenotype. Subjects were classified according to the onset of microvascular complications, diabetic retinopathy, diabetic nephropathy and retinopathy, diabetic retinopathy and neuropathy, diabetic neuropathy, full house patients and uncomplicated. For each allele the % frequency and the actual number of alleles (in parenthesis) is given. n represents the number of subjects in each group. Comparisons were made for each allele and genotype between patient and control groups using 2 x 2 contingency tables and the χ^2 test. P values were corrected for the number of comparisons made.

[2] **vs. Uncomplicated diabetic controls;**
        CC; χ^2 = 5.1, p= 0.03 (1df), Pc = ns
        C or T; χ^2 = 4.3, p= 0.04 (1df), Pc = ns

[3] **vs. Uncomplicated diabetic controls;**
        C or T; χ^2 = 3.8, p= 0.05 (1df), Pc = ns

[5] **vs. Uncomplicated diabetic controls;**
        CC; χ^2 = 6.6, p= 0.01 (1df), Pc = 0.03
        C or T; χ^2 = 6.3, p= 0.01 (1df), Pc = ns

	[1]Diabetic Retinopathy (DR)	[2]Diabetic Nephropathy (DN)	[3]Diabetic Neuropathy (Dnu)	Uncomplicated (DC)	Normal Controls (NC)
C(-106)T Genotype	104	65	67	39	120
CC	48.1	50.8	46.3	23.1	28.3
	(50)	(33)	(31)	(9)	(34)
CT	49.0	46.2	50.8	64.1	55.8
	(51)	(30)	(34)	(25)	(67)
TT	2.9	3.1	2.99	12.8	15.8
	(3)	(2)	(2)	(5)	(19)
C(-106)T Alleles	208	130	134	78	240
C	72.6	73.9	71.6	55.1	56.3
	(151)	(96)	(96)	(43)	(135)
T	27.4	26.2	28.4	44.9	43.8
	(57)	(34)	(38)	(35)	(105)

Table 48. C(-106)T allele and genotype frequencies in normal controls and T1DM patients according to onset of diabetic complications. This table displays C(-106)T allele frequencies in control and patient subjects. T1DM subjects are grouped according to the presence of diabetic nephropathy, retinopathy or neuropathy. The retinopathy (n=104) group is an amalgamation of the retinopathy (n=23), nephropathy and retinopathy (n=26), retinopathy and neuropathy (n=16) and full house patients (n=39) groups previously identified. The nephropathy group (n=65) is an amalgamation of the nephropathy and retinopathy (n=26), and full house patients' (n=39) subgroups previously identified. The neuropathy group (n=67) is an amalgamation of the retinopathy and neuropathy (n=16), the neuropathy (n=12) and the full house patients' (n=39) groups also previously identified. For each allele the % frequency and the actual number of alleles (in parenthesis) are given. n represents the number of subjects in each group. Comparisons were made for each allele and genotype between patient and control groups using 2 x 2 contingency tables and the χ^2 test. P values were corrected for the number of comparisons made.

[1] vs. Uncomplicated diabetic controls;
        CC; χ^2 = 7.3, p= 0.007 (1df), Pc = 0.02
        TT; χ^2 = 5.3, p= 0.02 (1df), Pc = ns
        C or T; χ^2 = 7.9, p= 0.005 (1df), Pc = 0.02

[2] vs. Uncomplicated diabetic controls;
        CC; χ^2 = 7.8, p= 0.005 (1df), Pc = 0.02
        C or T; χ^2 = 7.7, p= 0.006 (1df), Pc = 0.02

[3] vs. Uncomplicated diabetic controls;
        CC; χ^2 = 5.7, p= 0.02 (1df), Pc = ns
        TT; χ^2 = 3.9, p= 0.05 (1df), Pc = ns
        C or T; χ^2 = 5.95, p= 0.01 (1df), Pc = 0.02

C(-106)T polymorphism in a T1DM Caucasoid family study

In order to study the transmission of the C(-106)T alleles of the aldose reductase promoter region the 'Diabetes UK Warren 3 nephropathy family trios' were investigated for the polymorphism. A total of 109 family trios that had been previously typed for the 5'ALR2 microsatellite polymorphism were genotyped for the C(-106)T polymorphism. The allele and genotype frequencies detected in the proband are shown in table 49, a total of 90 probands were successfully genotyped in this study. Out of the available 172 family trios only 90 trios were successfully genotyped due to several reasons. Firstly, some of the DNA stock had been used up in the previous 5'ALR2 assay, therefore preventing the completion of the family trio for the C-106T assay. Secondly the assay had a very high failure rate and reliable data was only obtained in a fraction of the subjects investigated, for reasons previously explained. The results of which are not included in the analysis. From the 109 family trios genotyped for the C(-106)T polymorphism 77 trios were non-informative. Trios were classified as non-informative when both parents in the trio were heterozygous for the allele marker and transmission could not be determined, or if the parents genotype could not be confidently predicted from incomplete family sets. The informative 32 family trios were analysed using the transmission disequilibrium test. Table 49 shows the frequency of transmission of the C(-106)T alleles from parents who were heterozygous for the allele to affected offspring. If there is no linkage, the expected number of transmitted versus non-transmitted alleles should be equal. The difference therefore between the expected number of transmissions and observed transmission of alleles was calculated.

C(-106)T polymorphism in British Caucasoid families where proband has T1DM and diabetic nephropathy

Table 49 presents the frequency of the C(-106)T allele and genotypes detected in the 90 diabetic nephropathy probands of the DUK-Warren nephropathy collection. The CC genotype occurred in 30% of the subjects studied, the CT genotype occurred in 53.3% of the subjects studied and the TT genotype in 16.7% of the subjects studied. The C allele occurred in 56.7% of the subjects and the C allele in 43.3% of the subjects. The frequency of the CC, CT and TT genotypes conformed to the Hardy Weinberg equilibrium (table 50).

Table 51 presents the frequency of the transmission of the C(-106)T alleles from parents to proband in the DUK-Warren Nephropathy Collection. There was no significant deviation from the expected 50% transmission of the alleles from parents to proband.

	T1DM Nephropathy
C(-106)T Genotype	n=90
CC	30.0 (27)
TT	16.7 (15)
CT	53.3 (48)
C(-106)T Alleles	n=180
C	56.7 (102)
T	43.3 (78)

Table 49. C(-106)T allele and genotype frequencies in Diabetes UK- Warren nephropathy probands. This table displays C(-106)T allele and genotype frequencies in nephropathy proband subjects. For each allele and genotype detected the % frequency and the actual number of alleles and genotypes (in parenthesis) are given. n represents the number of subjects in each group.

		Predicted	Observed	χ^2	p-value	Pc-value
DUK Warren T1DM Nephropathy		(n=90)	(n=90)			
	CC	29	27	0.1	0.7	ns
	CT	44	48	0.4	0.6	ns
	TT	17	15	0.2	0.7	ns

Table 50. Comparison between the expected and observed frequency % of C(-106)T polymorphism genotypes in DUK- Warren British Caucasoid T1DM subjects with diabetic nephropathy.

This table shows the expected and observed frequencies of C(-106)T genotypes in T1DM subjects with diabetic nephropathy obtained from the DUK Warren Nephropathy collection. This demonstrated that the distribution in the T1DM nephropathy subjects conforms to Hardy-Weinberg equilibrium principal ($P^2+2pq+q^2=1$). There were no significant differences between the expected and observed frequencies according to the χ^2 test for significance.

		Transmitted	Non-transmitted	Total	χ^2	p-value	Pc
C	**Observed**	50 (34)	50 (34)	(68)	0.0	1.0	ns
	Expected	50 (34)	50 (34)	(68)			
T	**Observed**	50 (34)	50 (34)	(68)	0.0	1.0	ns
	Expected	50 (34)	50 (34)	(68)			

Table 51. Frequency of transmission of the C(-106)T alleles from parents of affected offspring to affected offspring in family proband study.

This table shows the frequency of transmission of the C and T alleles from parents to affected offspring with respect to diabetic nephropathy. The frequency of transmission of the alleles from parents who were heterozygous for the C(-106)T allele, to affected offspring was determined in a total of 32 families. The number in the transmitted column in the observed row is the actual number of copies of the allele that was transmitted from parents to offspring. The non-transmitted value in the observed column is the actual number of times that the allele was not transmitted from parent to affected offspring. The expected number of C and T alleles to be transmitted and not-transmitted is 50% of the total number of alleles. The deviation from 50% transmission or non-transmission of the alleles from parents to affected offspring was determined using the χ^2 test and 2x2 contingency tables.

A(+11842)C polymorphism marker

An adenine to cytosine single base polymorphism situated +11842 base pairs downstream of the ALR2 gene ATG transcription start sequence was investigated. Polymerase chain reaction was carried out to amplify the 252bp region of intron 8 of ALR2 gene containing the A(+11842)C polymorphism creating a *Bam HI* restriction site as described in chapter 3. Amplification products were cut by *Bam HI* restriction enzyme digestion, and separated on a 2.5% agarose/ethidium bromide gel at 100V for 1 hour. The fragment sizes were checked by running a 100bp molecular weight marker alongside. Bands were detected by UV transillumination (320nm) (figure 26) and subjects were assigned a genotype according to restriction fragments identified. A PCR control band was also present. After *Bam* HI digestion, the homozygote genotype CC produced a 252 bp fragment, while the homozygote genotype AA produced 174 bp and 78 bp fragments. The heterozygote AC genotype produced all three fragments. The A(+11842)C polymorphism was investigated in the T1DM subjects and normal controls of British Caucasoid origin.
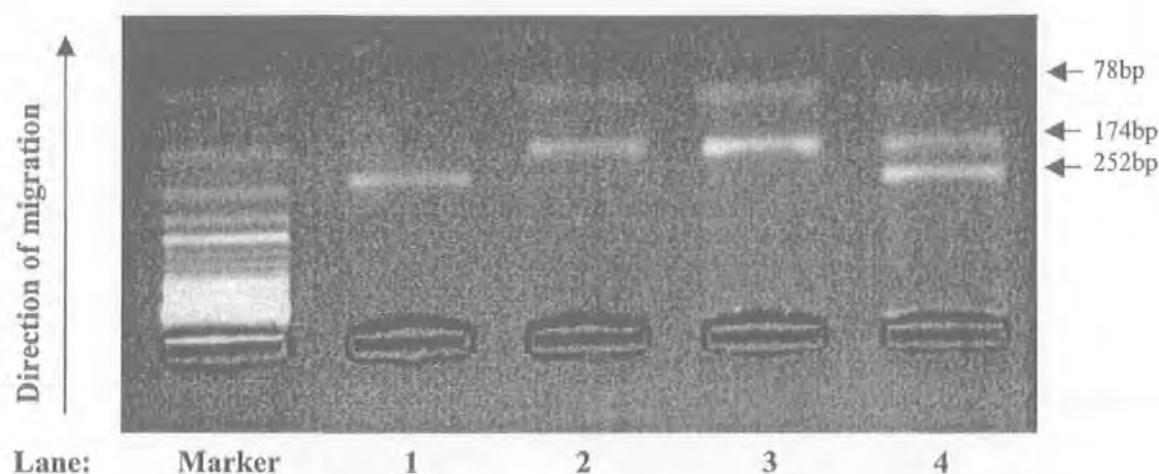
Figure 26. A(+11842)C polymorphism by Bam HI restriction fragment separation on 2.5% agarose/ethidium bromide gel

Polymerase Chain Reaction was carried out to amplify a 252 bp region of the aldose reductase gene. The region included an intragenic polymorphic site, a single base substitution of A to C at 95th nucleotide of Intron 8. The polymorphism abolishes a *Bam* HI restriction site.

Five microlitres of the PCR product was digested for 4 hours at 37°C with 10 units *Bam* HI (Promega). The genotypes were identified by 2.5% agarose gel electrophoresis alongside a 100bp molecular weight marker and ethidium bromide staining.

After *Bam* HI digestion, the homozygote *CC* produced a 252 bp fragment, while the homozygote *AA* produced 174 bp and 78 bp fragments. The heterozygote *AC* produced all three fragments. All samples were analysed in duplicate to reduce the risk of erroneous results.

Three genotypes were identified, homozygous *AA*, homozygous *CC* and heterozygous *AC*. Allocated genotypes for each of the subjects shown in this figure (lanes 1-4) are as follows:

> Lane 1: *CC*
> Lane 2: *AA*
> Lane 3: *AA*
> Lane 4: *CA*

A(+11842)C polymorphism in British Caucasoid T1DM subjects and normal controls

The A(+11842)C polymorphism located within the ALR2 gene itself, +11842bp downstream of the ATG start sequence was investigated using 244 T1DM subjects and 120 normal controls of British Caucasoid origin (clinical demographics shown in table 6). Within the T1DM subject population studied there were 66 uncomplicated, 62 full house patients, 18 neuropathy, 24 retinopathy and neuropathy, 30 nephropathy and retinopathy, and 44 retinopathy patients.

The frequency of the A(+11842)C alleles in both T1DM and normal populations is shown in table 52. In this study we were able to find subjects who were homozygous CC, homozygous AA and heterozygous CA. In the analysis of allelic frequencies homozygotes were counted as 2 alleles. Tables show the percentage frequency of detected alleles and genotypes with actual numbers detected shown in parenthesis. Possible associations were investigated with respect to allelic frequency and genotype and comparisons were made between groups using the χ^2 test and 2 x 2 contingency tables. P-values were corrected for the number of comparisons made, and where the observed frequencies were <5 Fishers exact test was applied.

All of the data obtained from this study is presented in the following tables. Possible associations were investigated with respect to allelic and genotypic frequencies and the onset of diabetic complications. The expected frequencies for A(+11842)C genotypes were calculated using 2xn analysis to determine whether frequencies of heterozygotes and homozygotes in control groups were in Hardy Weinberg equilibrium (table 53).

Allele and genotype frequencies of the A(+11842)C polymorphism in British Caucasoid T1DM subjects and normal controls

The A(+11842)C polymorphism was investigated in 244 T1DM subjects and 120 normal controls of British Caucasoid origin. Table 52 presents the percentage frequency of the detected A(+11842)C alleles and genotypes for all T1DM subjects studied compared to the normal controls. There is a significant increase in the frequency of the AA (+11842)–ALR2 genotype in the T1DM subjects compared to the normal controls (NC), (41.8 vs. 6.7%, χ^2 = 47.1, p=<0.0001, Pc=<0.0001). Consequently, there is also a significant increase in the frequency of the A (+11842) allele in the T1DM subjects compared to the NC group (62.3 vs. 30.0%, χ^2 = 67.2, p=<0.0001, Pc=<0.0001). Both the normal and control groups complied with the Hardy Weinberg equilibrium (table 53). Table 54 presents the alleles and genotypes for the T1DM subjects according to the presence or absence of diabetic microvascular disease, where T1DM subjects with DN/DR/DNu are compared with the uncomplicated DC and NC groups. A significant increase in the AA (+11842)-ALR2 genotype was seen in the DN/DR/DNu group compared to the uncomplicated DC group (52.3 vs. 13.7%, χ^2 = 29.5, p=<0.0001, Pc=<0.0001). There was a significant decrease in the frequency of the CC genotype, whereby the frequency was higher in the DC group compared to the T1DM DN/DR/DNu (40.9 vs. 8.4%, χ^2 = 35.7, p=<0.0001, Pc=<0.0001). There was also a significant increase in the A (+11842)-ALR2 allele in the T1DM DN/DR/DNU group compared to the uncomplicated DC group (71.9 vs. 36.4%, χ^2 = 51.8, p=<0.0001, Pc=<0.0001). Table 55 presents the frequency of the ALR2 A(+11842)C alleles and genotypes where the T1DM group has been subdivided according to the precise diabetic complications observed. A significant increase in the AA (+11842) genotype was observed in all T1DM complication groups compared to the DC group. This was accompanied by an unequivocal increase in the A (+11842) allele in the T1DM complication subgroups compared to the DC group. Table 56 presents the A(+11842)C ALR2 alleles and genotypes detected in T1DM subjects according to the presence of retinopathy, or nephropathy or neuropathy. In all three groups the frequency of

the AA genotype and the A allele was significantly increased in the complications groups compared to the uncomplicated DC group. In the case of the DR vs. uncomplicated DC group the frequency of the AA genotype was 51.3 vs. 13.6%, χ^2 = 27.5, p=<0.0001, Pc=<0.001, and the A allele was 70.9 vs. 36.4%, χ^2 = 46.9, p=<0.0001, Pc=<0.001. In the case of the DN vs. uncomplicated DC group the frequency of the AA genotype was 45.7 vs. 13.6%, χ^2 = 18.0, p=<0.0001, Pc=<0.001, and the A allele was 68.5 vs. 36.4%, χ^2 = 32.0, p=<0.0001, Pc=<0.001). In the case of the DNU vs. uncomplicated DC group the frequency of the AA genotype was 54.8 vs. 13.6%, χ^2 = 28.8, p=<0.0001, Pc=<0.001, and the A allele was 73.6 vs. 36.4%, χ^2 = 46.2, p=<0.0001, Pc=<0.001. In all three groups the frequency of the CC genotype and the C allele was significantly decreased in the complications groups compared to the uncomplicated DC group.

	T1DM Subjects	Normal Controls	χ^2	p-value	*P*c-value
A(+11842)C Genotype	n=244	n=120			
AA	41.8 (102)	6.7 (8)	47.09	<0.0001	<0.0001
AC	41.0 (100)	46.7 (56)	1.06	ns	ns
CC	17.2 (42)	46.7 (56)	35.5	<0.0001	<0.0001
Allele	n=488	n=240			
A	62.3 (304)	30.0 (72)	67.2	<0.0001	<0.0001
C	37.7 (184)	70.0 (168)			

Table 52. Percentage frequency of the detected A(+11842)C alleles and genotypes in all British Caucasoid T1DM subjects studied, compared to normal control subjects. This table displays A(+11842)C allele and genotype frequencies in T1DM subjects and normal control subjects.

	Predicted	Observed	χ^2	p-value	*P*c-value
Normal Controls (NC)	(n=120)	(n=120)			
AA	11	8	0.5	0.5	ns
AC	50	56	0.6	0.4	ns
CC	59	56	0.2	0.7	ns
T1DM Patients	(n=244)	(n=244)			
AA	95	102	0.4	0.5	ns
AC	115	100	1.9	0.2	ns
CC	34	42	1.0	0.3	ns

Table 53. Comparison between the expected and observed incidence of A(+11842)C polymorphism genotypes in British Caucasoid T1DM and normal subjects.

This table shows the expected and observed frequencies of A(+11842)C genotypes in T1DM subjects and normal control subjects. This demonstrates that the distribution in the normal controls (NC) conforms to Hardy-Weinberg equilibrium principal ($P^2+2pq+q^2=1$). There were no significant differences between the expected and observed frequencies in the normal controls and T1DM subjects according to the Chi-squared test for significance.

	T1DM Nephropathy and/or retinopathy and/or neuropathy	Uncomplicated (DC)	Normal Controls (NC)
A(+11842)C Genotype	**n=178**	**n=66**	**n=120**
AA	52.3 (93)	13.6 (9)	6.7 (8)
AC	39.3 (70)	45.5 (30)	46.7 (56)
CC	8.4 (15)	40.9 (27)	46.7 (56)
A(+11842)C Alleles	**n=356**	**n=132**	**n=240**
A	71.9 (256)	36.4 (48)	30.0 (72)
C	28.1 (100)	63.6 (84)	70.0 (168)

Table 54. A(+11842)C allele and genotype frequencies in normal controls and diabetic patients according to the presence of microvascular complications of diabetes. This table displays A(+11842)C allele frequencies in normal controls (NC), uncomplicated (DC) and T1DM Nephropathy/Retinopathy/Neuropathy. For each allele the percentage frequency and the actual number of alleles (in parenthesis) is given. n represents the number of subjects in each group. Comparisons were made for each allele and genotype between patient and control groups using 2 x 2 contingency tables and the χ^2 test. P values were corrected for the number of comparisons made. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5 Fishers exact test was applied.

T1DM DR/N/Nu vs. Uncomplicated diabetic controls;

AA; χ^2 = 29.5, p= <0.0001 (1df), Pc = <0.0001
CC; χ^2 = 35.7, p= <0.0001 (1df), Pc = <0.0001
A or C; χ^2 = 51.8, p= <0.0001 (1df), Pc = <0.0001

	[1] Diabetic Retinopathy	[2] Diabetic Nephropathy and Retinopathy	[3] Diabetic Retinopathy and Neuropathy	[4] Diabetic Neuropathy	[5] Full House Patients	Uncomplicated (DC)	Normal Controls (NC)
A(+11842)C Genotype	n=44	n=30	n=24	n=18	n=62	n=66	n=120
AA	52.3	43.3	70.8	61.1	46.8	13.6	6.7
	(23)	(13)	(17)	(11)	(29)	(9)	(8)
AC	36.4	50.0	20.8	38.9	43.6	45.5	46.7
	(16)	(15)	(5)	(7)	(27)	(30)	(56)
CC	11.4	6.7	8.3	-	9.7	40.9	46.7
	(5)	(2)	(2)	(0)	(6)	(27)	(56)
A(+11842)C Allele	n=88	n=60	n=48	n=36	n=124	n=132	n=240
A	70.5	68.3	81.3	80.6	68.6	36.4	30.0
	(62)	(41)	(39)	(29)	(85)	(48)	(72)
C	29.6	31.7	18.8	19.4	31.5	63.6	70.0
	(26)	(19)	(9)	(7)	(39)	(84)	(168)

Table 55. A(+11842)C allele and genotype frequencies in normal controls and diabetic subjects according to onset of diabetic complications. This table displays A(+11842)C allele and genotype frequencies in control and patient subjects according to their diabetic complication phenotype. Subjects were classified according to the onset of microvascular complications, diabetic retinopathy, diabetic nephropathy and retinopathy, diabetic retinopathy and neuropathy, diabetic neuropathy, full house patients and uncomplicated. For each allele the % frequency and the actual number of alleles (in parenthesis) is given. n represents the number of subjects in each group. Comparisons were made for each allele and genotype between patient and control groups using 2 x 2 contingency tables and the χ^2 test. P values were corrected for the number of comparisons made. Only significant differences by χ^2 and correction by multiplying by the number of comparisons made are shown. Where observed numbers were <5, Fishers exact test was applied.

[1] vs. UDC;   AA; χ^2 = 19.1, p= <0.0001 (1df), Pc = <0.001
CC; χ^2 = 11.17, p= 0.0008 (1df), Pc = 0.002
A or C; χ^2 = 24.0, p= <0.0001 (1df), Pc = <0.001

[2] vs. UDC;   AA; χ^2 = 10.3, p= 0.001 (1df), Pc = 0.003
CC; χ^2 = 16.2, p= <0.0001 (1df), Pc = <0.001
A or C; χ^2 = 16.95, p= <0.0001 (1df), Pc = <0.001

[3] vs. UDC;   AA; χ^2 = 28.0, p= <0.0001 (1df), Pc = <0.001
AC; χ^2 = 4.5, p= 0.03 (1df), Pc = ns
CC; χ^2 = 8.6, p= 0.004 (1df), Pc = 0.01
A or C; χ^2 = 28.4, p= <0.0001 (1df), Pc = <0.001

[4] vs. UDC;   AA; χ^2 = 17.6, p= <0.0001 (1df), Pc = 0.001
CC; χ^2 = 10.9, p= 0.0009 (1df), Pc = 0.003
A or C; χ^2 = 22.3, p= <0.0001 (1df), Pc = <0.0001

[5] vs. UDC;   AA; χ^2 = 16.8, p= <0.0001 (1df), Pc = <0.0001
CC; χ^2 = 16.3, p= <0.0001 (1df), Pc = 0.0001
A or C; χ^2 = 26.5, p= <0.0001 (1df), Pc = <0.0001

	[1]Diabetic Retinopathy (DR)	[2]Diabetic Nephropathy (DN)	[3]Diabetic Neuropathy (DNu)	Uncomplicated (DC)	Normal Controls (NC)
A(+11842)C Genotype	n=160	n=92	n=104	n=66	n=120
AA	51.3 (82)	45.7 (42)	54.8 (57)	13.6 (9)	6.7 (8)
AC	39.4 (63)	45.7 (42)	37.5 (39)	45.5 (30)	46.7 (56)
CC	9.4 (15)	8.7 (8)	7.7 (8)	40.9 (27)	46.7 (56)
Allele	n=320	n=184	n=208	n=132	n=240
A	70.9 (227)	68.5 (126)	73.6 (153)	36.4 (48)	30.0 (72)
C	29.1 (93)	31.5 (58)	26.4 (55)	63.6 (84)	70.0 (168)

Table 56. A(+11842)C allele and genotype frequencies in normal controls and T1DM patients classified according to onset of diabetic complications. This table displays A(+11842)T allele and genotype frequencies in control and patient subjects. T1DM subjects are grouped according to the presence of diabetic nephropathy, retinopathy or neuropathy. The retinopathy group is an amalgamation of the retinopathy, nephropathy and retinopathy, retinopathy and neuropathy and full house patients groups previously identified. The nephropathy group is an amalgamation of the nephropathy and retinopathy, and full house patients' subgroups previously identified. The neuropathy group is an amalgamation of the retinopathy and neuropathy, the neuropathy and the full house patients' groups also previously identified. For each allele the % frequency and the actual number of alleles (in parenthesis) are given. n represents the number of subjects in each group. Comparisons were made for each allele and genotype between patient and control groups using 2 x 2 contingency tables and the χ^2 test. P values were corrected for the number of comparisons made. Only significant differences by χ^2 and correction are shown.

[1] vs. Uncomplicated diabetic controls;
> AA; χ^2 = 27.5, p= <0.0001 (1df), Pc = <0.001
> CC; χ^2 = 30.7, p= <0.0001 (1df), Pc = <0.001
> A or C; χ^2 = 46.9, p= <0.0001 (1df), Pc = <0.001

[2] vs. Uncomplicated diabetic controls;
> AA; χ^2 = 18.0, p= <0.0001 (1df), Pc = <0.001
> CC; χ^2 = 23.1, p= <0.0001 (1df), Pc = <0.001
> A or C; χ^2 = 32.0, p= <0.0001 (1df), Pc = <0.001

[3] vs. Uncomplicated diabetic controls;
> AA; χ^2 = 28.8, p= <0.0001 (1df), Pc = <0.001
> CC; χ^2 = 27.3, p= <0.0001 (1df), Pc = <0.001
> A or C; χ^2 = 46.2, p= <0.0001 (1df), Pc = <0.001

Combined genotype analysis of ALR2 polymorphisms investigated.

Combined genotype analysis in patients with T1DM and normal controls of British Caucasoid origin

The frequency of the combined genotypes for the 5'ALR2, C(-106)T and A(+11842)C polymorphism's were investigated in the T1DM subjects of British Caucasoid origin. Firstly, the 5'ALR2/C(-106)T combined genotypes were counted and analysed. Secondly, the 5'ALR2/A(+11842)C and thirdly C(-106)T/A(+11842)C combined genotypes were counted and analysed. The three polymorphism's were however not investigated together in a combined genotype analysis (5'ALR2/C(-106)T/A(+11842)C) as the number of subjects in each group with a particular genotype was very small and a significant difference in the percentage frequency between groups would be difficult to find. Comparisons were made between the frequencies of detected combined genotypes using the χ^2 test and 2x2 contingency tables. Combined genotype frequencies were investigated with respect to the onset of diabetic microvascular complications.

Combined genotype analysis of the 5'ALR2/C(-106)T polymorphism's in T1DM subjects of British Caucasoid origin

Tables 57-60 present the combined genotype frequencies for the 5'ALR2/C(-106)T genotype in T1DM subjects and normal controls of British Caucasoid origin. Table 57 presents the T1DM subjects vs. normal controls. Following the application of the Fishers exact test for small numbers the Z-2/X.T/T combined genotype was significantly higher in the normal controls vs. the T1DM subjects. Table 58 compares DR/DN/DNU and the uncomplicated DC group. A significant association was observed between the DR/DN/DNU and uncomplicated DC group with respect to the Z-2/X.C/C genotype whereby it was increased in the complicated group (20.7% vs. 2.6%, χ^2=7.1, p=0.008, Pc=0.02). This however involved small numbers and more data would be required in order to confirm an association. Table 59 compares the 5'ALR2/C(-106)T genotype in T1DM subjects according to the precise onset of complications. After correction the Z-2/X.C/C genotype was significantly increased in the diabetic nephropathy with retinopathy and the full house patients groups compared to the uncomplicated diabetic controls. In the case of the DN/DR vs. uncomplicated DC groups (34.6% vs. 2.6%, χ^2=12.3, p=0.0005, Pc=0.002, Fishers exact = 0.0007), and the FHP vs. uncomplicated DC groups (20.5% vs. 2.6%, χ^2=6.2, p=0.01, Pc=0.03, Fishers exact = 0.01). Table 60 presents the 5'ALR2/C(-106)T genotype in T1DM subjects according to the onset of nephropathy or retinopathy or neuropathy. In the case of the DNu patients vs. DC patients there was a significant association between the Z-2/X.C/C and the X/X.C/C combined genotypes and the onset of diabetic neuropathy according to the Fishers exact test for small numbers. Larger numbers will be required to confirm this association.

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5'ALR2/C(-106)T genotype	T1DM Subjects	Normal Controls (NC)
n	155	120
Z-2/X.C/T	10.9	10.8
	(17)	(13)
Z-2/X.C/C	16.1	7.5
	(23)	(9)
Z-2/X.T/T	-	6.7
	(-)	(8)
Z+2/X C/T	14.2	13.3
	(22)	(16)
Z+2/X.C/C	7.1	6.7
	(11)	(8)
Z+2/X.T/T	1.3	1.7
	(2)	(2)
Z-2/Z+2.C/T	5.8	2.5
	(9)	(3)
Z-2/Z+2.C/C	3.2	1.7
	(5)	(2)
Z-2/Z+2.T/T	-	-
	(-)	(-)
X/X.CT	17.4	17.5
	(27)	(21)
X/X.C/C	6.5	10.0
	(10)	(12)
X/X.T/T	1.9	6.7
	(3)	(8)
Other	15.5	15.0
	(24)	(18)

Table 57. Frequency of 5'ALR2/ C(-106)T combined genotypes in patients with T1DM of British Caucasoid origin. The combined genotype frequencies were obtained by using the gene counting method. This table presents the combined genotypes detected in the T1DM and normal control (NC) subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotype frequencies detected for T1DM and NC subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis. X= patient has neither Z-2 nor Z+2 5'ALR2 alleles. Where observed numbers were <5, Fishers exact test for small numbers was applied.

T1DM vs. NC;

Z-2/X.C/C; χ^2= 4.7, p= 0.03, Pc= ns
Z-2/X.T/T; χ^2= 10.6, p= 0.001, Pc= 0.01, Fishers exact= 0.001.
X/X.T/T; χ^2= 3.9, p= 0.05, Pc= ns, Fishers exact= 0.05

5'ALR2/C(-106)T genotype	T1DM Nephropathy and/or Retinopathy and/or Neuropathy (DR/DN/DNu)	Uncomplicated (DC)	Normal Controls (NC)
n	116	39	120
Z-2/X.C/T	11.2 (13)	10.3 (4)	10.8 (13)
Z-2/X.C/C	20.7 (24)	2.6 (1)	7.5 (9)
Z-2/X.T/T	- (-)	- (-)	6.7 (8)
Z+2/X C/T	11.2 (13)	23.1 (9)	13.3 (16)
Z+2/X.C/C	5.2 (6)	12.8 (5)	6.7 (8)
Z+2/X.T/T	- (-)	5.1 (2)	1.7 (2)
Z-2/Z+2.C/T	5.2 (6)	7.7 (3)	2.5 (3)
Z-2/Z+2.C/C	4.3 (5)	- (-)	1.7 (2)
Z-2/Z+2.T/T	- (-)	- (-)	- (-)
X/X.CT	18.1 (21)	15.4 (6)	17.5 (21)
X/X.C/C	8.6 (10)	- (-)	10.0 (12)
X/X.T/T	2.6 (3)	- (-)	6.7 (8)
Other	12.9 (15)	23.1 (9)	15.0 (18)

Table 58. Frequency of 5'ALR2/ C(-106)T combined genotypes in patients with T1DM of British Caucasoid origin. The combined genotype frequencies were obtained by using the gene counting method. This table presents the combined genotypes detected in the T1DM subjects with microvascular disease (nephropathy, retinopathy, neuropathy) compared to normal control (NC) subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotypes detected for T1DM and NC subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis. X= patient has neither Z-2 nor Z+2 5'ALR2 alleles. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5, Fishers exact test for small numbers was applied.

DR/DN/DNU vs. Uncomplicated diabetic controls;
  Z-2/X.C/C; χ^2= 7.1, p= 0.008, Pc= 0.02
  Z+2/X.T/T; χ^2= 6.03, p= 0.01, Pc= ns, Fishers= 0.06

5'ALR2/ C(-106)T genotype	Diabetic Retinopathy	Diabetic Nephropathy and Retinopathy	Diabetic Retinopathy and Neuropathy	Diabetic Neuropathy	Full House Patients	Uncomplicated (DC)	Normal Controls (NC)
n	23	26	16	12	39	39	120
Z-2/X.C/T	17.4 (4)	7.7 (2)	6.3 (1)	33.3 (4)	5.1 (2)	10.3 (4)	10.8 (13)
Z-2/X.C/C	17.4 (4)	34.6 (9)	6.3 (1)	16.7 (2)	20.5 (8)	2.6 (1)	7.5 (9)
Z-2/X.T/T	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	6.7 (8)
Z+2/X.C/T	8.7 (2)	7.7 (2)	12.5 (2)	16.7 (2)	12.8 (5)	23.1 (9)	13.3 (16)
Z+2/X.C/C	13.0 (3)	- (-)	6.3 (1)	- (-)	5.1 (2)	12.8 (5)	6.7 (8)
Z+2/X.T/T	- (-)	- (-)	- (-)	- (-)	- (-)	5.1 (2)	1.7 (2)
Z-2/Z+2.C/T	8.7 (2)	3.9 (1)	6.3 (1)	- (-)	5.1 (2)	7.7 (3)	2.5 (3)
Z-2/Z+2.C/C	4.4 (1)	- (-)	12.5 (2)	- (-)	5.1 (2)	- (-)	1.7 (2)
Z-2/Z+2.T/T	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)
X/X.CT	17.4 (4)	19.2 (5)	25 (4)	8.3 (1)	17.95 (7)	15.4 (6)	17.5 (21)
X/X.C/C	4.4 (1)	7.7 (2)	6.3 (1)	8.3 (1)	12.8 (5)	- (-)	10.0 (12)
X/X.T/T	- (-)	3.9 (1)	- (-)	8.3 (1)	2.6 (1)	- (-)	6.7 (8)
Other	8.7 (2)	15.4 (4)	18.8 (3)	8.3 (1)	12.8 (5)	23.1 (9)	15.0 (18)

Table 59. Frequency of 5'ALR2/ C(-106)T combined genotypes in patients with T1DM of British Caucasoid origin. The combined frequencies were obtained by using the gene counting method. This table presents the genotypes detected in the T1DM subjects with retinopathy (DR), nephropathy and retinopathy (DN/DR), retinopathy and neuropathy (DN/DNu), full house complications (FHC). Also shown are the T1DM uncomplicated diabetic controls (DC) and normal control (NC) subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotypes detected for T1DM complication groups and the uncomplicated DC subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis. X= patient has neither Z-2 nor Z+2 5'ALR2 alleles. Where observed numbers were <5, Fishers exact test for small numbers was applied.

DR vs. DC; Z-2/X.C/C; χ^2= 4.3, p= 0.04, Pc= ns, Fishers= 0.06.

DN/DR vs. DC; Z-2/X.C/C; χ^2= 12.3, p= 0.0005, Pc= 0.002, Fishers= 0.0007.

DR/DNU vs. DC; Z-2/Z+2.C/C; χ^2= 5.1, p= 0.02, Pc= ns, Fishers = 0.08.

FHP vs. DC; Z-2/X.C/C; χ^2= 6.2, p= 0.01, Pc= 0.03, Fishers = 0.01.
X/X.C/C; χ^2= 5.3, p= 0.02, Pc= ns, Fishers = 0.03

5'ALR2/C(-106)T genotype	Diabetic Retinopathy (DR)	Diabetic Nephropathy (DN)	Diabetic Neuropathy (DNu)	Uncomplicated (DC)	Normal Controls (NC)
n	104	65	67	39	120
Z-2/X.C/T	8.7	6.2	10.5	10.3	10.8
	(9)	(4)	(7)	(4)	(13)
Z-2/X.C/C	21.2	26.2	16.4	2.6	7.5
	(22)	(17)	(11)	(1)	(9)
Z-2/X.T/T	-	-	-	-	6.7
	(-)	(-)	(-)	(-)	(8)
Z+2/X.C/T	10.6	10.8	13.4	23.1	13.3
	(11)	(7)	(9)	(9)	(16)
Z+2/X.C/C	5.8	3.1	4.5	12.8	6.7
	(6)	(2)	(3)	(5)	(8)
Z+2/X.T/T	-	-	-	5.1	1.7
	(-)	(-)	(-)	(2)	(2)
Z-2/Z+2.C/T	5.8	4.6	4.5	7.7	2.5
	(6)	(3)	(3)	(3)	(3)
Z-2/Z+2.C/C	4.8	3.1	5.97	-	1.7
	(5)	(2)	(4)	(-)	(2)
Z-2/Z+2.T/T	-	-	-	-	-
	(-)	(-)	(-)	(-)	(-)
X/X.CT	19.2	18.5	17.9	15.4	17.5
	(20)	(12)	(12)	(6)	(21)
X/X.C/C	8.7	10.8	10.5	-	10.0
	(9)	(7)	(7)	(-)	(12)
X/X.T/T	1.9	3.1	2.99	-	6.7
	(2)	(2)	(2)	(-)	(8)
Other	13.5	13.9	13.4	23.1	15.0
	(14)	(9)	(9)	(9)	(18)

Table 60. Frequency of 5'ALR2/ C(-106)T combined genotypes in patients with T1DM of British Caucasoid origin. The combined genotype frequencies were obtained by using the gene counting method with those subjects who were homozygous for either the 5'ALR2 or the C(-106)T locus or both. X= neither Z-2 nor Z+2 allele. This table presents the haplotypes detected in the T1DM normal control (NC) subjects. Subjects with microvascular disease are classified in accordance to onset of retinopathy, nephropathy or neuropathy. The nephropathy group (n=65) is an amalgamation of the nephropathy and retinopathy (n=26) and full house subjects (n=39) groups previously identified. The retinopathy group (n=104) is an amalgamation of the retinopathy (n=23), nephropathy with retinopathy (n=26), retinopathy and neuropathy (n=16) and full house patients (n=39) groups previously identified. The neuropathy group (n=67) consists of subjects diagnosed as having neuropathy in the absence of any other microvascular complication (n=12), the diabetic retinopathy with neuropathy (n=16) and full house patients (n=39) groups. Also presented is the allelic frequency in uncomplicated and normal controls. Frequencies are expressed as the percentage incidence out of the total number of haplotypes detected. Comparisons were made between the haplotypes detected for T1DM subjects with complications and the uncomplicated subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5, Fishers exact test for small numbers was applied.

DR vs. Uncomplicated diabetic controls;

$\quad$ **Z-2/X.C/C**; $\chi^2 = 7.3$, p= 0.007, Pc= ns.

$\quad$ **Z+2/X.T/T**; $\chi^2 = 5.4$, p= 0.02, Pc= 0.07.

DN vs. Uncomplicated diabetic controls;

$\quad$ **Z-2/X.C/C**; $\chi^2 = 9.5$, p= 0.002, Pc= ns.

DNU vs. Uncomplicated diabetic controls;

$\quad$ **Z-2/X.C/C**; $\chi^2 = 4.7$, p= 0.03, Pc= ns, Fishers = 0.03.

$\quad$ **X/X.C/C**; $\chi^2 = 4.4$, p= 0.04, Pc= ns, Fishers = 0.04.

Combined genotype analysis of the 5'ALR2/A(+11842)C polymorphisms in T1DM subjects of British Caucasoid origin

Tables 61-64 present the frequencies of the 5'ALR2/A(+11842)C genotypes in T1DM subjects and normal controls of British Caucasoid origin. Table 61 presents the frequencies in the T1DM vs. the normal controls, and no significant correlation's were found between the two groups by the χ^2 test, or Fishers exact test for small numbers. Table 62 presents the 5'ALR2/A(+11842)C genotypes in T1DM subjects with DN/DR/DNU vs. uncomplicated DC group. The Z+2/X.C/A genotype was found to be significantly increased in the T1DM control group (6.7% vs. 22.7%, χ^2=12.5, p=0.0004, Pc= 0.005). Table 63 presents the 5'ALR2/A(+11842)C genotypes in T1DM subjects according to the precise onset of diabetic complications. No significant differences were found by the Chi squared test , however, Fishers exact test found an increased frequency of the Z+2/X.C/A and Z-2/Z+2.A/A combined genotypes in DC vs. DR/DNu. Table 64 presents the 5'ALR2/A(+11842)C genotypes in T1DM subjects according to the onset of nephropathy or retinopathy or neuropathy. In the case of the DR vs. uncomplicated DC groups the Z+2/X.C/A genotype was lower in the DR group (5.6% vs. 22.7%, χ^2=14.4, p= 0.0002, Pc= 0.003). In the case of the DN vs. uncomplicated DC groups the Z+2/X.C/A genotype was lower in the DN group (4.4% vs. 22.7%, χ^2=12.3, p=0.0005, Pc=0.007). In the case of the DNU vs. uncomplicated DC groups the Z+2/X.C/A genotype was lower in the DNU group (6.7% vs. 22.7%, χ^2=9.2, p=0.003, Pc=0.04).

5'ALR2/A(+11842)C genotype	T1DM Subjects	Normal Controls (NC)
n	244	120
Z-2/X.C/A	13.5 (33)	10.0 (12)
Z-2/X.C/C	4.5 (11)	1.7 (2)
Z-2/X.A/A	11.5 (28)	13.3 (16)
Z+2/X.C/A	11.1 (27)	13.3 (16)
Z+2/X.C/C	2.1 (5)	0.8 (1)
Z+2/X.A/A	13.5 (33)	8.3 (10)
Z-2/Z+2.C/A	1.6 (4)	3.3 (4)
Z-2/Z+2.C/C	0.4 (1)	- (-)
Z-2/Z+2.A/A	4.1 (10)	0.8 (1)
X/X.CA	10.2 (25)	14.2 (17)
X/X.C/C	2.9 (7)	3.3 (4)
X/X.A/A	12.3 (30)	15.8 (19)
Other	12.3 (30)	15.0 (18)

Table 61. Frequency of 5'ALR2/A(+11842)C combined genotypes in patients with T1DM of British Caucasoid origin. The combined genotype frequencies were obtained by using the gene counting method. This table presents the combined genotypes detected in the T1DM and normal control (NC) subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotype frequencies detected for T1DM and NC subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis.

No significant differences were detected between the T1DM and the normal control populations with respect to the 5'ALR2/A(+11842)C combined genotype.

5'ALR2/A(+11842)C, genotype	T1DM Nephropathy and/or Retinopathy and/or Neuropathy (DR/DN/DNu)	Uncomplicated (DC)	Normal Controls (NC)
n	178	66	120
Z-2/X.C/A	15.2	9.1	10.0
	(27)	(6)	(12)
Z-2/X.C/C	5.1	3.0	1.7
	(9)	(2)	(2)
Z-2/X.A/A	13.5	6.1	13.3
	(24)	(4)	(16)
Z+2/X.C/A	6.7	22.7	13.3
	(12)	(15)	(16)
Z+2/X.C/C	1.1	4.6	0.83
	(2)	(3)	(1)
Z+2/X.A/A	13.5	13.6	8.3
	(24)	(9)	(10)
Z-2/Z+2.C/A	1.7	1.5	3.3
	(3)	(1)	(4)
Z-2/Z+2.C/C	0.6	-	-
	(1)	-	(-)
Z-2/Z+2.A/A	4.5	3.0	0.8
	(8)	(2)	(1)
X/X.CA	11.8	6.1	14.2
	(21)	(4)	(17)
X/X.C/C	1.7	6.1	3.3
	(3)	(4)	(4)
X/X.A/A	14.0	7.6	15.8
	(25)	(5)	(19)
Other	10.7	16.7	15.0
	(19)	(11)	(18)

Table 62. Frequency of 5'ALR2/ A(+11842)C combined genotypes in patients with T1DM of British Caucasoid origin. The combined genotype frequencies were obtained by using the gene counting method. This table presents the combined genotypes detected in the T1DM subjects with microvascular disease (nephropathy, retinopathy, neuropathy) compared to uncomplicated and normal control (NC) subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotypes detected for T1DM and DC subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5, Fishers exact test for small numbers was applied.

DR/DN/DNU vs. Uncomplicated diabetic controls;
   Z+2/X.C/A; χ^2= 12.5, p= 0.0004, Pc= 0.005*.

5'ALR2/ A(+11842)C genotype	[1]Diabetic Retinopathy	[2]Diabetic Nephropathy and Retinopathy	[3]Diabetic Retinopathy and Neuropathy	[4]Diabetic Neuropathy	[5]Full House Patients	Uncomplicated (DC)	Normal Controls (NC)
n	44	30	24	18	62	66	120
Z-2/X.C/A	13.6 (6)	23.3 (7)	4.2 (1)	16.7 (3)	16.1 (10)	9.1 (6)	10.0 (12)
Z-2/X.C/C	2.3 (1)	6.7 (2)	8.3 (2)	- (-)	6.5 (4)	3.0 (2)	1.7 (2)
Z-2/X.A/A	13.6 (6)	10.0 (3)	12.5 (3)	22.2 (4)	12.9 (8)	6.1 (4)	13.3 (16)
Z+2/X.C/A	9.1 (4)	3.3 (1)	4.2 (1)	16.7 (3)	4.8 (3)	22.7 (15)	13.3 (16)
Z+2/X.C/C	2.3 (1)	- (-)	- (-)	- (-)	1.6 (1)	4.6 (3)	0.8 (1)
Z+2/X.A/A	18.2 (8)	6.7 (2)	20.8 (5)	16.7 (3)	9.7 (6)	13.6 (9)	8.3 (10)
Z-2/Z+2.C/A	- (-)	3.3 (1)	- (-)	- (-)	3.2 (2)	1.5 (1)	3.3 (4)
Z-2/Z+2.C/C	2.3 (1)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)
Z-2/Z+2.A/A	4.6 (2)	- (-)	16.7 (4)	- (-)	3.2 (2)	3.0 (2)	0.83 (1)
X/X.CA	11.4 (5)	16.7 (5)	8.3 (2)	5.6 (1)	12.9 (8)	6.1 (4)	14.2 (17)
X/X.C/C	4.6 (2)	- (-)	- (-)	- (-)	1.6 (1)	6.1 (4)	3.3 (4)
X/X.A/A	9.1 (4)	16.7 (5)	12.5 (3)	16.7 (3)	16.1 (10)	7.6 (5)	15.8 (19)
Other	9.1 (4)	13.3 (4)	12.5 (3)	5.6 (1)	11.3 (7)	16.7 (11)	15.0 (18)

Table 63. Frequency of 5'ALR2/ A(+11842)C combined genotypes in patients with T1DM of British Caucasoid origin. The combined frequencies were obtained by using the gene counting method. This table presents the genotypes detected in the T1DM subjects with retinopathy (DR), nephropathy and retinopathy (DN/DR), retinopathy and neuropathy (DN/DNu), full house complications (FHC). Also shown are the T1DM diabetic controls (DC) and normal control (NC) subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotypes detected for T1DM complication groups and the uncomplicated DC subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5, Fishers exact test for small numbers was applied.

[2] vs. Uncomplicated diabetic controls;
      Z+2/X.C/A; χ^2=5.59, p=0.02, Pc=ns.

[3] vs. Uncomplicated diabetic controls;
      Z+2/X.C/A; χ^2=4.15, p=0.04, Pc=ns, Fishers=0.03.
      Z-2/Z+2.A/A; χ^2=5.26, p=0.02, Pc=ns, Fishers = 0.04.

[4] vs. Uncomplicated diabetic controls;

   Z-2/X.A/A; χ^2=4.29, p=0.04, Pc=ns, 0.06.

[5] vs. Uncomplicated diabetic controls;

   Z+2/X.C/A; χ^2=8.47, p=0.004, Pc=ns.

5'ALR2/A(+11842)C genotype	Diabetic Retinopathy (DR)	Diabetic Nephropathy (DN)	Diabetic Neuropathy (DNu)	Uncomplicated (DC)	Normal Controls (NC)
n	160	92	104	66	120
Z-2/X.C/A	15.0	18.5	13.5	9.1	10.0
	(24)	(17)	(14)	(6)	(12)
Z-2/X.C/C	5.6	6.5	5.8	3.0	1.7
	(9)	(6)	(6)	(2)	(2)
Z-2/X.A/A	12.5	11.96	14.4	6.1	13.3
	(20)	(11)	(15)	(4)	(16)
Z+2/X.C/A	5.6	4.4	6.7	22.7	13.3
	(9)	(4)	(7)	(15)	(16)
Z+2/X.C/C	1.3	1.1	0.96	4.6	0.8
	(2)	(1)	(1)	(3)	(1)
Z+2/X.A/A	13.1	8.7	13.5	13.6	8.3
	(21)	(8)	(14)	(9)	(10)
Z-2/Z+2.C/A	1.9	3.3	1.9	1.5	3.3
	(3)	(3)	(2)	(1)	(4)
Z-2/Z+2.C/C	0.6	-	-	-	-
	(1)	(-)	(-)	(-)	(-)
Z-2/Z+2.A/A	5.0	2.2	5.8	3.0	0.8
	(8)	(2)	(6)	(2)	(1)
X/X.CA	12.5	14.1	10.6	6.1	14.2
	(20)	(13)	(11)	(4)	(17)
X/X.C/C	1.9	1.1	0.96	6.1	3.3
	(3)	(1)	(1)	(4)	(4)
X/X.A/A	13.8	16.3	15.4	7.6	15.8
	(22)	(15)	(16)	(5)	(19)
Other	11.3	11.96	10.6	16.7	15.0
	(18)	(11)	(11)	(11)	(18)

Table 64. Frequency of 5'ALR2/ A(+11842)C combined genotypes in patients with T1DM of British Caucasoid origin. The combined genotype frequencies were obtained by using the gene counting method. X= neither Z-2 nor Z+2 allele. Subjects with microvascular disease are classified in accordance to onset of retinopathy, nephropathy or neuropathy. The nephropathy group (n=92) is an amalgamation of the nephropathy and retinopathy (n=30) and full house subjects (n=62) groups previously identified. The retinopathy group (n=160) is an amalgamation of the retinopathy (n=44), nephropathy with retinopathy (n=30), retinopathy and neuropathy (n=24) and full house patients (n=62) groups previously identified. The neuropathy group (n=104) consists of subjects diagnosed as having neuropathy in the absence of any other microvascular complication (n=18), the diabetic retinopathy with neuropathy (n=24) and full house patients (n=62) groups. Also presented is the allelic frequency in uncomplicated and normal controls. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotypes detected for T1DM subjects with complications and uncomplicated subjects using the χ^2 test and 2x2 contingency tables. n= number of alleles detected, number of subjects studied is shown in parenthesis. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5, Fishers exact test for small numbers was applied.

DR vs. DC;   Z+2/X.C/A; χ^2= 14.4, p= 0.0002, Pc= 0.003.
DN vs. DC;   Z+2/X.C/A; χ^2= 12.3, p= 0.0005, Pc= 0.007.
DNU vs. DC; Z+2/X.C/A; χ^2= 9.2, p= 0.003, Pc= 0.04.

Combined genotype analysis of the C(-106)T/A(+11842)C polymorphism's in T1DM subjects of British Caucasoid origin

Tables 65-68 present the C(-106)T/A(+11842)C combined genotypes observed in the T1DM subjects and normal controls of British Caucasoid origin. Table 65 compares the T1DM subjects with the normal control subjects frequencies. There was a significant increase in the T/T.A/C combined genotype in the normal controls compared to the T1DM subjects (7.5% vs. 0.6%, χ^2=9.07, p=0.003, Pc=0.03). Table 66 compares the T1DM DR/DN/DNU subjects and the diabetic controls. Following correction no significant associations were found. Table 67 compares the uncomplicated DC group with the T1DM subjects according to the precise onset of complications. Following correction no significant associations were found. Table 68 compares the T1DM subjects with the uncomplicated DC subjects according to the onset on nephropathy or retinopathy or neuropathy. Following correction no significant differences were found, however in all groups the C/C.A/A was borderline of significance where there was a higher frequency in the complication groups compared to the uncomplicated DC group.

C(-106)T/A(+11842)C genotype	T1DM Subjects	Normal Controls (NC)
n	155	120
C/C – A/C	21.3 (33)	10.8 (13)
C/C – A/A	16.1 (25)	11.7 (14)
C/C – C/C	2.6 (4)	5.0 (6)
T/T – A/C	0.6 (1)	7.5 (9)
T/T – A/A	4.5 (7)	7.5 (9)
T/T – C/C	- (-)	- (-)
C/T – A/C	25.2 (39)	27.5 (33)
C/T – A/A	26.5 (41)	28.3 (34)
C/T – C/C	3.2 (5)	1.7 (2)
C(-106)T		
C allele present (%)	75.5 (117)	85.0 (102)
T allele present (%)	60.0 (93)	72.5 (87)
A(+11842)C		
C allele present (%)	52.9 (82)	52.5 (63)
A allele present (%)	94.2 (146)	93.3 (112)

Table 65. Frequency of C(-106)T/ A(+11842)C combined genotypes in patients with T1DM of British Caucasoid origin. The combined genotype frequencies were obtained by using the gene counting method. This table presents the combined genotypes detected in the T1DM and normal control (NC) subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotype frequencies detected for T1DM and NC subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5, Fishers exact test for small numbers was applied.

T1DM vs. NC;

C/C.A/C; χ^2= 5.3, p= 0.02, Pc= ns.
T/T.A/C; χ^2= 9.07, p= 0.003, Pc= 0.03.

C(-106)T/A(+11842)C genotype	T1DM Nephropathy and/or Retinopathy and/or Neuropathy (DR/DN/DNu)	Uncomplicated (DC)	Normal Controls (NC)
n	116	39	120
C/C – A/C	23.3 (27)	15.4 (6)	10.8 (13)
C/C – A/A	19.8 (23)	5.1 (2)	11.7 (14)
C/C – C/C	2.6 (3)	2.6 (1)	5.0 (6)
T/T – A/C	- (-)	2.6 (1)	7.5 (9)
T/T – A/A	3.5 (4)	7.7 (3)	7.5 (9)
T/T – C/C	- (-)	- (-)	- (-)
C/T – A/C	21.6 (25)	35.9 (14)	27.5 (33)
C/T – A/A	26.7 (31)	25.6 (10)	28.3 (34)
C/T – C/C	2.6 (3)	5.1 (2)	1.7 (2)
C(-106)T			
C allele present (%)	96.6 (112)	89.7 (35)	85.0 (102)
T allele present (%)	54.3 (63)	76.9 (30)	72.5 (87)
A(+11842)C			
C allele present (%)	50.0 (58)	61.5 (24)	52.5 (63)
A allele present (%)	94.8 (110)	92.3 (36)	93.3 (112)

Table 66. Frequency of C(-106)T/ A(+11842)C combined genotypes in patients with T1DM of British Caucasoid origin. The combined genotype frequencies were obtained by using the gene counting method. This table presents the combined genotypes detected in the T1DM subjects with microvascular disease (nephropathy, retinopathy, neuropathy) compared to T1DM uncomplicated and normal control (NC) subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotypes detected for T1DM and NC subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis. Only significant differences by χ^2 and correction are shown.

DR/DN/DNU vs. Uncomplicated diabetic controls;
· C/C.A/A; χ^2= 4.7, p= 0.03, Pc= ns.

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C(-106)T/ A(+11842)C genotype	Diabetic Retinopathy	Diabetic Nephropathy and Retinopathy	Diabetic Retinopathy and Neuropathy	Diabetic Neuropathy	Full House Patients	Uncomplicated (DC)	Normal Controls (NC)
n	23	26	16	12	39	39	120
C/C – A/C	21.7	26.9	18.8	8.3	28.2	15.4	10.8
	(5)	(7)	(3)	(1)	(11)	(6)	(13)
C/C – A/A	17.4	19.2	25.0	16.7	20.5	5.1	11.7
	(4)	(5)	(4)	(2)	(8)	(2)	(14)
C/C – C/C	-	3.9	6.3	-	2.6	2.6	5.0
	(-)	(1)	(1)	(-)	(1)	(1)	(6)
T/T – A/C	-	-	-	-	-	2.6	7.5
	(-)	(-)	(-)	(-)	(-)	(1)	(9)
T/T – A/A	4.4	3.9	-	8.3	2.6	7.7	7.5
	(1)	(1)	(-)	(1)	(1)	(3)	(9)
T/T – C/C	-	-	-	-	-	-	-
	(-)	(-)	(-)	(-)	(-)	(-)	(-)
C/T – A/C	17.4	23.1	12.5	41.7	20.5	35.9	27.5
	(4)	(6)	(2)	(5)	(8)	(14)	(33)
C/T – A/A	30.4	19.2	37.5	25.0	25.6	25.6	28.3
	(7)	(5)	(6)	(3)	(10)	(10)	(34)
C/T – C/C	8.7	3.9	-	-	-	5.1	1.7
	(2)	(1)	(-)	(-)	(-)	(2)	(2)
C(-106)T							
C allele present (%)	95.7	96.2	100.0	91.7	97.4	89.7	85.0
	(22)	(25)	(16)	(11)	(38)	(35)	(102)
T allele present (%)	60.9	50.0	50.0	75.0	48.7	76.9	72.5
	(14)	(13)	(8)	(9)	(19)	(30)	(87)
A(+11842)C							
C allele present (%)	47.8	57.7	37.5	50.0	51.3	61.5	52.5
	(11)	(15)	(6)	(6)	(20)	(24)	(63)
A allele present (%)	91.3	92.3	93.8	100.0	97.4	92.3	93.3
	(21)	(24)	(15)	(12)	(38)	(36)	(112)

Table 67. Frequency of C(-106)T/ A(+11842)C combined genotypes in patients with T1DM of British Caucasoid origin. The combined frequencies were obtained by using the gene counting method. This table presents the genotypes detected in the T1DM subjects with retinopathy (DR), nephropathy and retinopathy (DN/DR), retinopathy and neuropathy (DN/DNu), full house complications (FHC). Also shown are the T1DM uncomplicated diabetic controls (DC) and normal control (NC) subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotypes detected for T1DM complication groups and the DC subjects using the χ^2 test and 2x2 contingency tables. n= number of genotypes detected, number of subjects studied is shown in parenthesis. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5, Fishers exact test for small numbers was applied.

DN/DR vs. Uncomplicated diabetic controls;
　　C/C.A/A; χ^2= 4.6, p= 0.03, Pc= ns.

No other significant differences were detected.

C(-106)T/A(+11842)C genotype	Diabetic Retinopathy (DR)	Diabetic Nephropathy (DN)	Diabetic Neuropathy (DNu)	Uncomplicated (DC)	Normal Controls (NC)
n	104	77	67	39	120
C/C – A/C	25.0	24.7	22.4	15.4	10.8
	(26)	(19)	(15)	(6)	(13)
C/C – A/A	20.2	19.5	20.9	5.1	11.7
	(21)	(15)	(14)	(2)	(14)
C/C – C/C	2.9	2.6	2.9	2.6	5.0
	(3)	(2)	(2)	(1)	(6)
T/T – A/C	-	-	-	2.6	7.5
	(-)	(-)	(-)	(1)	(9)
T/T – A/A	2.9	3.9	2.9	7.7	7.5
	(3)	(3)	(2)	(3)	(9)
T/T – C/C	-	-	-	-	-
	(-)	(-)	(-)	(-)	(-)
C/T – A/C	19.2	24.7	22.4	35.9	27.5
	(20)	(19)	(15)	(14)	(33)
C/T – A/A	26.9	23.4	28.4	25.6	28.3
	(28)	(18)	(19)	(10)	(34)
C/T – C/C	2.9	1.3	-	5.1	1.7
	(3)	(1)	(-)	(2)	(2)
C(-106)T					
C allele present	97.1	96.1	97.0	89.7	85.0
(%)	(101)	(74)	(65)	(35)	(102)
T allele present	51.9	53.3	53.7	76.9	72.5
(%)	(54)	(41)	(36)	(30)	(87)
A(+11842)C					
C allele present	50.0	53.3	47.8	61.5	52.5
(%)	(52)	(41)	(32)	(24)	(63)
A allele present	50.0	96.1	97.0	92.3	93.3
(%)	(52)	(74)	(65)	(36)	(112)

Table 68. Frequency of C(-106)T/ A(+11842)C combined genotypes in patients with T1DM of British Caucasoid origin. The combined genotype frequencies were obtained by using the gene counting method X= neither Z-2 nor Z+2 allele. Subjects with microvascular disease are classified in accordance to onset of retinopathy, nephropathy or neuropathy. The nephropathy group (n=77) is an amalgamation of the nephropathy and retinopathy (n=26) and full house subjects (n=39) groups previously identified. The retinopathy group (n=104) is an amalgamation of the retinopathy (n=23), nephropathy with retinopathy (n=26), retinopathy and neuropathy (n=16) and full house patients (n=39) groups previously identified. The neuropathy group (n=67) consists of subjects diagnosed as having neuropathy in the absence of any other microvascular complication (n=12), the diabetic retinopathy with neuropathy (n=16) and full house patients (n=39) groups. Also presented is the allelic frequency in uncomplicated and normal controls. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. Comparisons were made between the genotypes detected for T1DM and NC subjects using the χ^2 test and 2x2 contingency tables. n= number of alleles detected, number of subjects studied is shown in parenthesis. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5, Fishers exact test for small numbers was applied.

DR vs. Uncomplicated diabetic controls;
      C/C.A/A; χ^2= 4.8, p= 0.03, Pc= ns.
      C/T.A/C; χ^2= 4.4, p= 0.04, Pc= ns.

DN vs. Uncomplicated diabetic controls;
      C/C.A/A; χ^2= 4.3, p= 0.04, Pc= ns.

DNU vs. Uncomplicated diabetic controls;
      C/C.A/A; χ^2= 4.8, p= 0.03, Pc= ns.

Aldose Reductase Gene Study

Binding activity of OREBP to ALR2 promoter osmotic response elements by electrophoretic mobility shift assay in T1DM subjects and healthy adult controls.

Binding activity of OREBP to OREA,B and C of the ALR2 promoter region

Analysis of the binding activity of OREBP to the ORE sites within the aldose reductase gene promoter was performed by Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from peripheral blood mononuclear cells from whole blood donated from T1DM subjects and normal control subjects cultured under normoglycemic (11mM/L) and hyperglycemic (28mM/L) conditions. Within the T1DM subject group studied 6 were diagnosed as having diabetic nephropathy and retinopathy, 6 were full house patients and 3 were uncomplicated diabetic controls. Results were also obtained from 13 normal control subjects.

Nuclear extracts containing 5μg protein (determined by Coomassie protein assay) were incubated with a radio-labelled probe for 20 minutes at room temperature in appropriate buffer. Samples were then loaded onto a 4% bis-acrylamide urea gel and electrophoresed at 100V for 3-4 hours (refer to materials and methods). The gel was exposed to X-Omat photographic paper and bands were analysed and quantified using a phosphoimager (BioRad) with multi-analyst software (figure 27). The activation of OREBP binding was determined as a fold increase between the 11mM and 28mM glucose exposed samples, therefore above the level obtained under normal conditions.

Results are expressed a means ± SE. Statistical significance of comparisons were made between groups using the ANOVA test to detect variance and a P-value of the F-test, greater or equal to 0.05 was considered significant. The Student t test was also applied between groups.

A

D-glucose (mM);	11	28	11	28	11	28	11	28

Activation (fold); 1   1.28   1   1.005   1   2.16   1   1.69

B
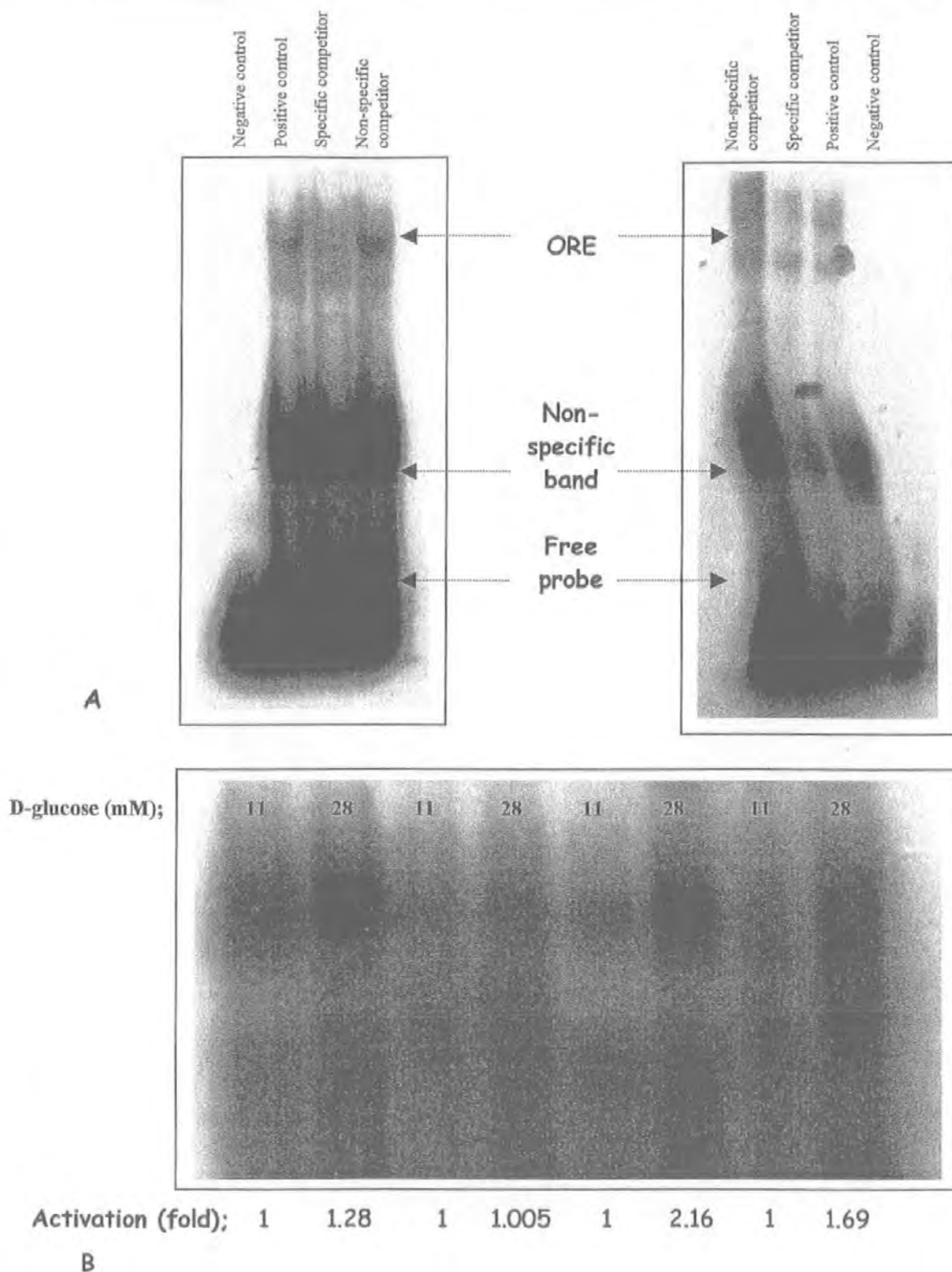
Figure 27. Electrophoretic Mobility Shift Assay analysis of binding activity to ORE of ALR2 promoter.

A. Competition experiments to demonstrate the specificity of the ORE band in Jurkat cells, the OREB and OREC competition experiments are shown here.

B This autoradiograph shows the expression of OREBPC in 4 patients with diabetic nephropathy. The level of OREBP binding was increased in samples stimulated with 28mM D-glucose and the increase is expressed as fold increases.

Binding activity of OREBP to OREA, B and C in T1DM subjects and normal controls of British Caucasoid origin

Figure 27 shows the EMSA analysis of the binding activity to Osmotic Response Elements of the aldose reductase promoter region. The fold increases of the ORE binding protein activity for OREA, B and C with respect to the onset of diabetic complications are shown in tables 69 and 70. No data was available for the diabetic control subjects for the OREA region. The results are expressed as fold increases in ORE/OREBP above the level obtained under normal conditions. In the T1DM subjects with microvascular complications the mean fold increase for OREA is 1.38 ($\pm$ 0.43), for OREB is 2.23 ($\pm$1.98), and for OREC is 2.23 ($\pm$3.12). In contrast to this, the uncomplicated diabetic controls show a slight reduction in ORE/OREBP compared to the level obtained under normal conditions, for OREB 0.78 ($\pm$0.22), and for OREC 0.89 ($\pm$0.22). The normal control subjects also show an increase in the ORE/OREBP above the level obtained under normal conditions for OREA and OREB. With respect to OREA the mean increase was 1.4 ($\pm$0.47), OREB the mean increase was 1.10 ($\pm$0.41), and for OREC the level was decreased 0.93 ($\pm$0.93). The differences in response of ORE/OREBP between T1DM with complications and the diabetic controls showed statistical significance following the ANOVA test and the P value of the F-test. With respect to the OREB the mean averages were 2.2 vs. 0.8 (P=0.02), and OREC the mean averages were 2.2 vs. 0.9 (P=0.01). The differences in response of ORE/OREBP between T1DM with complications and normal controls were also determined. With respect to OREA no significant difference was detected (1.4 vs. 1.4, P=0.9). With respect to OREB and OREC however there were significant differences detected, OREB 2.2 vs. 1.1, P=<0.001, OREC 2.2 vs. 0.9, P=<0.001.

Table 70 presents the fold increases in the ORE/OREBP levels above the level achieved under normal conditions with respect to the exact complications of diabetes. Two groups were studied, firstly, those with diabetic nephropathy and retinopathy, and secondly those with full house complications. In the case of the DN/DR group vs. uncomplicated DC

group the OREC showed a significant fold increase (1.4 vs. 0.9, P=<0.0001). For the full house patients the OREB fold increase was higher in the FHP group than the diabetic controls (3.0 vs. 0.8, P=0.015), and the normal controls (3.08 vs. 1.1, P= <0.0001). For the Full house patients the OREC fold increase is also higher in the FHP group than the uncomplicated DC (2.9 vs. 0.9, P=0.006), and the normal controls (2.9 vs. 0.9, P=<0.0001).

The data is also presented in box and whisker plots in figures 28 and 29. It is apparent from the box and whisker plots, however, that there is a large standard error with respect to the variation in binding activities between subjects within each of the groups studied. Although the mean averages are significantly different, there is significant overlap between the subject groups. It is therefore difficult to draw confident conclusions from this data, and a larger group now needs to be analysed in order to reduce the standard errors found here.

Glucose concentration	T1DM with microvascular complications			Uncomplicated Diabetic Controls			Normal controls		
	OREA n=9	OREB n=12	OREC n=11	OREA n=0	OREB n=3	OREC n=3	OREA n=13	OREB n=13	OREC n=12
Hyperglycae mia 28mM	1.38 ± 0.43 [a]	2.23 ±1.98 [b]	2.23 ± 3.12 [c]	-	0.78 ± 0.22	0.89 +0.22	1.40 ± 0.47	1.10 ± 0.41	0.93 ± 0.19

Table 69. Overall mean levels of OREBP binding activity to ALR2 OREA, OREB and OREC in T1DM subjects according to onset of microvascular disease, and normal adult controls. The results are expressed as a fold increases in ORE/OREBP activity above the level obtained under normal conditions (11mM). The results are expressed as mean values ± standard error.

a vs. uncomplicated, P = no data available, and vs. normal controls, $P = 0.85$
b vs. uncomplicated, $P = 0.02^*$, and vs. normal controls, $P = 0.000004^*$
c vs. uncomplicated, $P = 0.01^*$, and vs. normal controls, $P = <0.00001^*$

Glucose concentration	Jurkats			Diabetic nephropathy and retinopathy			Diabetic Full House Patients			Uncomplicated Diabetic Controls			Normal controls		
	OREA n=4	OREB n=4	OREC n=4	OREA n=5	OREB n=6	OREC n=5	OREA n=4	OREB n=6	OREC n=6	OREA n=0	OREB n=3	OREC n=3	OREA n=13	OREB n=13	OREC n=12
Hyper-glycaemia 28mM	0.61 ± 0.28	1.62 ± 0.98	1.21 ± 0.56	1.48 [a] ± 0.52	1.38 [b] ± 0.77	1.43 [c] ± 0.89	1.26 [d] ± 0.33	3.08 [e] ± 2.52	2.99 [f] ± 4.19	-	0.78 ± 0.22	0.89 ±0.22	1.40 ± 0.47	1.10 ± 0.41	0.93 ± 0.19

Table 70. Overall mean levels of OREBP binding activity to ALR2 OREA, OREB and OREC in Jurkat cells and T1DM subjects according to onset of microvascular disease, and normal adult controls. The results are expressed as a fold increases in ORE/OREBP activity above the level obtained under normal conditions (11mM). The results are expressed as mean values $\pm$ standard error.

a vs. uncomplicated, P = no data available, and vs. normal controls, P = 0.7
b vs. uncomplicated, P = 0.17, and vs. normal controls, P = 0.096
c vs. uncomplicated, P = 0.12, and vs. normal controls, P = 0.00009*
d vs. uncomplicated, P = no data available, and vs. normal controls, P = 0.62
e vs. uncomplicated, P = 0.015*, and vs. normal controls, P = 0.000001*
f vs. uncomplicated, P = 0.006*, and vs. normal controls, P = < 0.00001*
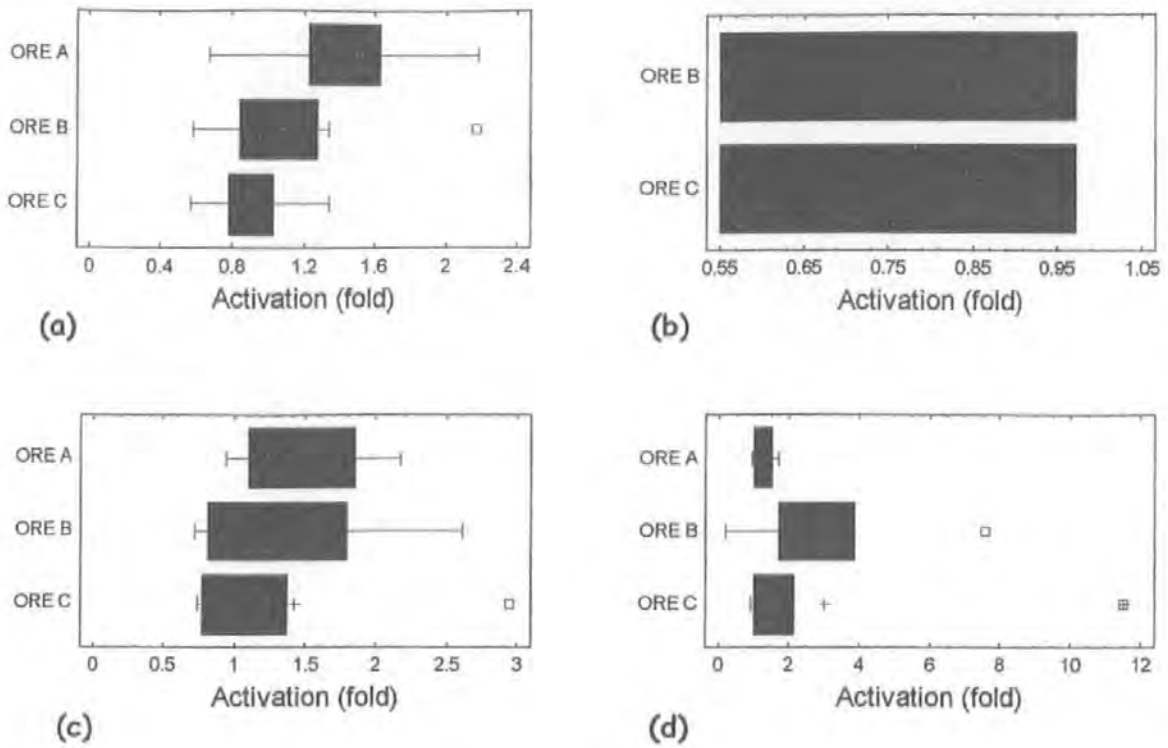
Figure 28.Box and whisker plots comparing the activation (fold) in the ORE A,B and C regions investigated according to onset of diabetic microvascular disease.

a) normal controls
b) uncomplicated diabetic controls
c) diabetic nephropathy with retinopathy
d) full house patients
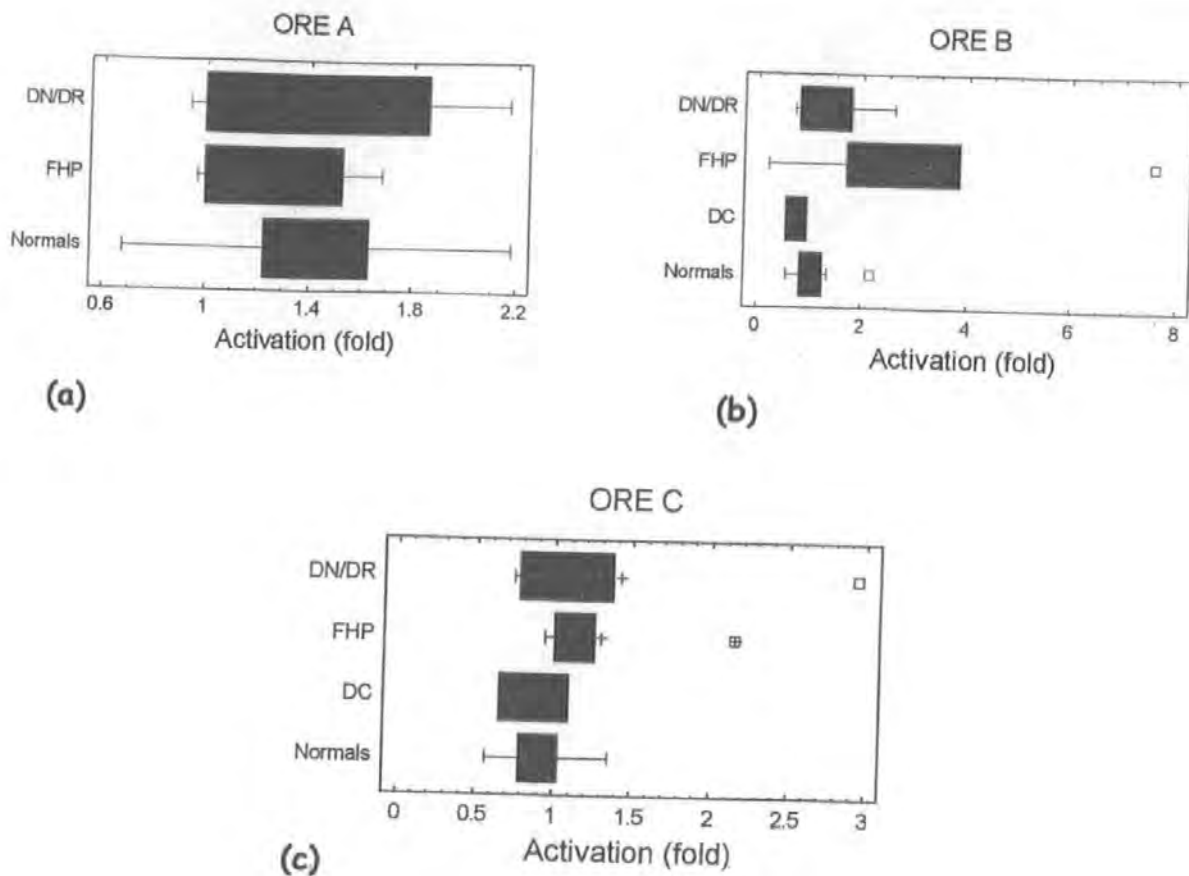
Figure 29. Box and whisker plots comparing the OREBP/ORE binding activity detected between groups according to ORE binding site investigated.

(a) Activation (fold) at ORE A
(b) Activation (fold) at ORE B
(c) Activation (fold) at ORE C

D17S934 Hypertension-Linked Gene Study

Associations of a polymorphism at D17S934 locus situated 18cM proximal to the ACE gene and the microvascular complications of T1DM and T2DM

Polymorphism at the D17S934 region

A microsatellite polymorphism located 18cM proximal to the ACE gene on chromosome 17q was investigated. The study incorporated two subject groups for which the case control study utilised a population of T1DM and normal control subjects of British Caucasoid origin, and a population of T2DM and normal control subjects of Southern Indian/Dravidian origin. A single microsatellite polymorphism was studied, a (CA)n repeat microsatellite polymorphic marker located 18cM proximal to the ACE gene. The results of this study are presented in tabular form in the following section. For the case-control studies the data was initially analysed for allelic and genotypic frequencies between subjects with diabetes and subjects without diabetes, and then with regard to subjects onset of diabetic microvascular disease. The data was then amalgamated and subject haplotype analysis was carried out with respect to diabetic complications. All subjects previously genotyped for the 5'ALR2 polymorphism were genotyped for the D17S934 polymorphism for ease of comparability.

Chromosome 17q EC 3.4.15.1

Angiotensin Converting Ezyme loci

D17S934/Hs256VB9/AFM256vb9
Essential hypertension susceptibility
loci

17q23

18 cM

17q21-22
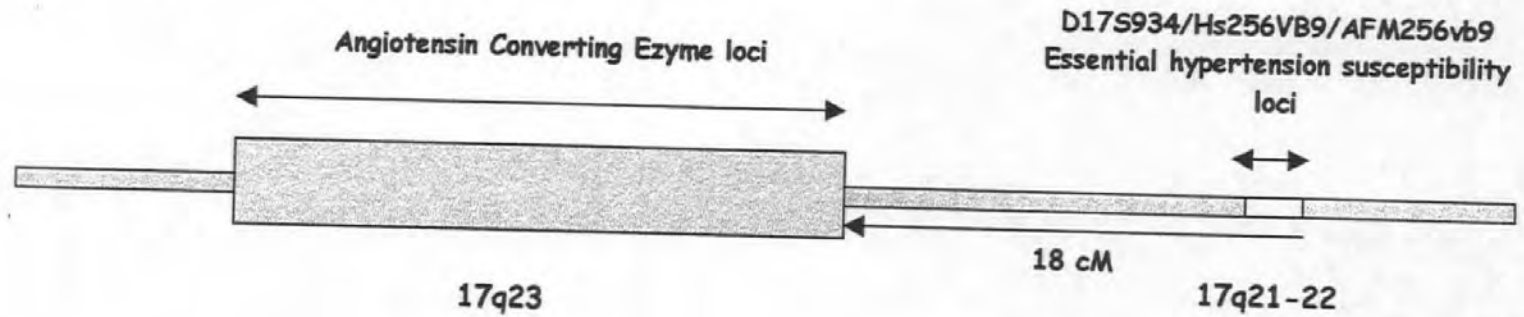
Figure 30. Diagrammatic representation of the polymorphic region studied within the region proximal to the ACE gene located on chromosome 17q

D17S934 (CA)n microsatellite polymorphic marker

The (CA)n dinucleotide repeat microsatellite situated 18cM proximal to the ACE gene was investigated. Polymerase chain reaction was carried out to amplify the region upstream of the ACE gene containing the (CA)n repeat microsatellite as described in chapter 3. Amplification products (6μl) mixed with stop solution (Amersham, UK) were separated on a 6% polyacrylamide gel at 1900V for 3 hours, and alleles were revealed by autoradiography (figure 31), and typed using the Fluor-s multi-imaging system (BioRad). The size of the bands was checked by running a [32P] ATP radio-labelled molecular weight marker alongside the subjects. The (CA)n microsatellite was investigated in T1DM subjects and normal controls of British Caucasoid origin and T2DM subjects and non-diabetic controls of Southern Indian/Dravidian origin. In these studies we were able to find 11 alleles which differed by the number of integral repeats. Subjects were assigned their allele and genotype according to the number of CA repeats which were identified (table 71).

(a)

Lane: 1 2 3 4 5 6 7 8

(b)
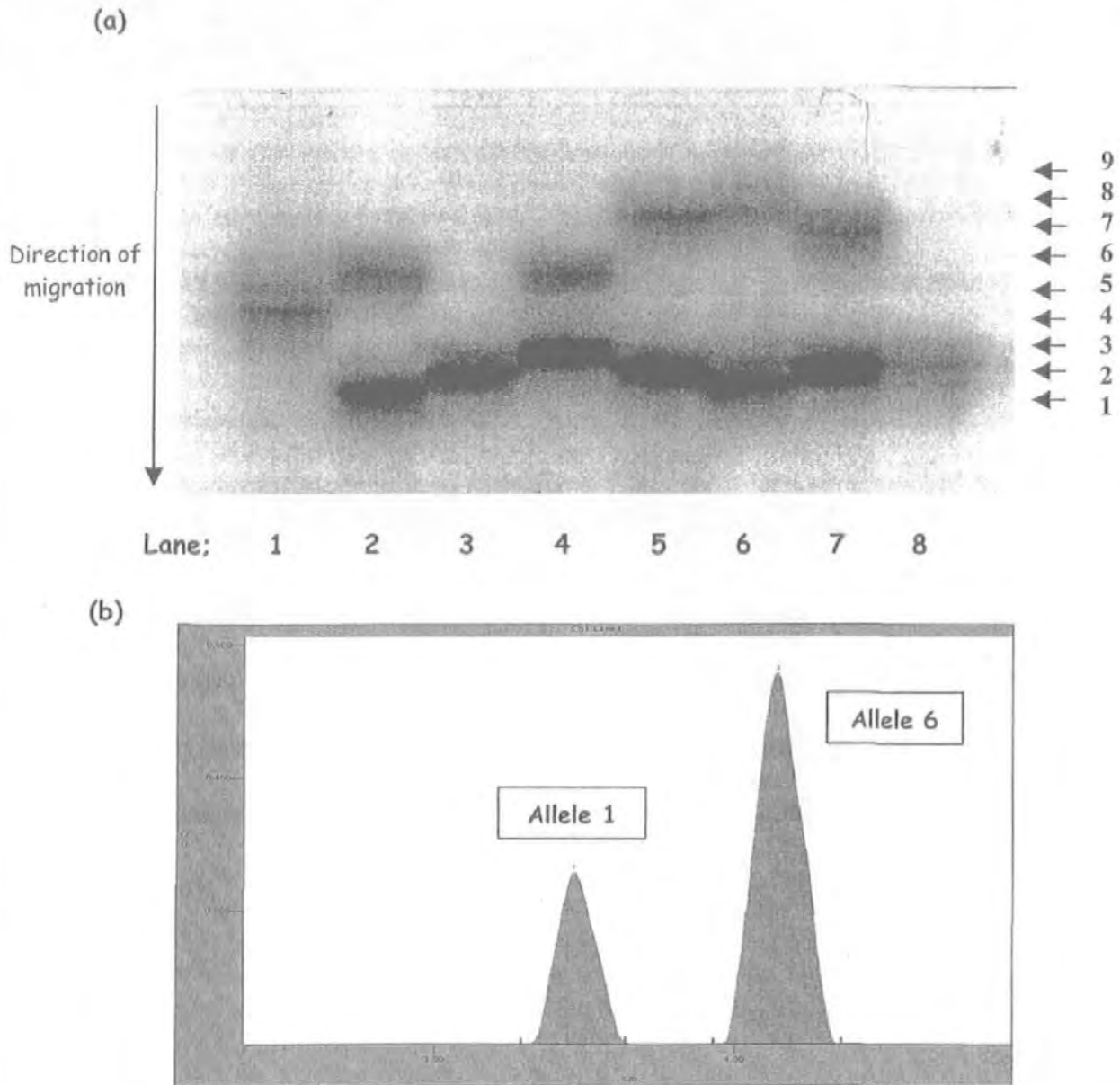
Figure 31. D17S934 microsatellite autoradiograph, and (b) quantification of bands using Fluor-s multi-imaging software.

(a)   D17S934 microsatellite autoradiograph. Nine alleles are identified in this autoradiograph and allocated genotypes for each of the subjects shown in lanes 1-8 are as follows:

Lane 1: 5.5
Lane 2: 1.6
Lane 3: 2.2
Lane 4: 3.6
Lane 5: 2.8
Lane 6: 1.8
Lane 7: 2.7
Lane 8: 2.2

(b) Image analysis quantification output for sample 2 in autoradiograph shown above. The bands appear as peaks in the graph which correspond to D17S934 alleles, shadow bands were also detected.

Allele	PCR product size (bp)	CA repeats
1	181	16
2	183	17
3	185	18
4	187	19
5	189	20
6	191	21
7	193	22
8	195	23
9	197	24
10	199	25
12	201	26

Table 71. Allele sizes in base pairs for the 11 different CA repeat polymorphisms identified. This table shows the size of the PCR amplified region corresponding to each allele, along with the number of dinucleotide (CA)n repeats.

D17S934 (CA)n microsatellite marker in British Caucasoid T1DM subjects and normal controls

The (CA)n microsatellite marker in the proximal region of the ACE gene was investigated using 244 subjects with T1DM of British Caucasoid origin as well as a collection of 120 British Caucasoid normal controls (clinical demographics shown in table 6). Within the T1DM population studies there were 66 uncomplicated diabetic controls, 62 full house patients, 18 with diabetic neuropathy, 24 with diabetic retinopathy and neuropathy, 30 with diabetic nephropathy and retinopathy and 44 with diabetic retinopathy.

The frequency of the (CA)n alleles in both T1DM and normal control populations is shown in table 72. There were 11 (CA)n alleles detected at the 5'ALR2 locus in the British Caucasoid T1DM and control population; 1,2,3,4,5,6,7,8,9,10 and 12. Eleven alleles gave rise to 66 possible genotypes of which 38 were detected in this study population. In the analysis of allelic frequencies homozygotes were counted as two alleles, and alleles and genotypes that were not detected are not shown. Tables show the percentage frequency of detected alleles and genotypes with actual numbers detected shown in parenthesis. Possible associations were investigated with respect to allelic frequency and genotype and comparisons were made between groups using the χ^2 test and 2x2 contingency tables.

D17S934 (CA)n allelic frequencies in British Caucasoid T1DM and normal control subjects

The frequency of the D17S934 alleles in T1DM British Caucasoid subjects is shown in tables 72 to 75. Table 72 shows the percentage frequency of the detected D17S934 (CA)n alleles in the total T1DM population studied compared to the normal control population studied. No significant differences were found in the frequencies of alleles between these two groups. In the case of allele 1 there was a higher frequency in the T1DM subjects compared to the normal controls (12.5% vs. 7.5%, $\chi^2 = 4.2$, p= 0.04, Pc= ns). In the case of allele 2 there was also a higher frequency in the T1DM subjects compared to the NC (28.7% vs. 19.9%, $\chi^2 = 6.34$, p= 0.01, Pc= ns). In the case of allele 6 there was a lower frequency in the T1DM subjects compared to the NC (7.9% vs. 13.3%, $\chi^2 = 5.2$, p= 0.02, Pc= ns). In the case of allele 8 there was a lower incidence in the T1DM group compared to the NC (1.6% vs. 4.6%, $\chi^2 = 6.3$, p= 0.02, Pc= ns).

Table 73 compares the percentage frequencies of alleles detected in the T1DM subjects with DN/DR/DNU and the uncomplicated DC group. No significant differences were found. Table 74. presents the frequency of the detected D17S934 microsatellite marker alleles according to the precise complications detected. Upon application of the Chi-squared test for significance and Fishers exact test for numbers <5, no associations were found with respect to the onset of microvascular complications of T1DM. Table 75 presents the frequency of the detected D17S934 (CA)n microsatellite alleles in subjects according to the onset of diabetic nephropathy, retinopathy or neuropathy. There were no significant associations detected with respect to the onset of the microvascular complications of T1DM.

Alleles	T1DM subjects	Normal Controls (NC)
n	488	240
1	12.5	7.5
	(61)	(18)
2	28.7	19.9
	(140)	(48)
3	17.8	23.3
	(87)	(56)
4	15.9	18.8
	(78)	(45)
5	12.5	8.8
	(61)	(21)
6	7.9	13.3
	(39)	(32)
7	2.5	2.9
	(12)	(7)
8	1.6	4.6
	(8)	(11)
9	-	0.008
	(0)	(2)
10	0.2	-
	(1)	(-)
12	0.2	-
	(1)	(-)

Table 72. Percentage frequency of the detected D17S934 (CA)n marker alleles in British Caucasoid T1DM subjects compared to normal controls.

This table shows the frequency of detected alleles for T1DM subjects and normal control subjects. Frequencies are expressed as the percentage incidence out of the total number of alleles detected. Comparisons were made between the allelic frequencies for control and T1DM subjects using the χ^2 test and 2x2 contingency tables. n = number of alleles detected, number of subjects studied is shown in parenthesis. Only significant differences by χ^2 and correction are shown. Where observed numbers were <5, Fishers exact test for small numbers was applied.

T1DM vs. NC;   1; χ^2 = 4.16, p= 0.04 (1df), Pc = ns
2; χ^2 = 6.34, p= 0.01 (1df), Pc = ns
6; χ^2 = 5.22, p= 0.02 (1df), Pc = ns
8; χ^2 = 5.49, p= 0.02 (1df), Pc = ns

The remaining allele frequencies were not analysed as only a small number were detected and significant correlation's would be difficult to determine.

	TIDM Nephropathy and/or Retinopathy and/or Neuropathy (DR/N/Nu)	Uncomplicated (DC)	Normal Controls (NC)
n	356	132	240
	(178)	(66)	(120)
1	12.9	11.4	7.5
	(46)	(15)	(18)
2	29.8	25.8	19.9
	(106)	(34)	(48)
3	16.9	20.5	23.3
	(60)	(27)	(56)
4	16.3	15.2	18.8
	(58)	(20)	(45)
5	12.9	11.4	8.8
	(46)	(15)	(21)
6	7.9	8.3	13.3
	(28)	(11)	(32)
7	1.4	5.3	2.9
	(5)	(7)	(7)
8	1.4	2.3	4.6
	(5)	(3)	(11)
9	-	-	0.008
	(-)	(-)	(2)
10	0.3	-	-
	(1)	(-)	(-)
12	0.3	-	-
	(1)	(-)	(-)

Table 73. Percentage frequency of the detected D17S934 (CA)n microsatellite marker alleles in British Caucasoid T1DM subjects with diabetic nephropathy and/or retinopathy and/or neuropathy (DR/N/Nu) compared to uncomplicated (DC) and normal controls (NC).

This table shows the percentage frequency of detected D17S934 alleles for T1DM subjects with microvascular disease (nephropathy, retinopathy, neuropathy) compared to uncomplicated and normal controls. The percentage frequency is shown and the actual number of each of the alleles detected for each group is shown in parenthesis. n = number of alleles detected, the number of subjects is shown in parenthesis. Only significant differences by χ^2 and correction are shown.

DR/DN/DNU vs. Uncomplicated diabetic controls;
$$7; \chi^2 = 6.1, p= 0.01 \ (1df), Pc = ns$$

The remaining allele frequencies were not analysed as only a small number were detected and significant correlation's would be difficult to determine.

	1 Diabetic Retinopathy	2 Diabetic Nephropathy and Retinopathy	3 Diabetic Retinopathy and Neuropathy	4 Diabetic Neuropathy	5 Full House Patients	Uncomplicated (DC)	Normal Controls (NC)
n	88 (44)	60 (30)	48 (24)	36 (18)	124 (62)	132 (76)	240 (120)
1	7.95 (7)	11.7 (7)	22.9 (11)	16.7 (6)	12.1 (15)	11.4 (15)	7.5 (18)
2	34.1 (30)	28.3 (17)	33.3 (16)	30.6 (11)	25.8 (32)	25.8 (34)	19.9 (48)
3	18.2 (16)	14.99 (9)	12.5 (6)	11.1 (4)	20.2 (25)	20.5 (27)	23.3 (56)
4	17.1 (15)	19.99 (12)	12.5 (6)	19.4 (7)	14.5 (18)	15.2 (20)	18.8 (45)
5	10.2 (9)	9.99 (6)	8.3 (4)	13.9 (5)	17.7 (22)	11.4 (15)	8.8 (21)
6	6.8 (6)	4.99 (3)	10.4 (5)	8.3 (3)	8.9 (11)	8.3 (11)	13.3 (32)
7	3.4 (3)	1.7 (1)	- (-)	- (-)	0.81 (1)	5.3 (7)	2.9 (7)
8	2.3 (2)	4.99 (3)	- (-)	- (-)	- (-)	2.3 (3)	4.6 (11)
9	- (-)	1.7 (-)	- (-)	- (-)	- (-)	- (-)	0.008 (2)
10	- (-)	1.7 (1)	- (-)	- (-)	- (-)	- (-)	- (-)
12	- (-)	1.7 (1)	- (-)	- (-)	- (-)	- (-)	- (-)

Table 74. Percentage frequency of the detected D17S934 (CA)n microsatellite marker alleles in British Caucasoid T1DM subjects categorised in accordance to the onset of diabetic complications and normal control subjects.

This table shows the percentage frequency of detected alleles for T1DM subjects with retinopathy (DR), nephropathy and retinopathy (DN/DR), retinopathy and neuropathy (DR/DNu), neuropathy (DNu), full house complications patients (FHC) and uncomplicated diabetics controls (DC). Also shown here is the allelic frequency of normal healthy controls.

n = number of alleles detected, the number of subjects is shown in parenthesis.

Upon application of the Chi squared test for significance and Fishers exact test for numbers <5, no associations were found between the frequencies of each of the alleles detected and onset of microvascular complications of T1DM.

	[1] Diabetic Nephropathy (DN)	[2] Diabetic Retinopathy (DR)	[3] Diabetic Neuropathy (DNu)	Uncomplicated (DC)	Normal Controls (NC)
n	184	320	208	132	240
	(92)	(160)	(104)	(76)	(120)
1	11.96	12.5	15.4	11.4	7.5
	(22)	(40)	(32)	(15)	(18)
2	26.6	29.7	28.4	25.8	19.9
	(49)	(95)	(59)	(34)	(48)
3	18.0	17.5	16.8	20.5	23.3
	(34)	(56)	(35)	(27)	(56)
4	16.3	15.9	14.9	15.2	18.8
	(30)	(51)	(31)	(20)	(45)
5	15.2	12.8	14.9	11.4	8.8
	(28)	(41)	(31)	(15)	(21)
6	7.6	7.8	9.1	8.3	13.3
	(14)	(25)	(19)	(11)	(32)
7	1.09	1.6	0.5	5.3	2.9
	(2)	(5)	(1)	(7)	(7)
8	1.6	1.6	-	2.3	4.6
	(3)	(5)	(-)	(3)	(11)
9	-	-	-	-	0.008
	(-)	(-)	(-)	(-)	(2)
10	0.5	0.3	-	-	-
	(1)	(1)	(-)	(-)	(-)
12	0.5	0.3	-	-	-
	(-)	(-)	(-)	(-)	(-)

Table 75. Percentage frequency of the detected D17S934 (CA)n microsatellite alleles in Caucasoid T1DM subjects categorised in accordance to onset of diabetic nephropathy, retinopathy or neuropathy.

This table presents the allelic frequency of the D17S934 (CA)n microsatellite in T1DM subjects of British Caucasoid origin. Subjects are classified in accordance to onset of retinopathy, nephropathy or neuropathy. The nephropathy group (n=92) is an amalgamation of the nephropathy and retinopathy (n=30) and full house subjects (n=62) groups previously identified. The retinopathy group (n=160) is an amalgamation of the retinopathy (n=44), nephropathy with retinopathy (n=30), retinopathy and neuropathy (n=24) and full house patients (n=62) groups previously identified. The neuropathy group (n=104) consists of subjects diagnosed as having neuropathy in the absence of any other microvascular complication (n=18), the diabetic retinopathy with neuropathy (n=24) and full house patients (n=62) groups. Also presented is the allelic frequency in uncomplicated and normal controls. n = number of alleles detected, the number of subjects is shown in parenthesis.

Upon application of the Chi squared test for significance and Fishers exact test where numbers were <5, no associations were found between the frequencies of each of the alleles detected and onset of microvascular complications of T1DM.

D17S934- (CA)n genotype frequencies in British Caucasoid T1DM and normal control subjects

The frequencies of the D17S934 genotype frequencies in British Caucasoid T1DM and normal controls are shown in tables 76-79. Table 76 shows the detected frequency of the D17S934 genotypes in all T1DM subjects studied compared to the normal controls. There were no significant differences in the frequencies of the detected genotypes between the two groups. Table 77 shows the frequency of the detected genotypes in the subgroups of the subjects studied; T1DM diabetic microvascular disease, uncomplicated diabetic controls and normal controls. There were no significant differences found when the Chi-squared test was applied. Table 78 shows the frequency of each of the genotypes detected in all of the patient subgroups according to the precise phenotype of diabetic microvascular complications. Comparisons were made between each of the complication subgroups and the T1DM uncomplicated controls. There were no significant differences detected between the frequencies of any of the genotypes detected between these groups. Equally, in table 79, whereby the patient subgroups were amalgamated according to the presence of nephropathy or retinopathy or neuropathy no significant differences were found indicating that this polymorphism is not associated with the onset of microvascular complications of diabetes in the population studied here.

Genotype	T1DM subjects	Normal Controls (NC)
n	244	120
1.1	2.0	1.7
	(5)	(2)
1.2	6.6	1.7
	(16)	(2)
1.3	4.5	3.3
	(11)	(4)
1.4	4.1	3.3
	(10)	(4)
1.5	2.9	2.5
	(7)	(3)
1.6	2.0	-
	(5)	(-)
1.7	0.4	0.8
	(1)	(1)
1.8	0.4	-
	(1)	(-)
2.2	10.2	8.3
	(25)	(10)
2.3	9.0	4.99
	(22)	(6)
2.4	9.0	7.5
	(22)	(9)
2.5	6.9	2.5
	(17)	(3)
2.6	3.3	4.2
	(8)	(5)
2.7	1.2	0.8
	(3)	(1)
2.8	0.8	0.8
	(2)	(1)
2.9	-	0.8
	(-)	(1)
3.3	2.9	7.5
	(7)	(9)
3.4	6.1	12.5
	(15)	(15)
3.5	5.3	4.2
	(13)	(5)
3.6	3.3	5.8
	(8)	(7)
3.7	1.2	-
	(3)	(-)
3.8	0.4	0.8
	(1)	(1)
4.4	1.6	3.3
	(4)	(4)
4.5	5.7	2.5
	(14)	(3)
4.6	2.5	4.2
	(6)	(5)
4.7	0.4	0.8
	(1)	(1)
4.8	0.8	-
	(2)	(-)
5.5	0.4	2.5
	(1)	(3)
5.6	2.5	-
	(6)	(-)
5.7	T0.8	-
	(2)	(-)
5.8	-	0.8
	(-)	(1)

6.6	0.8	4.99
	(2)	(6)
6.7	-	1.7
	(-)	(2)
6.8	0.8	0.8
	(2)	(1)
7.7	0.4	0.8
	(1)	(1)
8.8	-	2.5
	(-)	(3)
8.9	-	0.8
	(0)	(1)
10.12	0.4	-
	(1)	(-)

Table 76. Percentage frequency of the detected D17S934 (CA)n microsatellite marker genotypes in British Caucasoid T1DM subjects compared to normal controls.
This table shows the frequency of detected genotypes for T1DM subjects and normal control subjects. Frequencies are expressed as the percentage incidence out of the total number of genotypes detected. n = number of genotypes detected. Comparisons were made between the genotype frequencies for normal controls and T1DM subjects using the χ^2 test and 2 x 2 contingency tables. P-values were corrected for the number of comparisons made. Where observed values were <5 Fishers exact test was used. No significant differences were found.

Genotype	T1DM Nephropathy and/or Retinopathy and/or Neuropathy (DR/N/Nu)	Uncomplicated (DC)	Normal Controls (NC)
n	178	66	120
1.1	2.3 (4)	1.52 (1)	1.7 (2)
1.2	7.3 (13)	4.6 (3)	1.7 (2)
1.3	5.6 (10)	1.5 (1)	3.3 (4)
1.4	3.9 (7)	4.6 (3)	3.3 (4)
1.5	2.8 (5)	3.0 (2)	2.5 (3)
1.6	1.1 (2)	4.6 (3)	- (-)
1.7	- (-)	1.5 (1)	0.8 (1)
1.8	0.6 (1)	- (-)	- (-)
2.2	10.7 (19)	9.1 (6)	8.3 (10)
2.3	8.99 (16)	9.1 (6)	4.99 (6)
2.4	10.1 (18)	6.1 (4)	7.5 (9)
2.5	6.2 (11)	9.1 (6)	2.5 (3)
2.6	3.4 (6)	3.0 (2)	4.2 (5)
2.7	1.1 (2)	1.5 (1)	0.8 (1)
2.8	1.1 (2)	- (-)	0.8 (1)
2.9	- (-)	- (-)	0.8 (1)
3.3	1.7 (3)	6.1 (4)	7.5 (9)
3.4	6.2 (11)	6.1 (4)	12.5 (15)
3.5	6.2 (11)	3.0 (2)	4.2 (5)
3.6	3.4 (6)	3.0 (2)	5.8 (7)
3.7	- (-)	4.6 (3)	- (-)
3.8	- (-)	1.5 (1)	0.8 (1)
4.4	1.7 (3)	1.5 (1)	3.3 (4)
4.5	6.7 (12)	3.0 (2)	2.5 (3)
4.6	2.3 (4)	3.0 (2)	4.2 (5)
4.7	- (-)	1.5 (1)	0.8 (1)
4.8	- (-)	3.0 (2)	- (-)
5.5	0.6 (1)	- (-)	2.5 (3)
5.6	2.3 (4)	3.0 (2)	- (-)
5.7	0.6 (1)	1.5 (1)	- (-)

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5.8	-	-	0.8
	(-)	(-)	(1)
6.6	1.1	-	4.99
	(2)	(-)	(6)
6.7	-	-	1.7
	(-)	(-)	(2)
6.8	1.1	-	0.8
	(2)	(-)	(1)
7.7	0.6	-	0.8
	(1)	(-)	(1)
7.9	-	-	-
	(-)	(-)	(-)
8.8	-	-	2.5
	(-)	(-)	(3)
8.9	-	-	0.8
	(-)	(-)	(1)
10.12	0.6	-	-
	(1)	(-)	(-)

Table 77. Percentage frequency of the detected D17S934 (CA)n microsatellite marker genotypes in British Caucasoid T1DM subjects with nephropathy and/or retinopathy and/or neuropathy (DR/N/Nu) compared to uncomplicated (DC) and normal controls (NC). This table shows the percentage frequency of detected genotypes for T1DM subjects with microvascular disease (nephropathy, retinopathy, neuropathy) compared to uncomplicated and normal controls. The percentage frequency is shown and the actual number of genotypes detected for each group is shown in parenthesis. n = number of genotypes detected.

Genotype	Diabetic Retinopathy[1]	Diabetic Nephropathy and Retinopathy[2]	Diabetic Retinopathy and Neuropathy[3]	Diabetic Neuropathy[4]	Full House Patients[5]	Uncomplicated (DC)	Normal Controls (NC)
n	44	30	24	18	62	66	120
1.1	-	-	12.5	-	1.6	1.5	1.7
	(-)	(-)	(3)	(-)	(1)	(1)	(2)
1.2	4.6	6.7	16.7	5.6	6.5	4.6	1.7
	(2)	(2)	(4)	(1)	(4)	(3)	(2)
1.3	4.6	9.99	4.2	5.6	4.8	1.5	3.3
	(2)	(3)	(1)	(1)	(3)	(1)	(4)
1.4	-	-	-	22.2	4.8	4.6	3.3
	(-)	(-)	(-)	(4)	(3)	(3)	(4)
1.5	6.8	3.3	-	-	1.6	3.0	2.5
	(3)	(1)	(-)	(-)	(1)	(2)	(3)
1.6	-	-	-	-	3.2	4.6	-
	(-)	(-)	(-)	(-)	(2)	(3)	(-)
1.7	-	-	-	-	-	1.5	0.8
	(-)	(-)	(-)	(-)	(-)	(1)	(1)
1.8	-	3.3	-	-	-	-	-
	(-)	(1)	(-)	(-)	(-)	(-)	(-)
2.2	15.9	6.7	12.5	16.7	6.5	9.1	8.3
	(7)	(2)	(3)	(3)	(4)	(6)	(10)
2.3	4.6	6.7	4.2	5.6	16.1	9.1	4.99
	(2)	(2)	(1)	(1)	(10)	(6)	(6)
2.4	13.6	16.7	12.5	-	6.5	6.1	7.5
	(6)	(5)	(3)	(-)	(4)	(4)	(9)
2.5	9.1	6.7	4.2	11.1	3.2	9.1	2.5
	(4)	(2)	(1)	(2)	(2)	(6)	(3)
2.6	2.3	-	4.2	5.6	4.8	3.0	4.2
	(1)	(-)	(1)	(1)	(3)	(2)	(5)
2.7	-	3.3	-	-	1.6	1.5	0.8
	(-)	(1)	(-)	(-)	(1)	(1)	(1)
2.8	2.3	3.3	-	-	-	-	0.8
	(1)	(1)	(-)	(-)	(-)	(-)	(1)
2.9	-	-	-	-	-	-	0.8
	(-)	(-)	(-)	(-)	(-)	(-)	(1)
3.3	4.6	-	-	-	1.6	6.1	7.5
	(2)	(-)	(-)	(-)	(1)	(4)	(9)
3.4	11.4	6.7	4.2	-	4.8	6.1	12.5
	(5)	(2)	(1)	(-)	(3)	(4)	(15)
3.5	-	3.3	8.3	5.6	11.3	3.0	4.2
	(-)	(1)	(2)	(1)	(7)	(2)	(5)
3.6	6.8	3.3	4.2	5.6	-	3.0	5.8
	(3)	(1)	(1)	(1)	(-)	(2)	(7)
3.7	-	-	-	-	-	4.6	-
	(-)	(-)	(-)	(-)	(-)	(3)	(-)
3.8	-	-	-	-	-	1.5	0.8
	(-)	(-)	(-)	(-)	(-)	(1)	(1)
4.4	2.3	3.3	-	-	1.6	1.5	3.3
	(1)	(1)	(-)	(-)	(1)	(1)	(4)
4.5	2.3	6.7	4.2	11.1	9.7	3.0	2.5
	(1)	(2)	(1)	(2)	(6)	(2)	(3)
4.6	2.3	3.3	4.2	5.6	-	3.0	4.2
	(1)	(1)	(1)	(1)	(-)	(2)	(5)
4.7	-	-	-	-	-	1.5	0.8
	(-)	(-)	(-)	(-)	(-)	(1)	(1)
4.8	-	-	-	-	-	3.0	-
	(-)	(-)	(-)	(-)	(-)	(2)	(-)
5.5	-	-	-	-	1.6	-	2.5
	(-)	(-)	(-)	(-)	(1)	(-)	(3)
5.6	-	-	-	-	6.5	3.0	-
	(-)	(-)	(-)	(-)	(4)	(2)	(-)
5.7	2.3	-	-	-	-	1.5	-
	(1)	(-)	(-)	(-)	(-)	(1)	(-)

5.8	-	-	-	-	-	-	0.8
	(-)	(-)	(-)	(-)	(-)	(-)	(1)
6.6	-	-	4.2	-	1.6	-	4.99
	(-)	(-)	(1)	(-)	(1)	(-)	(6)
6.7	-	-	-	-	-	-	1.7
	(-)	(-)	(-)	(-)	(-)	(-)	(2)
6.8	2.3	3.3	-	-	-	-	0.8
	(1)	(1)	(-)	(-)	(-)	(-)	(1)
7.7	2.3	-	-	-	-	-	0.8
	(1)	(-)	(-)	(-)	(-)	(-)	(1)
7.9	-	-	-	-	-	-	-
	(-)	(-)	(-)	(-)	(-)	(-)	(-)
8.8	-	-	-	-	-	-	2.5
	(-)	(-)	(-)	(-)	(-)	(-)	(3)
8.9	-	-	-	-	-	-	0.8
	(-)	(-)	(-)	(-)	(-)	(-)	(1)
10.12	-	3.3	-	-	-	-	-
	(-)	(1)	(-)	(-)	(-)	(-)	(-)

Table 78. Percentage frequency of the detected D17S934 (CA)n microsatellite marker genotypes in British Caucasoid T1DM subjects categorised in accordance to the onset of diabetic complications and uncomplicated (DC) and normal controls (NC).

This table shows the percentage frequency of detected genotypes for T1DM subjects with respect to onset of microvascular complications, and normal control subjects. The percentage frequency is shown and the actual number of genotypes detected for each group is shown in parenthesis. n = number of subjects studied and genotypes detected.

Genotype	[1] Diabetic Nephropathy (DN)	[2] Diabetic Retinopathy (DR)	[3] Diabetic Neuropathy (DNu)	Uncomplicated (DC)	Normal Controls (NC)
n	92	160	104	66	120
1.1	1.1	2.5	3.9	1.5	1.7
	(1)	(4)	(4)	(1)	(2)
1.2	6.5	7.5	8.7	4.6	1.7
	(6)	(12)	(9)	(3)	(2)
1.3	6.5	5.6	4.8	1.5	3.3
	(6)	(9)	(5)	(1)	(4)
1.4	3.3	1.9	6.7	4.6	3.3
	(3)	(3)	(7)	(3)	(4)
1.5	2.2	3.1	0.9	3.0	2.5
	(2)	(5)	(1)	(2)	(3)
1.6	2.2	1.3	1.9	4.6	-
	(2)	(2)	(2)	(3)	(-)
1.7	-	-	-	1.5	0.8
	(-)	(-)	(-)	(1)	(1)
1.8	1.1	0.6	-	-	-
	(1)	(1)	(-)	(-)	(-)
2.2	6.5	10.0	9.6	9.1	8.3
	(6)	(16)	(10)	(6)	(10)
2.3	13.0	9.4	11.5	9.1	4.99
	(12)	(15)	(12)	(6)	(6)
2.4	9.8	11.3	6.7	6.1	7.5
	(9)	(18)	(7)	(4)	(9)
2.5	4.4	5.6	4.8	9.1	2.5
	(4)	(9)	(5)	(6)	(3)
2.6	3.3	3.1	4.8	3.0	4.2
	(3)	(5)	(5)	(2)	(5)
2.7	2.2	1.3	0.96	1.5	0.8
	(2)	(2)	(1)	(1)	(1)
2.8	1.1	1.3	-	-	0.8
	(1)	(2)	(-)	(-)	(1)
2.9	-	-	-	-	0.8
	(-)	(-)	(-)	(-)	(1)
3.3	1.1	1.9	0.96	6.1	7.5
	(1)	(3)	(1)	(4)	(9)
3.4	5.4	6.9	3.8	6.1	12.5
	(5)	(11)	(4)	(4)	(15)
3.5	8.7	6.3	9.6	3.0	4.2
	(8)	(10)	(10)	(2)	(5)
3.6	1.1	3.1	1.9	3.0	5.8
	(1)	(5)	(2)	(2)	(7)
3.7	-	-	-	4.6	-
	(-)	(-)	(-)	(3)	(-)
3.8	-	-	-	1.5	0.8
	(-)	(-)	(-)	(1)	(1)
4.4	2.2	1.9	0.96	1.5	3.3
	(2)	(3)	(1)	(1)	(4)
4.5	8.7	6.3	8.7	3.0	2.5
	(8)	(10)	(9)	(2)	(3)
4.6	1.1	1.9	1.9	3.0	4.2
	(1)	(3)	(2)	(2)	(5)
4.7	-	-	-	1.5	0.8
	(-)	(-)	(-)	(1)	(1)
4.8	-	-	-	3.0	-
	(-)	(-)	(-)	(2)	(-)
5.5	1.1	0.6	0.96	-	2.5
	(1)	(1)	(1)	(-)	(3)
5.6	4.4	2.5	3.9	3.0	-
	(4)	(4)	(4)	(2)	(-)
5.7	-	0.6	-	1.5	-
	(-)	(1)	(-)	(1)	(-)

5.8	-	-	-	-	0.8
	(-)	(-)	(-)	(-)	(1)
6.6	1.1	1.3	1.9	-	4.99
	(1)	(2)	(2)	(-)	(6)
6.7	-	-	-	-	1.7
	(-)	(-)	(-)	(-)	(2)
6.8	1.1	1.3	-	-	0.8
	(1)	(2)	(-)	(-)	(1)
7.7	-	0.6	-	-	0.8
	(-)	(1)	(-)	(-)	(1)
8.8	-	-	-	-	2.5
	(-)	(-)	(-)	(-)	(3)
8.9	-	-	-	-	0.8
	(-)	(-)	(-)	(-)	(1)
10.12	1.1	0.6	-	-	-
	(1)	(1)	(-)	(-)	(-)

Table 79. Percentage frequency of the detected D17S934 (CA)n microsatellite marker genotypes in British Caucasoid T1DM subjects categorised in accordance to onset of diabetic nephropathy, retinopathy or neuropathy.

This table presents the genotype frequency of the D17S934 (CA)n microsatellite in T1DM subjects of British Caucasoid origin. Subjects are classified in accordance to onset of retinopathy, nephropathy or neuropathy. The nephropathy group (n=92) is an amalgamation of the nephropathy and retinopathy (n=30) and full house subjects (n=62) groups previously identified. The retinopathy group (n=160) is an amalgamation of the retinopathy (n=44), nephropathy with retinopathy (n=30), retinopathy and neuropathy (n=24) and full house patients (n=62) groups previously identified. The neuropathy group (n=104) consists of subjects diagnosed as having neuropathy in the absence of any other microvascular complication (n=18), the diabetic retinopathy with neuropathy (n=24) and full house patients (n=62) groups. Also presented is the genotype frequency in uncomplicated and normal controls. n = number of subjects analysed and genotypes detected. The percentage frequency is shown and the actual number of each genotype detected for each subjects group is shown in parenthesis.

D17S934- (CA)n microsatellite marker in Southern Indian T2DM subjects and non-diabetic controls

The (CA)n microsatellite marker at D17S934 in the proximal region of the ACE gene was investigated using 60 subjects with T2DM of Southern Indian origin as well as a collection of 42 Southern Indian/Dravidian non-diabetic controls (clinical demographics shown in table 7). Within the T2DM subject population studied, there were 28 subjects that were normoalbuminuric without retinopathy and 32 subjects with proteinuria. The frequency of the (CA)n alleles in both patient and control populations is shown in tables 80 to 81. There were 9 (CA)n alleles detected at the D17S934 locus in the Southern Indian T2DM and non-diabetic control population; 1,2,3,4,5,6,7,8 from which 29 genotypes were also identified. Tables show the percentage frequency of detected alleles and genotypes, with actual numbers detected shown in parenthesis. Possible associations were investigated with respect to allelic frequency and genotype and comparisons were made between groups using the Chi-squared test and 2x2 contingency tables. All of the data obtained from the D17S934 study of the Southern Indian population is presented in the following tables. Possible associations with the D17S934 (CA)n allelic frequencies and genotypes and the onset of proteinuria were investigated.

D17S934 (CA)n allelic frequencies in Southern Indian T2DM and non-diabetic control subjects

The frequency of the D17S934 alleles in T2DM Southern Indian/Dravidian subjects is shown in tables 80 to 81. Table 80 shows the percentage frequency of the D17S934 (CA)n alleles in the total T2DM population studied compared to the non-diabetic control population studied. Several significant differences were found in the frequencies of the detected alleles between the two groups. Significant differences were found between the two groups with respect to alleles 3,6 and 7. Table 81 compares the percentage frequencies of alleles detected in the T2DM population with proteinuria and with normoalbuminuria. There were no significant differences found in the frequencies of the detected alleles between the two groups.

Alleles	T2DM subjects	Non-diabetic Controls (NDC)
n	120	84
	(60)	(42)
1	9.2	1.2
	(11)	(1)
2	4.99	15.5
	(6)	(13)
3	4.2	20.2
	(5)	(17)
4	21.7	30.95
	(26)	(26)
5	17.5	27.4
	(21)	(23)
6	18.3	3.6
	(22)	(3)
7	13.3	-
	(16)	(-)
8	6.7	1.2
	(8)	(1)
9	4.2	-
	(5)	(-)

Table 80. Percentage frequency of the detected D17S934 (CA)n microsatellite marker alleles in Southern Indian/Dravidian T2DM subjects compared to non-diabetic controls.

This table shows the frequency of detected D17S934 alleles for T2DM subjects and non-diabetic control subjects. Frequencies are expressed as the percentage incidence out of the total number of alleles detected. Actual numbers of alleles detected is shown in parenthesis.

n = total number of alleles detected, number of subjects studied is shown in parentheses. Only significant differences by χ^2 and correction are shown.

T2DM vs. NDC;
1; χ^2 = 5.7, p= 0.02 (1df), Pc = ns
2; χ^2 = 6.4, p= 0.01 (1df), Pc = ns
3; χ^2 = 13.3, p= 0.0003 (1df), Pc = 0.003 *
6; χ^2 = 10.01, p= 0.002 (1df), Pc = 0.02 *
7; χ^2 = 12.2, p= 0.0005 (1df), Pc = 0.005 *
8; χ^2 = 3.98, p= 0.05 (1df), Pc = ns

n	T2DM with Proteinuria	T2DM with Normoalbuminuria	Non-diabetic Controls (NDC)
n	56	64	84
	(28)	(32)	(42)
1	7.1	10.9	1.2
	(4)	(7)	(1)
2	1.8	7.8	15.5
	(1)	(5)	(13)
3	3.6	4.7	20.2
	(2)	(3)	(17)
4	19.6	23.4	30.95
	(11)	(15)	(26)
5	14.3	20.3	27.4
	(8)	(13)	(23)
6	17.9	18.8	3.6
	(10)	(12)	(3)
7	19.6	7.8	-
	(11)	(5)	(-)
8	7.1	6.3	1.2
	(4)	(4)	(1)
9	8.9	-	-
	(5)	(-)	(-)

Table 81. Percentage frequency of the detected D17S934 microsatellite marker alleles in Southern Indian/Dravidian T2DM subjects with proteinuria compared to T2DM subjects with normoalbuminuria and non-diabetic control subjects. This table shows the percentage frequency of the detected alleles for T2DM subjects with normoalbuminuria and T2DM subjects with proteinuria as well as non-diabetic controls. The actual number of alleles within each group is shown in parentheses.

n = number of alleles detected, number of subjects studied is shown in parentheses.

No significant correlations were found.

D17S934 (CA)n genotype frequencies in Southern Indian T2DM and non-diabetic control subjects

Table 82 presents the frequency of the detected genotypes in the T2DM Southern Indian subjects compared with the non-diabetic controls, all genotypes detected are presented in the tables. Due to the low frequencies of each of the genotypes detected, no significant correlations could be made between the T2DM and non-diabetic control groups. Table 83. presents the genotypes detected for the T2DM subjects according to the onset of proteinuria and normoalbuminuria. There were no significant differences found in the frequencies of the detected genotypes in these groups. There was a low frequency of each of the genotypes detected and consequently no significant correlations could be made between the T2DM normoalbuminuria and proteinuria groups and the non-diabetic controls.

Genotype	T2DM subjects	Non-diabetic Controls (NDC)
n	60	42
1.1	1.7 (1)	- (-)
1.2	1.7 (1)	- (-)
1.3	3.3 (2)	- (-)
1.4	3.3 (2)	- (-)
1.5	4.99 (3)	2.4 (1)
1.6	1.7 (1)	- (-)
2.2	- (-)	2.4 (1)
2.4	3.3 (2)	19.1 (8)
2.5	- (-)	7.1 (3)
2.6	3.3 (2)	- (-)
2.9	1.7 (1)	- (-)
3.3	- (-)	4.8 (2)
3.4	- (-)	14.3 (6)
3.5	- (-)	14.3 (6)
3.6	3.3 (2)	- (-)
3.8	1.7 (1)	2.4 (1)
4.4	6.7 (4)	9.5 (4)
4.5	9.99 (6)	4.8 (2)
4.6	4.99 (3)	4.8 (2)
4.7	6.7 (4)	- (-)
4.9	1.7 (1)	- (-)
5.5	3.3 (2)	11.9 (5)
5.6	9.99 (6)	2.4 (1)
5.8	3.3 (2)	- (-)
6.6	1.7 (1)	- (-)
6.7	9.99 (6)	- (-)
7.8	6.7 (4)	- (-)
7.9	3.3 (2)	- (-)
8.9	1.7 (1)	- (-)

Table 82. Percentage frequency of the detected D17S934 (CA)n microsatellite genotypes in T2DM subjects compared to non-diabetic control subjects of Southern Indian/Dravidian origin. This table shows the percentage frequency of detected genotypes for T2DM subjects with respect to onset of diabetes, and non-diabetic control subjects. n = number of genotypes detected. Due to the low frequencies of each of the genotypes detected no significant correlation's could be made between the T2DM group and the non-diabetic controls.

Genotype	T2DM Proteinuria	T2DM Normoalbuminuria	Non-diabetic Controls (NDC)
n	28	32	42
1.1	3.6 (1)	- (-)	- (-)
1.2	- (-)	3.1 (1)	- (-)
1.3	3.6 (1)	3.1 (1)	- (-)
1.4	3.6 (1)	3.1 (1)	- (-)
1.5	- (-)	9.4 (3)	2.4 (1)
1.6	- (-)	3.1 (1)	- (-)
2.2	- (-)	- (-)	2.4 (1)
2.4	- (-)	6.3 (2)	19.1 (8)
2.5	- (-)	- (-)	7.1 (3)
2.6	- (-)	6.3 (2)	- (-)
2.9	3.6 (1)	- (-)	- (-)
3.3	- (-)	- (-)	4.8 (2)
3.4	- (-)	- (-)	14.3 (6)
3.5	- (-)	- (-)	14.3 (6)
3.6	3.6 (1)	3.1 (1)	- (-)
3.8	- (-)	3.1 (1)	2.4 (1)
4.4	7.1 (2)	6.3 (2)	9.5 (4)
4.5	10.7 (3)	9.4 (3)	4.8 (2)
4.6	- (-)	9.4 (3)	4.8 (2)
4.7	7.1 (2)	6.3 (2)	- (-)
4.9	3.6 (1)	- (-)	- (-)
5.5	- (-)	6.3 (2)	11.9 (5)
5.6	17.9 (5)	3.1 (1)	2.4 (1)
5.8	- (-)	6.3 (2)	- (-)
6.6	- (-)	3.1 (1)	- (-)
6.7	14.3 (4)	6.3 (2)	- (-)
7.8	10.7 (3)	3.1 (1)	- (-)
7.9	7.1 (2)	- (-)	- (-)
8.9	3.6 (1)	- (-)	- (-)

Table 83. Percentage frequency of the detected (CA)n D17S934 microsatellite marker genotypes in Southern Indian T2DM subjects classified and compared in accordance to presence of proteinuria or normoalbuminuria against non-diabetic controls.
This table shows the percentage frequency of detected genotypes for T2DM subjects with respect to onset of proteinuria, and non-diabetic control subjects. n = number of genotypes detected. Due to the low frequencies of each of the genotypes detected no significant correlation's could be made between the T2DM normoalbuminuria and proteinuria groups and the non-diabetic controls.

Mitochondrial genome study

Associations of polymorphism's within the mitochondrial genome and the microvascular complications of T1DM

Polymorphism at the C(Mt5178)A region

The Mt5178A/C polymorphism situated at position 5178 of the mitochondrial genome was investigated. Polymerase Chain Reaction (PCR) was carried out to amplify the region within the NADH dehydrogenase (ND2) region containing the polymorphism as described in chapter 3 (figure 32). Amplification products were digested overnight at 37°C with Alu1 restriction enzyme, and separated on a 2.5% agarose/ethidium gel at 100V for 1 hour. The fragment sizes were checked by running a 100bp molecular weight marker alongside. Bands were revealed by UV transillumination (320nm) (figure 33) and subjects were assigned a genotype according to the restriction fragments identified. A PCR control band was also present.

The Mt5178A/C polymorphism located within NADH dehydrogenase (ND2) region of the mitochondrial genome was investigated using 126 T1DM subjects and 91 normal controls of British Caucasoid origin (clinical demographics shown in table 6). Within the T1DM subject population studied there were 32 uncomplicated, 30 full house patients, 0 neuropathy patients, 19 retinopathy and neuropathy patients, 13 nephropathy and retinopathy and 29 retinopathy. In this study we were only able to identify subject who were homozygous CC. The A polymorphism was not detected in any of the subjects investigated. It was not felt necessary to analyse further patients or controls for this polymorphism because the A polymorphism had not been detected in a single subject. Therefore felt to be unlikely to be a significant polymorphism in the subjects investigated. No association could therefore be made with respect to the mitochondrial gene region and diabetic microvascular complications.
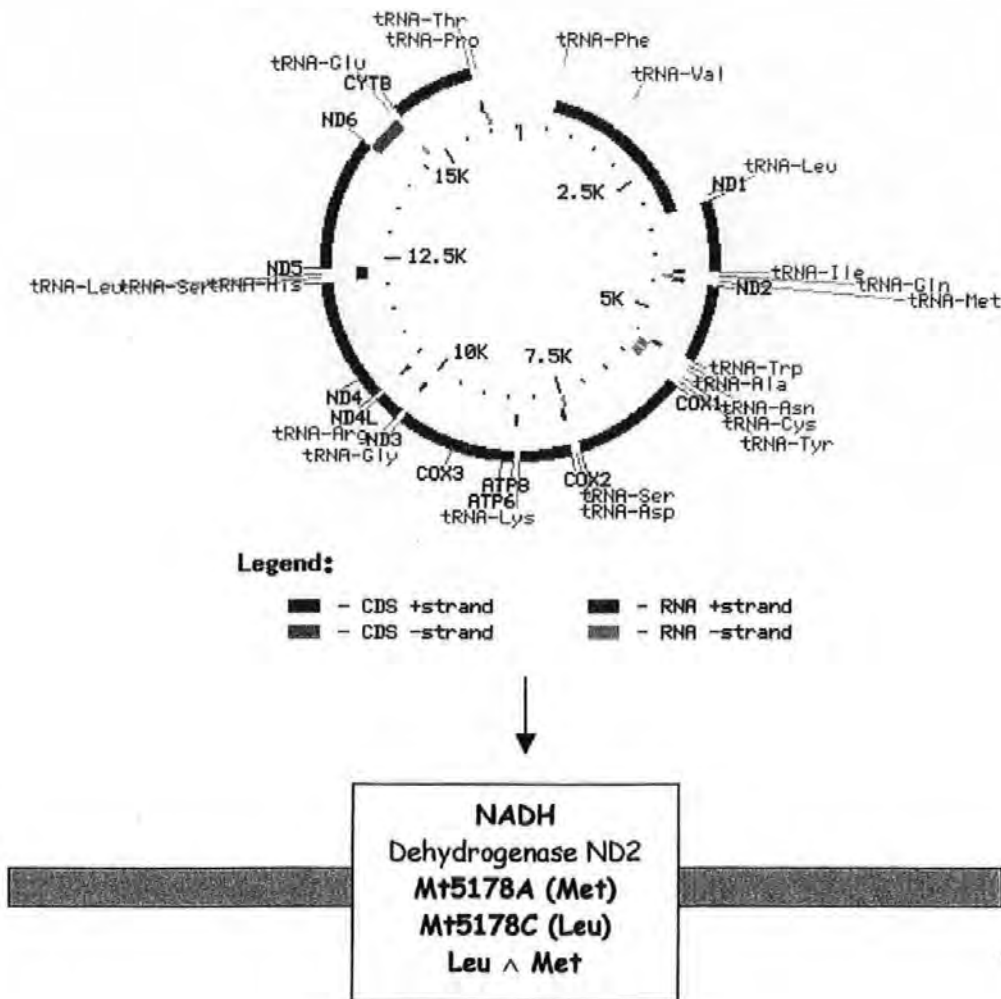
Figure 32. Diagrammatic representation of the polymorphic region studied within the mitochondrial genome at position 5178. The C to A polymorphism altered the amino acid configuration by changing from Leu to Met.
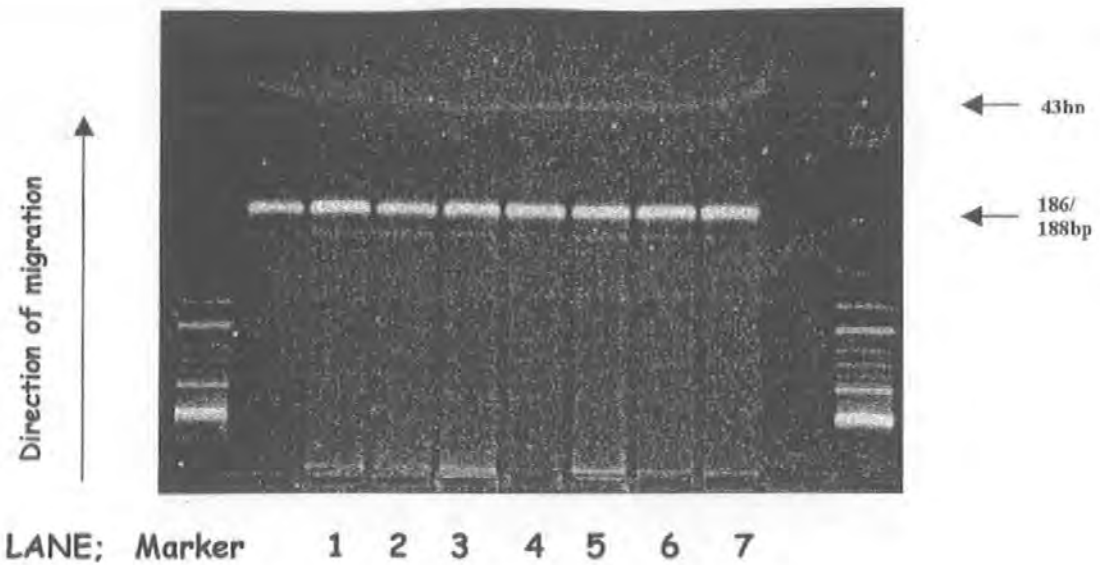
Figure 33. C(Mt5178)A polymorphism by restriction enzyme digestion and fragment separation on 2.5% agarose gel with ethidium bromide staining. Only one genotype was identified; homozygous CC. All subjects analysed in this figure have the CC genotype.

PCR was carried out to amplify the 417bp region of the mitochondrial genome. The region included a polymorphic site, a single base substitution of C to A and resultant amino acid exchange from Leu to Met. Five microlitres of the PCR product was digested overnight at 37°C with 10 units of Alu1 restriction enzyme (Promega, UK). The genotypes were identified by 2.5% agarose gel electrophoresis alongside a 100bp molecular weight marker and ethidium bromide staining. After Alu1 digestion the homozygous CC produced 43bp, 188bp and 186bp fragments. All samples were analysed in duplicated to reduce the risk of error.

C(Mt5178)A polymorphic marker in British Caucasoid T1DM subjects and normal controls

The polymorphism was not detected in the British Caucasoid T1DM or normal control populations studied here. No analysis could therefore be made with regard to the onset of diabetic microvascular disease with respect to this polymorphism.

C(Mt5178)A polymorphic marker in British Caucasoid T1DM subjects and normal controls

The polymorphism was not detected in the British Caucasoid T1DM or normal control populations studied here.

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