Autopsy Study of Islet Amyloidosis and Diabetic Glomerulopathy in Relation to Candidate Genetic Markers

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胰岛淀粉样变性和糖尿病肾小球病的遗传标志研究

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ABSTRACT

BACKGROUND AND OBJECTIVES: Type 2 diabetes mellitus (T2DM) is a complex disease with genetic predisposition and histopathological characterization. Pancreatic islet amyloidosis, hyaline arteriolosclerosis, and diabetic glomerulopathy are histopathological hallmarks of T2DM at autopsy examination. The associations of genetic variants with diabetic amyloidosis, arteriosclerosis and glomerulopathy have not been fully elucidated. Several candidate genes including apolipoprotein E (*ApoE*), insulin degrading-enzyme (*IDE*) and glucose transporter-1 (*GLUT1*) have been reported to increase risk of T2DM in human studies although results are not always consistent. Capitalizing on the pathological hallmarks of T2DM, I used autopsy specimens to investigate the risk associations of polymorphisms of *ApoE* (rs429358 and rs7412), *IDE* (rs6583813) and *GLUT1* (rs710218) genes with clinical features and specific pathological changes in diabetic kidney and pancreas. I further explored the mechanisms of these associations by evaluating the histopathological changes and protein expression in pancreas and kidney.

METHODS AND MATERIALS: Genomic DNA was extracted from white blood cell-concentrated paraffin embedded formalin fixed spleen tissues. Genotyping for *ApoE*, *IDE* and *GLUT1* polymorphisms was determined by polymerase chain reaction (PCR) and ligase detection reaction (LDR). The pathological changes were blindly assessed in pancreatic and kidney tissues of autopsy specimens. Protein expression of these genes was examined by immunostaining and quantified by using Metamorph image analysis system.

RESULTS: In a consecutive study population of 3693 autopsy specimens containing 328 T2DM and 209 control cases, the respective frequencies of genotypes were as follows: 1) TT of *GLUT1* rs710218: 11.2% vs. 11.3%; 2) *ApoE ε2*: 19.4% vs. 10.9%; 3) *ApoE ε4:* 12.1% vs. 9.1% and 4) C carriers of *IDE* rs6583813: 51.2% vs. 47.9%. The key genotype-phenotype correlations were as follows. 1) In the T2DM cases, *GLUT1* rs710218 TT genotype carriers (0% in TT genotype vs. 59.1% in AA genotype, P=0.0407) were less likely but *ApoE ε2* allele carriers (57.1% in *ε2* allele carriers vs. 23.5% in *ε3* allele carriers P=0.0382) were more likely to have diabetic glomerular hypertrophy than referential group. *ApoE ε2* carriers showed increased glomerular ApoE protein expression with the immunoreactivity found mainly in the mesangial regions and nodular lesions. On the other hand, *ApoE* ε3/ε4 cases had

diffuse ApoE expression in glomerular capillaries. 2) *ApoE ε4* carriers were more likely to have islet amyloidosis than non-carriers (62.5% in *ε4* allele carriers vs. 23.6% in *ε3* allele carriers P=0.0232). There was immunolocalization of the chaperone proteins, amyloid P and ApoE in both islet amyloid deposits and arterial walls with hyaline arteriolosclerosis. 3) In T2DM cases, *IDE* rs6583813 C allele carriers had higher prevalence of vascular disorders [hypertension (67.4% vs. 43.6%, P=0.0332), death due to cardiovascular disease (58.1% vs. 25.6%, P=0.0479) and cerebral vascular accident (CVA) (20.9% vs. 2.4%, P=0.0412)] than T allele carriers.

CONCLUSIONS: These findings suggest that genetic factors have important effects in the development of tissue-specific changes and chronic complications in T2DM. Islet amyloidosis, arteriosclerosis and glomerulosclerosis in T2DM may share common pathogenetic processes as suggested by the coexistence of chaperone proteins, amyloid P and ApoE. Genetic–pathologic correlation studies are useful in advancing our understanding of the mechanisms of complex diseases such as T2DM.

中文摘要

研究背景與目的：在屍檢中，胰腺胰島中的澱粉樣變性，血管玻璃樣變性和糖尿病性腎小球病是 II 型糖尿病的特征性病理改變。這些病理特征的發病機制目前尚未完全闡明。近年來，許多有關於糖尿病發生發展的新候選基因不斷被發現，比如載脂蛋白 E (ApoE),胰島素降解酶 (IDE) 以及葡萄糖運載體-1 (GLUT1)。臨床流行病學研究已經發現這些基因和糖尿病的關系，遺傳因子和糖尿病組織學改變的關系從未報道。本研究使用了屍檢標本探討 ApoE (rs429358 和 rs7412), IDE (rs6583813)以及 GLUT1 (rs710218)的基因多態性與 T2DM 的病理改變關系。

研究方法：首先，從脾臟組織中提取基因組 DNA。然後通過聚合酶鏈反應和連接酶檢測反應確定 ApoE, IDE and GLUT1 基因多態性的基因分型。同時，觀察胰腺和腎臟的系統病理改變。最後，免疫組織化學或免疫熒光染色後，使用 Metamorph 軟件對這些基因的蛋白原位表達水平進行定量。

研究結果： 1）在二型糖尿病人中，攜帶 GLUT1 rs710218 TT 基因型的患者腎小球肥大的比例較低，携带 ApoE ε2 等位基因的，腎小球肥大的比例較高，而且腎小球中 ApoE 蛋白的表達水平較高。ApoE ε2 等位基因攜帶者的 ApoE 蛋白在腎小球系膜區和結節性病變中表達明顯，相對而言 ApoE ε3/ε4 基因型患者的腎小球中 ApoE 蛋白彌漫性表達在腎小球毛細血管壁上。 2）T2DM 患者中，ApoE ε4 等位基因攜帶者更容易發生胰島澱粉樣變性。作為澱粉樣變性的伴侶蛋白，澱粉樣物質 P 和 ApoE 蛋白也表達在血管壁的玻璃樣變性病變中。這一發現說明玻璃樣變性和澱粉樣變性也許有著相似的伴侶分子以及生物化學特性。3）IDE C 等位基因攜帶者有著較高的心腦血管疾病（高血壓，冠狀動脈性心臟病，冠狀動脈硬化，心血管疾病死因和腦血管意外）發生率。心腦血管疾病在 T2DM 中也十分重要。

結論：本研究顯示遺傳因子對於 T2DM 的特征性病變的發生有著重要的作用。T2DM 的特征病理改變如胰島澱粉樣變性和小動脈玻璃樣變性可能有著共同的分子伴侶。由於 II 型糖尿病是多因子復雜性疾病，遺傳因子和病理改變的相關性研究可揭示基因多態性導致的組織結構損傷，從而明確 T2DM 及其並發癥的發生機制。

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ABBREVIATIONS

ACE	Angiotensin-converting enzyme
AD	Alzheimer's disease
AGE	Advanced glycosylation end product
ANOVA	One-way analysis of variance
ANP	Atrial natriuretic peptide
ApoA	Apolipoprotein A
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ARIC	Atherosclerosis Risk in Communities Study
BMI	Body mass index
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CAD	Coronary artery disease
CARE	Cholesterol and Recurrent Events Study
CAS	Coronary arteriosclerosis
CCK	Cholecystokinin octapeptide
CHD	Coronary heart disease
CI	Confidence interval
CM	Chylomicron
CPT-1	Carnitine palmitoyl transferase-1
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
DAB	3,3'-Diaminobenzidine
DCCT	Diabetes Control and Complications Trial
DECODE	Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria
DGS	Diffuse glomerulosclerosis
DM	Diabetes mellitus
DN	Diabetic nephropathy
ECM	Extracellular martrix
EDC	Epidemiology of Diabetes Complications Study
ER	Endoplasmic reticulum

ESRD	End stage renal disease
EURODIAB	European Diabetes studies
FAM	Carboxyfluorescein
FFA	Free fatty acid
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
FITC	Fluorescein isothiocyanate
FPG	Fasting plasma glucose
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
GGS	Global glomerulosclerosis
GIGT	Gestational impaired glucose tolerance
GK	Goto-Kakizaki
GLUT1	Glucose transporter-1
GWAS	Genome Wide Association Study
H&E	Haemtoxylin and eosin
HbA1c	Glycosylated hemoglobin
HDL	High-density lipoprotein
HDS	Hypertension in Diabetes Study
HNF	Hepatocyte nuclear factor
HOPE	Heart Outcomes and Prevention Evaluation Study
HOT	Hypertension Optimal Treatment Trial
HRP	Horseradish peroxidase
HSPG	Heparan sulfate proteoglycan
IAPP	Islet amyloid polypeptide
IDE	Insulin degrading-enzyme
IDL	Intermediate density lipoprotein
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IMT	Intimal-medial wall thickness
IPF	Insulin promoter factor
KWN	Kimmelstiel-Wilson nodule
LDL	Low-density lipoprotein
sd-LDL	Small dense low-density lipoprotein

LDR	Ligase detection reaction
LPL	Lipoprotein lipase
LVH	Left ventricular hypertrophy
MDRD	Modification of Diet in Renal Disease
MODY	Maturity-onset diabetes of the young
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NGS	Nodular glomerulosclerosis
e-NO	Endothelial-nitric oxide
OGTT	Oral glucose tolerance test
OR	Odds ratio
PAS	Periodic acid-schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEACE	Prevention of Events with Angiotensin-Converting Enzyme Inhibition Trial
PG	Plasma glucose
PK	Proteinase K
PKC	Protein kinase C
PPARG	Peroxisome proliferator-activated receptor G
PWH	Prince of Wales Hospital
RAAS	Renin-angiotensin-aldosterone system
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
SNS	Sympathetic nervous system
SPSS	Statistics Package for the Social Sciences
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCF	Transcription factor
TCF7L2	Transcription factor 7-like 2
TG	Triglyceride
TGF-β	Transforming growth factor-β

TGF-α	Transforming growth factor-α
TNF-α	Tumor necrosis factor-α
UAE	Urinary albumin excretion
UCP2	Uncoupling protein-2
UKPDS	United Kingdom Prospective Diabetes Study
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cells
WHO	World Health Organization
ZDF	Zuker diabetic fatty

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CHAPTER 1 INTRODUCTION

1. INTRODUCTION

1.1. Background

1.1.1. Overview of diabetes

1.1.1.1. Classification of diabetes

Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia, usually resulting from defects in insulin secretion or insulin action, or both [1]. Many hereditable and environmental factors are involved in the development and progression of diabetes. Based on aetiopathological processes, diabetes mellitus can be classified into four main categories: type 1 diabetes (T1DM), type 2 diabetes (T2DM), gestational diabetes (first diagnosed during pregnancy) and other specific types. Both T1DM and T2DM have strong genetic backgrounds often precipitated by lifestyle or environmental factors [2]. Type 1 diabetes, previously known as insulin-dependent diabetes, often results from autoimmune destruction of pancreatic islet beta-cells mediated by CD8+ T cells and macrophages, triggered by external factors, such as viruses or cow's milk antigens [3]. Thus, T1DM is characterized by proneness to ketoacidosis due to severe insulin deficiency [4]. Type 2 diabetes, previously known as non-insulin-dependent diabetes, is the major form of diabetes characterized by insulin resistance in various target tissues and beta-cell dysfunction. While genetic factors are important predisposing factors of T2DM, other precipitating and perpetuating factors include glucotoxicity, lipotoxicity, mitochondrial dysfunction, insulin resistance and amyloid formation, which can worsen beta-cell dysfunction. Gestational diabetes encompasses subgroups formerly classified as gestational impaired glucose tolerance (GIGT) and gestational diabetes [5]. Other specific types of diabetes include monogenic disease due to genetic defects implicated in beta-cell development, structure and function as well as insulin action. Other aetiologies include diseases of the exocrine pancreas, endocrinopathies, drug- or chemical-induced diabetes, infection-induced diabetes, uncommon forms of immune-mediated diabetes and other genetic syndromes with diabetes as a component feature [6].

Over time, undiagnosed or suboptimally managed diabetes can result in acute and chronic complications. Acute complications include diabetic ketoacidosis, nonketotic hyperosmolar coma, hypoglycemia and diabetic coma. Chronic hyperglycemia leads to widespread angiopathies through a number of mechanisms including the formation of glycosylated protein in the surface of endothelial cells. Damage to small blood vessels (microvascular disease) leads to retinopathy, neuropathy, nephropathy and

cardiomyopathy. On the other hand, destruction of large blood vessels (macrovacular disease) results in coronary artery disease (CAD), stroke, peripheral vascular disease and diabetic myonecrosis. Both vasculopathy and neuropathy contribute to diabetic foot or/and skin ulcers, infection and necrosis. Among these complications, cardiovascular and kidney diseases are the two most devastating complications leading to multiple morbidities and premature mortality [7]. In this thesis, my main focus is on T2DM which is referred to as 'diabetes' throughout the text, unless otherwise stated.

1.1.1.2. History of diabetes as an amyloid disease

Diabetes mellitus is known to human beings even in the prehistoric times. Diabetes was first mentioned by Egyptians about 3500 years ago. An Egyptian papyrus described a disease characterized by rapid weight loss and frequent urination. The term, 'Diabetes', was first mentioned by a Greek physician (30-90 Christian Era) who identified a disease with symptoms of polydipsia, polyuria and weight loss. Similar descriptions have also been recorded in the ancient civilizations of Egypt, Greece, India, Rome and China. In the Middle Ages, a clinical diagnosis of diabetes was an invariable death sentence [8]. By the early 19^{th} century, availability of a chemical test made detection of excess sugar in urine possible. During the same period, the French physician, Bouchardat, noticed that a restricted diet helped to reduce glycosuria in diabetic patients [9]. In 1869, microscopic studies by the German Pathologist, Paul Langerhans, first revealed the composition of the endocrine pancreas although its function was unknown [10]. Two decades later, the endocrine pancreas was referred to the "islet of Langerhans" by Gustave-Edouard LaGuesse (1861-1922) when he discussed the anatomy of the pancreas.

In 1889, Oskar Minkowski found that dogs developed diabetes upon removal of their pancreas. Further studies led to the conclusion that the pancreas secreted a substance which might affect glucose metabolism [11]. In 1901, another major step was taken by an American pathologist, Eugene Opie, who clearly established the link between diabetes and deposits of hyaline materials in the islet of Langerhans [12]. The amyloid nature of these material was established by Ahronheim in 1943 [13]. In 1961, Ehrlich JC and Ratner IM stained these deposits using alkaline Congo red [14]. In 1973, Westermark P demonstrated the fibrillar structure of islet amyloid in diabetes using electron microscopy [15]. In 1987, the amyloidogenic protein in islet amyloid

was discovered in two separate laboratories and referred to as islet amyloid polypeptide (IAPP) by Westermark P [16] and amylin by Cooper GJS [17]. It is now clearly established that human IAPP is a 37-amino acid polypeptide co-produced and co-secreted with insulin from pancreatic islet beta-cells [18].

1.1.1.3. Histological hallmark in diabetic nephropathy

The other histopathological hallmark of diabetes is nodular glomerulosclerosis or the Kimmelstiel-Wilson nodule (KWN) in the kidney. The latter was named in honour of the two pathologists, Kimmelstiel P and Wilson C, who published their clinical and pathological study of diabetic nephropathy in 1936 [19]. From a histopathological standpoint, the detection of islet amyloid is pathognomonic of T2DM whereas that of KWN signifies a progressive kidney disease caused by longstanding diabetes. Somewhat paradoxically, despite these well characterized histological features and recent advancement in the field of the genetics of T2DM, there is a paucity of knowledge regarding the correlation between genetic variants and these hallmark histopathological changes which may provide important insights into the understanding of disease mechanisms.

1.1.2. Epidemiology of islet amyloidosis and diabetic glomerulosclerosis

1.1.2.1. Epidemiology of diabetes

Diabetes is a global epidemic which poses major threats to public health in the 21st century. The global prevalence of diabetes is approximately 5.9%, affecting 246 million people worldwide. According to the World Health Organization (WHO), the number of people with diabetes will increase to 360 million by 2030. The majority will have T2DM with less than 10% of affected people having T1DM and other types of diabetes. The proportion of T2DM may be as high as 85-95% in developing countries. Alarmingly, the onset age of T2DM is becoming younger and a large proportion of affected people are in their middle age [20]. For the same number of risk factors, people with T2DM have 2-4 fold higher risk of CHD [21] and stroke [22] compared to those without. Diabetes, mainly T2DM, is also the leading cause of end stage renal disease (ESRD) [23]. On an annual basis, approximately 3.8 million people die from diabetes and related causes. This number is expected to increase by more than 50% in the next decade if no urgent action is taken to curb this rising trend of diabetes prevalence [20].

1.1.2.2. Epidemiology of islet amyloidosis

Even before the discovery of insulin from the pancreatic islets, islet amyloid (islet hyalinization) had been linked to diabetes [24]. Nowadays, islet amyloid is considered a pathological hallmark of T2DM [25, 26]. Islet amyloid in diabetic human pancreas has been reported across different ethnic populations including Caucasians, Pima Indians, Indians, Chinese, Japanese and other Asians [27]. Table 1 summarises the prevalence of diabetes-associated islet amyloidosis in human autopsy studies reported from 1950 to May 2007 [28]. Meta-analysis of these studies showed that the prevalence of islet amyloid was 37% in diabetic cases and 7% in nondiabetic cases after adjusting for sample size in each study [odds ratio (OR)=7.82, 95%CI=6.83-8.95, P<0.0001). Asian diabetic patients appeared to have lower prevalence (48% versus 81%, OR=0.21, 95%CI=0.15-0.31, P<0.0001) of islet amyloid than their Caucasian counterparts.

Table 1 Prevalence of diabetes-associated islet amyloidosis in autopsy studies [28]

Diabetic Patients	Nondiabetic subjects	References
26 (1684)	7 (3959)	Bell, 1952
39 (72)	0 (0)	Mclean and Ogilvie, 1955
49 (91)	4 (178)	Erlich and Ratner, 1961
56 (16)	0 (20)	Vishwanathan et al, 1972
91 (11)	0 (16)	Westermark and Grimelius, 1973
77 (26)	27 (18)	Saito et al, 1979
59 (27)	12 (142)	Maloy et al, 1981
92 (13)	55 (11)	Westermark et al, 1987
92 (24)	0 (10)	Clark et al, 1987
87 (15)	0 (10)	Clark et al, 1988
77 (26)	0 (10)	Clark et al, 1990
90 (111)	45 (22)	Rochen et al, 1992
76 (37)	8.3 (12)	Ohsawa et al, 1992
57 (41)	28 (33)	Sempoux et al, 2001
83 (57)	11 (48)	Butler et al, 2003
40 (235)	3 (533)	Zhao et al, 2003
37 (2486)	7 (5021)	Sum

Data are expressed as percentage of total number of cases or control in parentheses.

1.1.2.3. Diabetic glomerulosclerosis

Diabetic nephropathy (DN) refers to a group of functional and structural renal abnormalities caused by diabetes. Clinical DN is classically defined by the presence of proteinuria >0.5g/24 hours [29]. It is the leading cause of ESRD and renal replacement therapy in USA [30]. In Hong Kong, over 30% of patients receiving dialysis have diabetes [31]. The structural changes in DN include mesangial expansion, glomerular hypertrophy, nodular glomerulosclerosis (NGS), diffuse glomerulosclerosis [32], arteriosclerosis, basement membrane thickening, tubulointerstitial atrophy and fibrosis. Renal structural alterations are correlated with clinical parameters including persistent proteinuria, systemic hypertension, and progressive loss of renal function [33-35]. In these lesions, the hallmark of diabetic glomerulopathy, NGS, firstly described by Kimmelstiel and Wilson [36] in 1936, was regarded as pathognomonic for DN [34]. In different ethnic populations, the prevalence of NGS varies from 17.6% to 77.1% (Table 2). The variance may be due to differences in inclusion criteria, study samples and detection methods.

Table 2 Prevalence of classical diabetic glomerulopathy in Type 2 diabetic patients

No of patients	Stage of DN	Nodular lesions	Reference
35	UAE>300mg/day	77.1	[37]
52	Overt nephropathy	36.5	[38]
65	Persistent proteinuria	46.2	[39]
33	Persistent proteinuria	60.6	[40]
205	Autopsy	17.6	[41]
53	Microalbuminuria	29.4	[42]
74	Autopsy	39.2	[43]
200	Proteinuria	40	[44]
36	Proteinuria	47.4	[45]
55	Autopsy	30.9	[46]
158	Diabetic nephropathy	27.2	[47]

UAE: urinary albumin excretion

1.1.3. Aetiological factors

Diabetes, often associated with dyslipidemia and hypertension, is considered a prototype of complex diseases due to interplay between genetic susceptibilities and unhealthy lifestyle. It is characterized by beta-cell dysfunction and insulin resistance in peripheral tissues including skeletal muscles [48], adipose tissue [49], liver [50] and pancreatic islet itself [51]. The additive effects of these metabolic, inflammatory and haemodyanmic perturbations can lead to abnormal structure and function of multiple organs resulting in widespread complications.

1.1.3.1. Genetic susceptibilities

1.1.3.1.1. Inherited diabetes

Familial segregation and disease concordance in twin studies strongly support the heritability of diabetes, especially T2DM. In monozygotic twin studies, T2DM showed high concordance with a disease incidence of 70% compared to 20-30% in dizygotic twins in USA [52] and in Finland [53]. Other evidence of genetic contribution include familial aggregation of diabetes with higher disease prevalence or incidence in subjects with positive family history compared to the general population [54]. The life-time risk of T2DM is approximately 40% in individuals with one diabetic parent. This risk increases to 70% if both parents are affected [55, 56]. These concordance rate of T2DM in affected families tend to increase with prolonged observation [53]. Compared to individuals without parental diabetes, the odds ratio of T2DM in the offspring of an affected father was 3.5 (95%CI=2.3-5.2) and that of an affected mother, 3.4 (95%CI=2.3-4.9) [57]. The odds ratio increased further to 6.1 (95%CI=2.9-13.0) if both parents were affected [57].

The marked variations in diabetes prevalence in different populations highlight the complex nature of diabetes [58]. While variations in distributions and frequencies of risk loci may contribute to differing inter-population disease susceptibilities, the variations in disease prevalence in populations with similar genetic background suggest that additional factors such as lifestyles or environment may also be important. Using Chinese as an example, the prevalence of diabetes at any one time tends to be higher in Chinese living in affluent societies such as Hong Kong and Taiwan compared to Mainland Chinese [59]. In admixed populations, the prevalence of T2DM also tends to be higher in full-blooded Nauruan than those with mixed blood due to inter-marriage. Similarly, the prevalence of T2DM tends to decrease in Pima

Indians after interbreeding with European Americans [60].

The genetic factors of T2DM can be divided into two groups: monogenic and polygenic. Monogenic factors cause diabetes by a single genetic mutation and are characterized by early onset of disease and high phenotypic penetrance. Polygenic T2DM typically results from interplay between genetic susceptibility and environmental adversity. Patients with polygenic diabetes often harbour multiple risk variants and develop disease during middle or old age [61].

1.1.3.1.2. Importance of genetic study

Genetic studies provide important insights into the understanding of molecular mechanisms of diabetes. These risk variants can lead to discovery of causal metabolic pathyways which will lead to targets for intervention including drug discovery. On a shorter term basis, such knowledge may enable clinicians to predict risk, classify disease and formulate the most appropriate strategy to maximize benefits and minimize harm.

There are specific categories of diabetes due to hereditary defects affecting a particular pathyway. These forms of disease, often referred to as monogenic diabetes, are due to rare mutations within the family and characterized by strong familial inheritance, early onset of disease and high penetrance of the phenotype. There are many forms of monogenic diabetes but the most recognized one is maturity-onset diabetes of the young (MODY 1-6) [62]. Here, apart from MODY 2 which is caused by genetic defects in the enzyme glucokinase, other forms of MODY are due to mutations in transcription factors which include hepatocyte nuclear factor (*HNF-4 alpha,* MODY 1), transcription factor 1 (*TCF1*, MODY 3), insulin promoter factor-1 (*IPF-1*, MODY 4), transcription factor 2 (*TCF2*, MODY 5) and NeuroD1 (MODY 6). Due to their strong familial penetrence and because many of these patients require insulin treatment to control hyperglycemia, genetic screening and diagnosis of MODY is implicated in subjects with typical presentation (i.e. young onset typically less than 25-years-old, non-ketotic presentation and positive history in multiple generations) [62].

1.1.3.1.3. Gene-environment interaction

In T2DM, structural variations (e.g. SNP, deletion/insertin polymorphisms, copy number variations, CNV) or dysregulation of genomic expression (e.g. chromatin

modification and DNA methylation) interact with lifestyle or environmental factors, notably obesity often due to physical inactivity and excessive caloric intake, to cause overt hyperglycemia [63].

James Neel first proposed the thrifty gene hypothesis to explain the role of gene-environment interaction in the evolutionary nature of T2DM. Based on the phenomenon that defects in erythrocytes protect the mutation bearers from malarial infection, it was theorized that the susceptibility genes of diabetes might enable the carriers to store calories efficiently. This genetic trait is conducive to survival during times of famines and thus evolutionally preserved. However, in modern societies, food abundance, technologies and reduced need of strenuous exercise contribute to a positive energy state rendering these preserved genes to become disadvantages resulting in obesity and diabetes [64].

In common chronic diseases, genetic effects can modify biological responses to environmental stressors, medications and disease processes. Although the effects of each and individual genetic variants may be modest on their own, they may be amplified in the presence of other causative/modifier genes or triggering environmental factors to precipitate clinical disease [63]. Thus, the genetic makeup of an individual may play a pivotal role in his/her responses to these acquired or endogenous processes (e.g. aging or disease state). One such example is the genetic variation of peroxisome proliferator-activated receptor gamma (*PPARG*) where different genotype carriers display different patterns of fat intake and obesity. A C/G variant in this gene predicts the substitution of Ala for Pro at position 12 in the *PPARG*2-specific exon B [65]. The protective effect of the Ala allele for T2DM is influenced by the degree of ingestion of saturated fat [66]. When the dietary polyunsaturated fat to saturated fat ratio is low, the body mass index (BMI) in Ala carriers is greater than the Pro homozygotes. On the other hand, when the dietary ratio is high, the opposite is seen [67]. Other examples are pharmacogenetic responses to drugs which may allow individualization of therapies. Carriers of risk alleles of TCF7L2 have reduced insulin response [68] and suboptimal response to sulfonylurea treatment [69].

1.1.3.1.4. Strategies of the genetic research in diabetes

Classically, association studies and genome scans have been used to discover the genetic basis of T2DM [61]. The association study based on selection of candidate

genes is primarily hypothesis-driven while the genome scan aims to discover novel genes or chromosomal loci linked to various disease traits. In association study, a candidate gene usually with known biological functions and gene structure is selected. This is then followed by examination of mutations or polymorphisms, usually located within the coding or regulatory region, in a case-control cohort for genotype-phenotype associations. Using these strategies, novel genes such as glucokinase which regulates glucose sensing in the beta cell and NEUROD which regulates beta cell neogenesis have been discovered [70, 71]. However, in complex and polygenic diseases, these association studies often have limited power due to the small effects of these risk variants and the heterogeneity of host and environmental/lifestyle factors. With the recent development of high throughput technology, Genome-Wide Association Study (GWAS) using microarrays containing more than 500,000 SNPs can be used to study large populations. To date, more than 30 T2DM genes have been discovered by GWAS. The majority of these novel genes are implicated in insulin secretion, beta-cell biology and cell cycle regulation [66]. Although microarrays used in GWAS cover most of the known SNPs in the human genome, most of these disease-associated SNPs only confer 15-20% increased risk and collectively, account for less than 20% of the heritability of T2DM. While more powerful tools such as whole genome or whole exon sequencing are now being advocated as preferred discovery tools, regulatory mechanisms of genomic expression and genomic architectures such as copy number variations, non-coding RNAs, DNA methylation and histone acetylation are also being intensively studied in the field of complex disease such as T2DM [72, 73].

1.1.3.2. Hyperglycemia

Hyperglycemia is characterized by chronic elevation of blood glucose. While there are multiple stress hormones which can elevate blood glucose (eg. catecholamines, glucagon, cortisol, growth hormone), insulin is the only hormone that can reduce blood glucose. Subjects who have defects either in insulin secretion or action are at risk of developing diabetes [74]. Other factors such as eating disorders [75], drug intake [76], critical illness [77], and physiological stress [78] may contribute to increased blood glucose.

Clinically, hyperglycemia is the defining characteristics of diabetes [74]. Persistent high blood glucose is contributed by both fasting and postprandial hyperglycemia in

varying degrees and can be measured by glycosylated hemoglobin (HbA_{1c}). In T2DM, hyperglycemia can lead to acute (e.g. hyperglycemic hyperosmolar nonketotic syndrome) and chronic complications, notably vasculopathy and neuropathy which play pivotal roles in maintaining normal structure and functions of tissues and organs. In the United Kingdom Prospective Diabetes Study (UKPDS), a 0.9% difference in HbA_{1c} between the intensively-treated (7%) and conventionally-treated group (7.9%) was translated to 10%-25% risk reduction in all diabetes-related endpoints including death, macro- and microvascular complications [79].

1.1.3.2.1. Hyperglycemia and beta-cell dysfunction

Apart from its adverse effects on vasculopathy, hyperglycemia also contributes to beta-cell dysfunction although it is not the initial factor for beta-cell death. In this respect, glucose desensitization, beta-cell exhaustion and glucotoxicity may contribute to progressive beta cell failure to set up a vicious cycle. Glucose desensitization is a physiological adaptive mechanism referring to rapid and reversible refractoriness of the beta-cell exocytotic machinery after exposure to elevated glucose for a short period of time [80]. The mechanism of desensitization may result from dysregulation of intracellular calcium [Ca^{2+}] homeostasis which is a crucial sigal for insulin secretion [81]. Beta-cell exhaustion refers to depletion of releasable insulin from vesicles after persistent and excessive secretory signals despite increased insulin synthesis [82]. Persistent glucotoxicity may cause impaired beta-cell function or/and loss of beta-cell mass due to apoptosis or endoplasmic reticulum stress [83, 84].

In support of this notion, there was little evidence to suggest insufficient insulin content or defective insulin secretion when insulin-secreting cells were cultured in medium with different glucose concentrations for a short period of time. However, with prolonged exposure, the insulin secretory capacity was reduced. Besides, the degree of beta cell recovery appears to be time-dependent with shorter the period of antecedent glucose toxicity, the greater the degree of recovery [85]. In sum, glucose toxicity to beta-cells appears to be a result of continuous exposure rather than a threshold function of blood glucose. Possible mechanisms of glucotoxicity include impaired insulin gene expression through diminished activity of beta-cell transcription factors, loss of differentiation of beta-cells, generation of reactive oxygen species and accumulation of advanced glycosylation end products [83]. Besides, glucotoxicity and lipotoxicity can act in concert to contribute to beta-cell dysfunction and development

of diabetes [83].

1.1.3.2.2. Hyperglycemia and diabetic complications

Hyperglycemia is one of the key causal factors in the development of diabetic vascular complications through multiple pathways. Long-term diabetic complications correlate with the severity and duration of hyperglycemia. Patients with suboptimal glycemic control are at higher risk of developing microvascular complications in the kidneys, eyes and peripheral nerves as well as macrovascular complications notably CVD, than those with good control [86].

1.1.3.2.2.1. Hyperglycemia and microvascular complications

In 1993, data from the Diabetes Control and Complications Trial (DCCT) in T1DM and in 1998, data from the UKPDS in T2DM demonstrated the linkage between hyperglycemia and microvascular complications in randomized clinical trial settings. In the DCCT, intensively-treated T1DM patients with a target to achieve near normoglycemia using multiple insulin injections or insulin pump, had 60% risk reduction in all microvascular complications compared to the conventionally-treated group. These effects were independent of age, sex or duration of diabetes. There was also no apparent threshold for the blood glucose value in delaying the onset or slowing the progression of these complications [87, 88].

Similar findings were also reported in the 7-year study of the UKPDS where T2DM patients treated intensively with an achieved A1c level of 7% had 25% risk reduction in all microvascular complications, compared to the conventionally-treated group with an attained A1c level of 7.9%, [79, 89]. In agreement with the DCCT and UKPDS, other studies including the Blue Mountains Eye Study, Australian Diabetes, Obesity and Life Style, and the Multi-Ethnic Study of Atherosclerosis conducted in four different diabetic groups (including T1DM and T2DM) also did not demonstrate clear glycemic threshold for onset or progression of diabetic retinopathy [90].

There are several mechanisms whereby hyperglycemia contributes to the development of microvascular complications. These include the sorbitol pathway, protein kinase C (PKC) pathway, the advanced glycosylation end product (AGE) hypothesis and oxidative stress. The sorbitol pathway was the first proposed mechanism link hyperglycemia to the complications [91, 92]. Sorbitol is one of the products of the aldose reductase pathway with nicotinamide adenine dinucleotide

phosphate (NADPH) as cofactor of the reaction. Under hyperglycemic condition, there is increased activity of aldose reductase with elevated sorbitol levels which can lead to dysregulation of cellular energy metabolism and cell-membrane integrity [89]. Hyperglycemia can also activate the PKC system through increased diacylglycerol signals. Activation of the PKC system can lead to dysergulation of growth factors with abnormal DNA synthesis and transcription as well as cellular turnover. Furthermore, PKC can promote expression of transforming growth factor-beta (TGF-beta) resulting in increased extracellular matrix production [93, 94]. In chronic hyperglycemia, excess glucose may bind to circulating proteins (early glycosylation) to form AGE after a series of rearrangements and oxidative reactions. AGE can cause cross-linking of proteins which affect cellular function and are widely found in plasma, cells, tissues, arterial wall, renal mesangium and basement membranes [89].

1.1.3.2.2.2. Hyperglycemia and macrovascular complications

In the primary results of the DCCT and UKPDS, intensively-treated T1DM and T2DM patients had lower risk of macrovascular events compared to the conventionally-treated group, albeit short of significance [79, 95]. However, post-trial data from both studies have recently confirmed the long term benefits of glycemic control on cardiovascular complications and all-cause mortality [96, 97]. These randomized trial data are in line with the observed risk associations between 2-hour post-challenge blood glucose level and risk of CVD in epidemiological surveys including the Hoorn Study in Netherlands [98], the Honolulu Heart Study in men of Japanese ancestry [98], the Chicago Heart Study in Americans, and the DECODE (Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe) study [98]. In meta-analysis, hyperglycemia, especially postprandial hyperglycemia, has been shown to be an independent risk factor for CVD [98].

Macrovascular complications may share similar mechanisms with microvascular complications, such as the adverse effects of AGE, oxidative stress and the PKC pathway albeit with different emphasis. The effects of hyperglycemia on endothelial cell dysfunction have been extensively investigated. High blood glucose can activate nuclear factor-κB (NF-κB) in endothelial cells, vacular smooth muscle cells (VSMC) and macrophages, which can lead to inflammation and atherosclerosis. Both hyperglycemia and AGE can activate the oxidative pathway and stimulate endothelial cells to produce superoxide [99, 100]. For VSMC, hyperglycemia activates

matrix-degrading metalloproteinase and stimulates VSMC proliferation and migration [101, 102]. Glucose can also activate NF-κB and PKC in monocytes, stimulate monocyte growth and release inflammatory factors [103].

1.1.3.3. Dyslipidemia

Dyslipidemia is characterized by abnormal quantity and quality of lipid particles and content in the blood. Apart from lipoprotein overproduction (hyperlipidemia) or deficiency (hypolipidemia), a large number of pathways are involved in the clearance and precessing of these lipid particles. The Fredrickson classification divides hyperlipidemias into five types with different patterns of distribution of lipoproteins [104], all of which can be found in T2DM. The commonest lipid phenotype is increased triglyceride (TG) and reduced high-density lipoprotein cholesterol (HDL-C) [105]. In the Framingham Heart Study, diabetic subjects had higher prevalence of hypertriglyceridemia (men: 19% versus 9%; women: 17% versus 8%) and low HDL-C level (men: 21% versus 12%; women: 25% versus 10%) than control subjects. Although both groups had similar LDL-C levels, diabetic patients are known to have increased numbers of small dense low-density lipoprotein (sd-LDL) particles [106]. Similar results have also been reported in the UKPDS [107].

Although the exact aetiological factors of diabetic dyslipidemia are unknown, several studies point to the important role of insulin resistance. Increased energy intake or high fat diet or excessive lipolytic activities can cause increased delivery of free fatty acid (FFA) to the liver resulting in excess production of TG-rich very low density lipoprotein cholesterol (VLDL-C) and ApoB rich lipoprotein particles. Insulin resistance, in part due to fuel competition between FFA and glucose as energy substrates, can cause reduced lipoprotein lipase (LPL) activity leading to increased TG, low intermediate density lipoprotein cholesterol (IDL-C) and low HDL-C. Insulin resistance can also enhance hepatic lipase (HL) activity to promote transfer of TG to LDL-C and HDL-C particles. This leads to increased production of atherogenic small dense LDL-C particles which are rich in apo B content as well as that of small dense HDL particles with reduced reverse lipid transport from lipid-laden macrophages from endothelial cells. Apart from this dyslipidemic pattern of high TG, low HDL-C, apo B rich LDL-C and TG-rich HDL-C particles, insulin resistant subjects also have reduced hepatic responsiveness to the inhibitory effects of insulin on ApoB secretion and VLDL-C synthesis. The resultant hepatic fatty infiltration can further impair the

clearance of circulating LDL-C, setting up a vicious cycle [105].

1.1.3.3.1. Dyslipidemia and beta-cell dysfunction

Like hyperglycemia, dyslipidemia also contributes to beta-cell dysfunction. Paolisso et al showed that high fasting FFAs predicted T2DM [108]. In experimental studies, increased levels of FFAs stimulate insulin secretion in normal individuals but lead to beta-cell failure in individuals with genetic susceptibility to diabetes [84]. In *in vitro* studies, physiological concentrations of FFAs are toxic to isolated islets and insulin-secreting INS-1E cells. Prolonged exposure of pancreatic beta-cells to FFAs increases basal insulin release but inhibits glucose-induced insulin secretion. Excessive FFAs induce beta-cell death by apoptosis both *in vitro* (isolated rat islets) and in Zuker diabetic fatty (ZDF) rats [83]. Several mechanisms underlie the lipotoxicity to beta-cells. Free fatty acid oxidation is normally mediated by carnitine palmitoyl transferase-1 (CPT-1), the latter normally inhibited by malonyl CoA. Under diabetic conditions, elevated FFAs result in increased malonyl CoA, reduced FFA acid oxidation and elevated long-chain acyl CoA esters [84]. The latter is proposed to mediate the deleterious effects of chronically elevated FFAs, such as suppression of glucose-induced depolarization in beta-cells [109]. Prolonged exposure to FFAs also enhances uncoupling protein-2 (UCP2) expression, increases production of reactive oxygen species (ROS) production [84], activates the ER stress pathway, such as inositol-requiring enzyme-1(IRE1), and causes electron-lucent clefts which extend throughout the cytoplasm and ER lumen in insulin-secreting INS-1 cells [110]. Since the ER is the main cell organelle responsible for processing protein, ER stress can lead to protein misfolding which may also underlie lipotoxicity to beta-cells [111]. In summary, dyslipidemia and hyperglycemia have independent and joint toxic effects on beta-cells through multiple mechanisms including inhibition of oxidation of FFAs, decreased insulin gene expression, accelerated cellular apoptosis and ER stress.

1.1.3.3.2. Dyslipidemia and diabetic complications

1.1.3.3.2.1. Dyslipidemia and macrovascular complication

Both epidemiological and interventional studies have firmly established the independent risk association between diabetes and CVD [112-114]. Diabetic patients had the same risk of myocardial infarction as non-diabetic patients with previous myocardial infarction [115]. In the epidemiological analysis of the UKPDS, high

LDL-C (hazard ratio of 1.57 for every 1 mmol/L increase) and decreased HDL-C (hazard ratio of 1.15 with every 0.1 mmol/L decrease) were the most important risk factors for CVD followed by HbA_{1c} (hazard ratio of 1.11 for every 1% increase) and systolic blood pressure (hazard ratio of 1.15 for every 10 mmHg increase) [116].

Large scale randomized clinical trials, such as Cholesterol and Recurrent Events (CARE) study, have confirmed the benefits of LDL-C lowering using statins in reducing CVD incidence [117]. In the Helsinki Heart Study, diabetic men treated with gemfibrozil, a fibrate, which predominantly lowers TG and to some extent, increases HDL-C, had lower incidence of CHD than placebo-treated patients [118]. In the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) [70] study, fenofibrate significantly reduced CVD risk in subjects with elevated TG and low HDL-C levels [119]. Based on this evidence, several international bodies such as the American Diabetes Association, have recommended the need to control blood lipid levels in diabetic patients asymptomatic of vascular disease [119].

1.1.3.3.2.2. Dyslipidemia and microvascular complication

Apart from macrovascular complications, dyslipidemia is also a risk factor for diabetic microvascular disease, including nephropathy [120, 121]. Patients with diabetic nephropathy have adverse lipid profiles characterized by increased VLDL-C and LDL-C levels, small dense LDL-C and HDL-C particles [122]. In prospective studies, elevated levels of LDL-C and TG predicted decline in renal function while a high level of HDL-C was protective [122]. The protective effects of HDL-C may be attributed to the reverse cholesterol transport, in which HDL-C shuttles cholesterol from cells in the arterial wall to the liver [123]. Of note, the prediction of dyslipidemia in diabetic nephropathy may be different in different stages of diabetic nephropathy. In the Pittsburgh Epidemiology of Diabetes Complications (EDC) study, dyslipidemia predicted microalbuminuria onset within five years. While elevated LDL-C predicted increased albuminuria in T1DM patients with short disease duration, TG was a better predictor in patients with long disease duration [124, 125].

These deleterious effects of dyslipidemia on diabetic nephropathy may be mediated by several mechanisms. These include alterations in the coagulation-fibrinolysis system, damage in basement membrane and endothelial cells with consequent artherosclerosis [126]. Dyslipidemia can promote oxidation of LDL-C which can promote VSMC and mesangial cells proliferation, endothelial cell apoptosis, foam

cell formation, smooth muscle cell migration, and decreased nitric oxide bioavailability [127]. Compared with circulating lipid particles, local lipid synthesis may play a more important role in pathogenesis of diabetic nephropathy. Increased lipid synthesis in the renal system can lead to extracellular matrix accumulation, mesangial expansion and glomerulosclerosis [128].

1.1.3.4. Hypertension

Hypertension is a cardiovascular risk factor which frequently coexists with diabetes. Depending on definition, hypertension is present in about 70% of T2DM patients which is approximately two-fold higher than in individuals without diabetes [129, 130]. On the other hand, individuals with hypertension have 2.5 times higher risk of T2DM than individuals without hypertension [130]. Coexistence of hypertension and diabetes was also found in populations with increased risk of CHD, chronic kidney disease, ischemic cerebrovascular disease and retinopathy [131]. In the "Hypertension in Diabetes Study" (HDS), 5-10 mmHg lowing in blood pressure in T2DM patients reduced one-quarter of all-related endpoints, one-third of related deaths, one-half of stroke and heart failure, and one-third of microvascular disease [132].

1.1.3.4.1. Hypertension and diabetic complications

Hypertension is a major risk factor for diabetic macrovascular complication [132] and contributes to approximately 75% of CVD which is a leading cause of death in T2DM. Given their additive effects on development of complications [133], intensive control of blood pressure has been shown to reduce CVD morbidity and mortality as well as end stage renal disease (ESRD) in T2DM. In elderly T2DM patients, control of systolic blood pressure also reduced CVD morbidity [134]. In the Hypertension Optimal Treatment [135] trial which aimed to define the optimal level for control of diastolic blood pressure, reducing level to 85-90 mmHg reduced CVD morbidity in patients with T2DM compared to 90 mmHg. Similar benefits were not found in non-diabetic patients [136]. In the Systolic Hypertension in Europe (Syst Eur), T2DM patients had greater reduction in CVD mortality compared with nondiabetic subjects for the same level of controlled diastolic blood pressure [137].

Hypertension is a major risk factor for microvascular complications including nephropathy, neuropathy and retinopathy [138, 139]. In addition to hypertension, renal function is an independent risk factor of CVD [140]. Diabetic patients with

chronic kidney disease (CKD) are more likely to die from CVD than from ESRD [141]. In the Heart Outcomes and Prevention Evaluation (HOPE) study, patients with mild renal insufficiency had higher frequency of CVD (22.2%) than individuals with normal renal function (15.0%) [140]. In the Prevention of Events with Angiotensin-Converting Enzyme Inhibition (PEACE) trial, incidence of CVD increased in a stepwise fasion with the reducing glomerular filtration rate (GFR) [142].

A large number of epidemiological studies indicate that insulin resistance is a common feature in subjects with hypertension. In one of these surveys, 25-47% of hypertensive individuals have insulin resistance or impaired glucose tolerance [143]. This evidence suggests that insulin resistance may be a common link for hypertension and T2DM. Apart from its effect on glucose and lipid metabolism, insulin resistance can impair endothelium-dependent vasodilatation, vascular recruitment, and vasodilator responses [144, 145]. Diabetic patients often have upregulation of the renin-angiotensin-aldosterone system (RAAS) with increased angiotensin-II production. The latter can lead to impaired signaling of insulin and insulin-like growth factor-1 (IGF-1) through the PI3K/AKt pathway with reduced vasodilatation and glucose transport [146].

1.1.4. Pathology

1.1.4.1. Diabetic pancreas

1.1.4.1.1. Normal pancreas

Description of the normal structure of pancreas is essential in understanding changes that take place in diabetic pancreas. Pancreas is a solid organ with coated connective tissue tegument. The connective tissue extends and separates parenchymatous tissues into distinct lobes and lobules. In human, distinction of the lobules is not apparent. Parenchyma tissues include exocrine pancreas and endocrine pancreas. The exocrine pancreas plays an important role in digestion and secretes multiple digestive enzymes which are drained into the duodenum. The endocrine pancreas or islet of Langerhans is distinct cellular clusters with abundant blood supply and vasculature. Various hormones are secreted from islets into the circulation to regulate glucose metabolism [114].

Exocrine pancreas includes a duct system and acinar cells. About 85% of the pancreas is made up of acina cells and 2-4% by the duct system [147, 148]. The acina

cells are the fundamental secretory unit of the exocrine pancreas. Acinar cells are arranged in rounded nests to form acinis which are surrounded by basement membrane. Glandular cell exhibits features of a protein-synthesizing cell. The basal cytoplasm is basophilic due to copious ER while the apical cytoplasm is eosinophilic due to enrichment of aymogen granules. The duct system of exocrine pancreas includes the centroacinar cell-lining smallest duct (intercellular canaliculi) and progressively larger ducts (intercalated duct, intralobular duct, interlobular duct, main pancreatic duct, and duct of Wirsung and Santorini) [149].

In adults, the total islet mass is about 1-4% of the total pancreatic weight. The islet of Langerhans comprises about 90% endocrine cells while the remaining 10% are scattered amongst the acina cells, located in or near pancreatic ducts [149]. The islet size ranges from 50 to 250 μm in adults. On average, there are about 1000 endocrine cells in an islet with an average size of 140 μm in diameter [150]. In tissue sections derived from body or tail of pancreas, the proportions of different endocrine cell types are 60-80% for insulin-containing beta-cells, 15-20% for glucagon-containing α cells, 5-10% for somatostatin-containing δ cells, and less than 2% for pancreatic polypeptide-containing PP cells [151]. Alpha-cells and δ cells have close anatomical relationships and are mainly located in the periphery of the islets while the beta-cells tend to aggregate in the center of islets. There are one to five arterioles in each islet. These arterioles form glomerulus in islets to distribute nutritive materials, oxygen and secretory products. The main innervation of pancreas is vagus and splanchinic nerves [149] (Figure 1)[152].

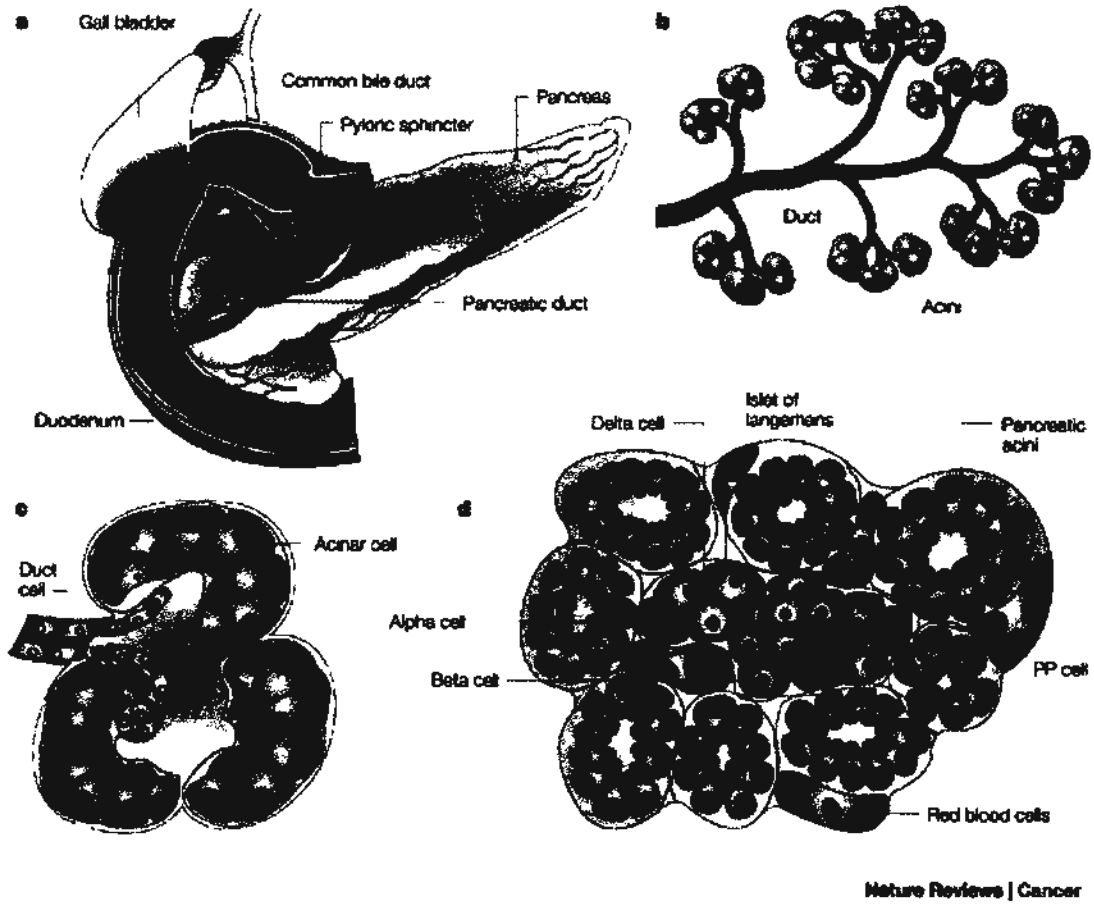


Figure 1 Anatomy of pancreas.

The exocrine pancreas consists of acinar and duct cells. The acinar cells produce digestive enzymes and constitute the bulk of the pancreatic tissue. The grape-like acini link to the centroacinar cells that are linking the smallest termini of the branching duct system. The endocrine pancreas consists of four specialized cell types (beta-cells, α cells, δ cells, and PP cells) which are organized into compact islets embedded within acinar tissue. These cells secrete various hormones into the bloodstream to regulate metabolism. (Panel a: gross anatomy of the pancreas; panel b: the exocrine pancreas; panel c: a single acinus; panel d: a pancreatic islet embedded in exocrine tissue) [152].

1.1.4.1.2. Diabetic pancreas

1.1.4.1.2.1. Diabetic islet

Grossly, the pancreatic weight and size were not significantly altered in T2DM [153]. However, the number of pancreatic islets has been reported to range from normal to 30-50% lower in T2DM patients than that of normal subjects [153, 154]. Using immunohistochemistry technique, researchers were able to identify individual cell types and reported 24% reduction in beta-cell population and 58% increase in glucagon-containing alpha-cells in T2DM patients [155]. The relative increase in alpha cells is associated with hyperglucagonemia which contributes to hyperglycemia in addition to insulin deficiency resulting from reduced beta-cell mass. The latter has been partially attributed to islet amyloidosis in T2DM [156].

Islet amyloidosis is one of the pathological hallmarks of T2DM and was firstly reported by Opie. In autopsy specimens of pancreas, islet of Langerhans showed peculiar hyaline degeneration [157] which are homogeneous pink materials in haemtoxylin and eosin (H&E) staining sections. In Congo red staining sections, these lesions are red-colored under light microscopy and show apple-green birefringence under polarizing microscopy. When observed under electron microscope, these amorphous materials appear as non-branching fibrils [158] (Figure 2). Amyloidosis occurs in many kinds of diseases involving multiple organs (systemic amyloidosis) or single organ (localized amyloidosis) [159]. Amyloidogenic protein or peptide undergoes structural transformation from soluble monomers, to oligomers, and eventually to fibrils which has predominantly beta-sheet structure [158, 160] (Figure 3) [161].

Each kind of amyloidosis has its unique protein. In T2DM pancreas, the major protein is a 37-amino acid polypeptide known as amylin or islet amyloid polypeptide (IAPP) [17, 162]. Amylin is an important neuroendocrine hormone colocalized and co-secreted with insulin in islet beta-cells [163]. Monomeric amylin regulates postprandial glycaemia level by reducing food intake, suppressing post-meal glucagon secretion and delaying stomach emptying time [164]. It is a highly conserved protein with only few changes in amino acids between species suggesting its biological importance. Interestingly, mouse and rat amylin do not form "beta-pleated sheet" and amyloid fibrils. By contrast, amylin from human, monkey, feline and canine share similar amino acid sequence 20-29 which can form amyloid [163], suggesting its possible pathogenetic role.

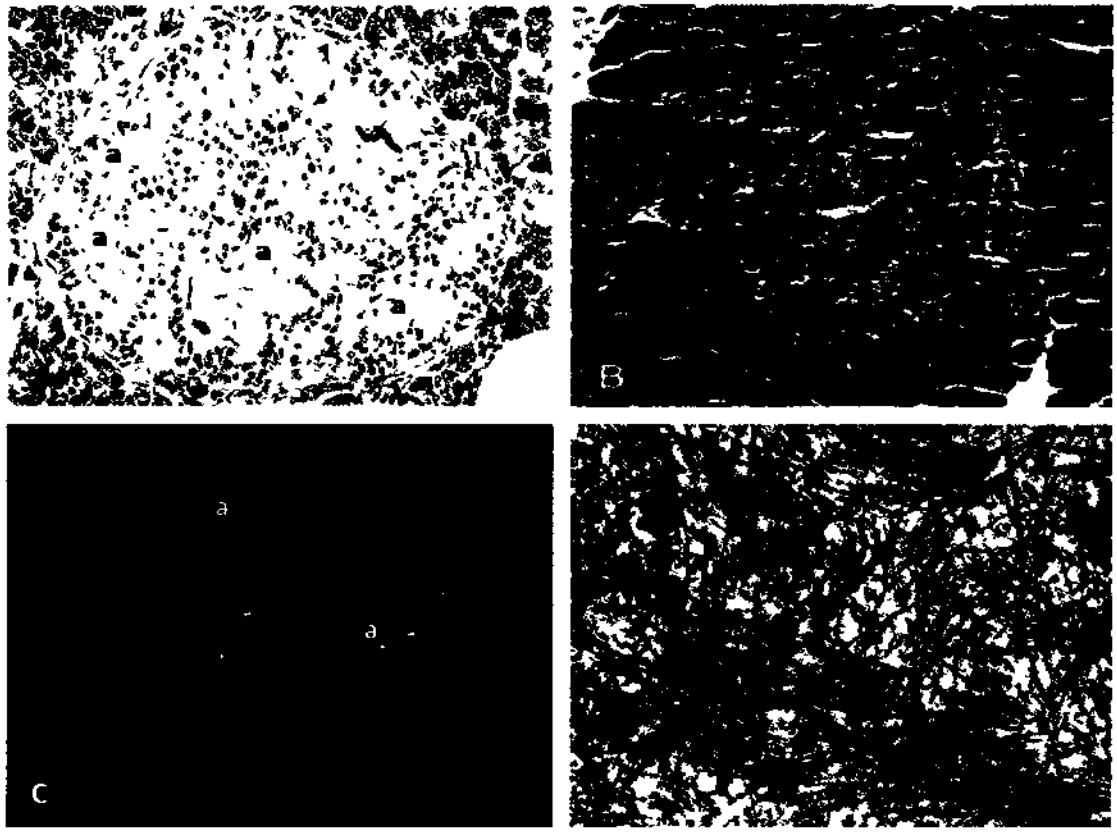


Figure 2 Islet amyloidosis

In autopsy specimens of pancreas, islet of Langerhans showed peculiar hyaline degeneration forming homogeneous pink materials (a) in H&E staining section (panel A). In Congo red staining section (panel B), these materials are red (a) under light microscopy and show apple-green birefringence (a) under polarizing microscopy (panel C). When observed under electron microscope (panel D), the amyloid deposits consist of non-branching fibrils

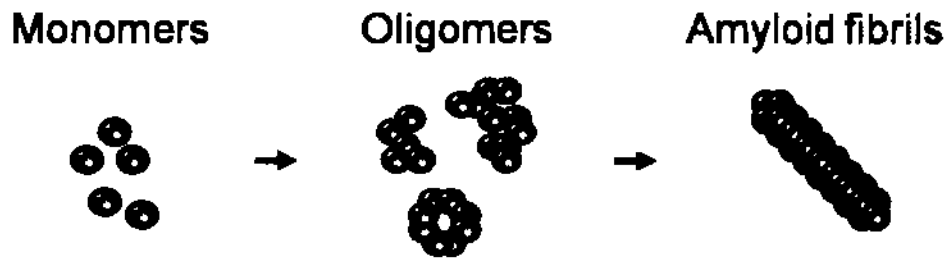


Figure 3 Proposed model for the aggregation states of hIAPP.

Amyloidogenic protein hIAPP undergoes structural transformation from soluble monomers, to oligomers, and eventually to fibrils which have predominantly beta-sheet structure.

1.1.4.1.2.2. Diabetic exocrine pancreas

Type 2 diabetic pancreas has a full spectrum of arteriosclerotic changes, including hyaline arteriolosclerosis (thickening of the arteriole wall due to deposition of glassy materials with scarred appearance) and atherosclerosis (vascular changes characterized by intimal thickening, lipid deposition and loss of elasticity of arterial walls). The histopathological changes of hyaline arteriolosclerosis include subendothelial exudation, intimal foam cell accumulation, and calcification. The prevalence of arteriosclerotic changes was slightly higher in pancreas with islet amyloid than those without. Fat infiltration, fibrosis, and chronic inflammatory infiltrates have also been found in diabetic pancreas. Furthermore, pancreatic fibrosis is often accompanied by islet amyloidosis, chronic inflammatory infiltrates, and acinar atrophy [27, 165]. Other interstitial lesions and exocrine atrophy can also distort the pancreatic microarchitecture with vascular insufficiency [166].

1.1.4.1.2.3. Pathogenesis of islet amyloidosis

1.1.4.1.2.3.1. Amyloid fibril formation

Although a number of studies focus on the pathogenesis of amyloidosis, the exact mechanism of amyloidogenesis remains elusive. In Macaca monkeys, islet amyloidosis precedes the spontaneous development of T2DM [167]. In both human and animal studies, the prevalence and degree of amyloidosis is positively correlated with clinical severity of T2DM [167, 168]. Islet amyloidosis is associated with decreased beta-cell function, loss of beta-cell mass, and increased beta-cell apoptosis [169]. In keeping with the potential toxic effects of amyloidosis on beta cells, mutation S20G in amylin gene is associated with high risk of T2DM, elevated amylin to insulin ratio, early occurrence of hyperglycemia, and severe T2DM [170]. Furthermore, synthetic S20G amylin has been shown to form amyloid more readily than the wild-type amylin and exerts greater biological cytotoxicity [171].

Despite these findings, the toxicity of amyloid deposition remains controversial. Some studies have failed to demonstrate relationship between amyloidosis and duration and/or severity of T2DM [158, 172]. In hIAPP homozygous transgenic model, mice developed diabetes due to increased beta-cell apoptosis without islet amyloidosis. In obese hemizygous hIAPP transgenic mice, no relationship was found between islet amyloidosis and beta-cell apoptosis. Other *in vitro* studies revealed that amyloid fibrils did not induce beta-cell apoptosis, but small non-fibril hIAPP

oligomers contribute to damage of cell membrane [158]. Thus, there is a need to distinguish the cytotoxic effects of these three distinct forms of amyloid: monomer, oligomer and fibrils. In this respect, increasing evidence implicates oligomers as the culprits for beta-cell dysfunction in T2DM [158] (Figure 3).

The islet amyloidosis in T2DM share common features with some neuro-degenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease, prion encephalopathy and amyotrophic lateral sclerosis. All these conditions show cell loss associated with aggregation of misfolded proteins [158]. Amyloid deposits in these diseases also share common components such as Apolipoprotein E (ApoE), serum amyloid P, heparan sulfate proteoglycan (HSPG), perlecan amongst other proteins and substances. It is likely that these components contribute to the organization of amyloid fibrils, some of which may be implicated in the pathogenesis of amyloidosis [173]. It is interesting to note these common components are structure specific but not amino acids specific, suggesting that common mechanisms may underlie amyloid fibril formation.

There is a growing body of evidence suggesting that ApoE may paly direct role in the fibril formation of amyloidosis with most of the evidence coming from studies in AD. ApoE is the lipid-binding protein of various lipoproteins including HDL, VLDL, and chylomicron (CM) in the regulation of TG and cholesterol metabolism. The ApoE gene exon 4 polymorphism is the major genetic variant associated with AD, in which Aβ is the amyloidogenic peptide [174]. ApoE has three common isoforms: ε2, ε3, and ε4. ApoE ε3 is the predominant isoform which accounts for about 80% of total ApoE content. The isoforms of ε2 and ε4 differ from ε3 by only one amino acid.

Compared to the reference isoform ε3 allele, there is a change from Cys to Arg in the 112^{th} residue of the ApoE ε4 isoform, which leads to arginine 61 being more exposed which facilitates its interaction with glutamic acid 255 in its carboxyl-terminal [174]. The ApoE ε4 carriers have higher level of TG and LDL-C than ApoE ε2 and ε3 carriers, Thus, its has been hypothesized that hypercholesterolemia in ApoE ε4 carriers may aggravate the accumulation of beta-amyloid [175]. Furthermore, ApoE ε4 has high affinity to beta-amyloid and amylin peptides and thus can promote aggregation process with possibly reduced transport and clearance of beta-amyloid [174]. In support of this notion, ApoE knock-out mice show decreased deposition of beta-amyloid [176]. Of note, the

association between T2DM and AD is particularly strong among ApoE ε4 allele carriers [177]. Given the shared risk factors of abnormal lipid metabolism and amyloid formation in both diseases, it is plausible that ApoE ε4 may exert similar effects on amyloid fibril formation in the pancreas.

1.1.4.1.2.3.2. Clearance of amyloid fibril

Amyloid deposition is dependent on the balance between fibril formation and fibril clearance. Insulin degredation enzyme (IDE) is a widely expressed zinc metalloprotease and was the first major enzyme discovered *in vivo* insulin proteolysis [178]. Glucagon is the next substrate found to be lysed by IDE [179]. Other small molecular weight proteins have also been found to be substrates of IDE which include amylin [180], atrial natriuretic peptide (ANP) [181], transforming growth factor-α (TGF-α) [182], insulin-like growth factor-I (IGF-I), and IGF-II [183]. Although the amino acid sequences of these substrates are significantly different, some of these proteins share three-dimensional structural properties similar to amyloid fibril.

In AD, IDE is found in pericytes, endothelial and cerebrovascular smooth muscle cells suggesting that it may contribute to cerebral amyloid angiopathy [184]. In the central nervous system and under physiological conditions, IDE is secreted at high levels from the microglial cells to degrade beta-amyloid in the extracellular compartments [185]. In AD, apart from reduced amount of hippocampal IDE protein, there is also reduced beta-amyloid degradation activity of IDE. In line with these findings, IDE knock-out mice have increased beta-amyloid load in the brain suggesting that IDE activity is critical in regulating beta-amyloid deposition *in vivo* [178]. Since amylin secreted by the islet β cells is also a substrate of IDE, it is plausible that genetic polymorphism of IDE may be implicated in islet amyloidosis in diabetic pancreas.

1.1.4.1.2.3.3. Islet homeostasis

In autopsy studies, islet amyloidosis is often accompanied by vascular changes and interstitial fibrosis. Here, alteration of the microarchitecture of the islets due to vasculopathy and neuropathy may contribute to amyloid formation due to ischemia and abnormal neuronal sensing. Notably, ApoE protects against the development of atherosclerosis, but this benefit depends on the ApoE isoform [174].

Abnormal lipid and lipoprotein metabolism is strongly implicated in atherosclerosis. Apo E is the main lipoprotein of TG rich lipid particles, such as CM and VLDL The presence of ApoE ε4 leads to rapid remnant catabolism with accelerated clearance of dietary fat and downregulation of LDL receptors [174]. The overall consequence is hypercholesterolaemia which is a major risk factor for atherosclerosis [174]. Many studies have demonstrated that ApoE ε4 is overrepresented in populations with hyperlipidemia, heart disease [174] and ischemic cerebral vascular disease [186]. Apart from elevating LDL level, ApoE ε4 can upregulate expression of angiotensin II receptor [187] and exhibit reduced efficiency in inhibiting proliferation of VSMC [188].

In the nervous system, ApoE plays an important role in tissue repair after nerve injury by redistributing lipids to regenerating axons and modulating neurite outgrowth *in vitro* [174]. In this connection, compared to non-carriers, ApoE ε4 carriers have poorer outcome after acute head trauma and stroke [189, 190], in part due to reduced ability for neuronal repair, remodeling and protection [191]. Based on these findings, we postulate that ApoE polymorphism may influence islet microenvironment by altering blood supply, innervation and interstitial condition to contribute to islet amyloidosis.

One possible mechanism underlying the close association between T2DM and CVD [192] may be related to the coupling of glucose metabolism and vasodilation [193]. Complementary to its role in glucose metabolism, insulin elicits vasodilating effects by promoting release of endothelial-nitric oxide (e-NO) [193]. Besides, insulin and IGF-1 share similar receptors and signaling pathways. While IGF-1 may possesse vasculoprotective effect [194], IGF-1 dysfunction has been associated with CVD [195]. Insulin is a pluripotent hormone with effects on cellular growth and sodium metabolism which may contribute to hypertension and CVD. In part due to activation of the sympathetic nervous system, hyperinsulinemia promotes sodium and water retention [196] which are often accompanied by increased natriuretic responses including release of atrial natriurteic peptide (ANP) from the right atrium [197, 198]. The latter can interact with the RAAS to increase glomerular filtration to increase urinary sodium and water excretion [199]. Adding to this complexity, many substrates of IDE including IGF1, e-NO, and ANP are implicated in cardiovascular and renal biology [200]. Intriguingly, amylin has also been linked to hypertension probably by the stimulation of the RAAS [201]. Hyperglucagonemia, which frequently coexists

with diabetes, also suppress NO production by coronary endothelial cells [202]. While the nature of these observations requires clarification, apart from having direct effects on clearance, IDE may also contribute to islet amyloid formation through vascular dysfunction.

1.1.4.2. Diabetic kidney

1.1.4.2.1. Normal kidney

The structure and functional unit of the kidney is the nephron comprising of glomerulus (mesangium, capillary and Bowman's capsule) and renal tubule (proximal tubule, thin limbs, and distal tubule). There are marked inter-individual variations in the number of nephrons [203]. There are two groups of nephrons: nephrons with a short and nephrons with a long loop. The length of the loop of Henle is generally related to the location of glomeruli in the cortex. Cortical nephrons located at the superficial renal cortex have a shorter medullary loop, while juxtamedullary nephrons locating near the renal medulla have a longer medullary loop [204].

The renal corpuscle consists of a tuft of interconnected capillaries and an enclosing capsule named after Bowman. The glomerulus presents a round configuration and the diameter is about 200μm [205]. The glomerular tuft originates from the afferent arteriole that enters the glomerulus at the vascular pole and divides into several lobules. The efferent arteriole is formed by rejoined capillaries and leaves the glomerulus at the vascular pole. The central region of the capillary tuft is occupied by mesangium that consists of intercapillary cells and their surrounding matrix materials. The capillaries are lined by a thin layer of endothelial cells with a basement membrane (GBM) and covered by podocytes. The glomerular filtration barrier includes the fenestrated endothelium, GBM, and the podocyte slit diaphragm. The permeability is high to water and small molecules but low to larger molecules such as albumin. At the urinary pole, the epithelial cell of the parietal layer in Bowman's capsule continues to form the lumen and epithelium of the proximal tubule [204] (Figure 4).

The renal tubule starts with the capsule of the glomerulus, and includes the proximal tubule, thin limbs of Henle's loop, distal tubule, connecting segment, and collecting duct. Each part of the renal tubule has its exclusive function in reabsorption, concentration, resorption of water, secretion and acid-base equilibrium function [204].

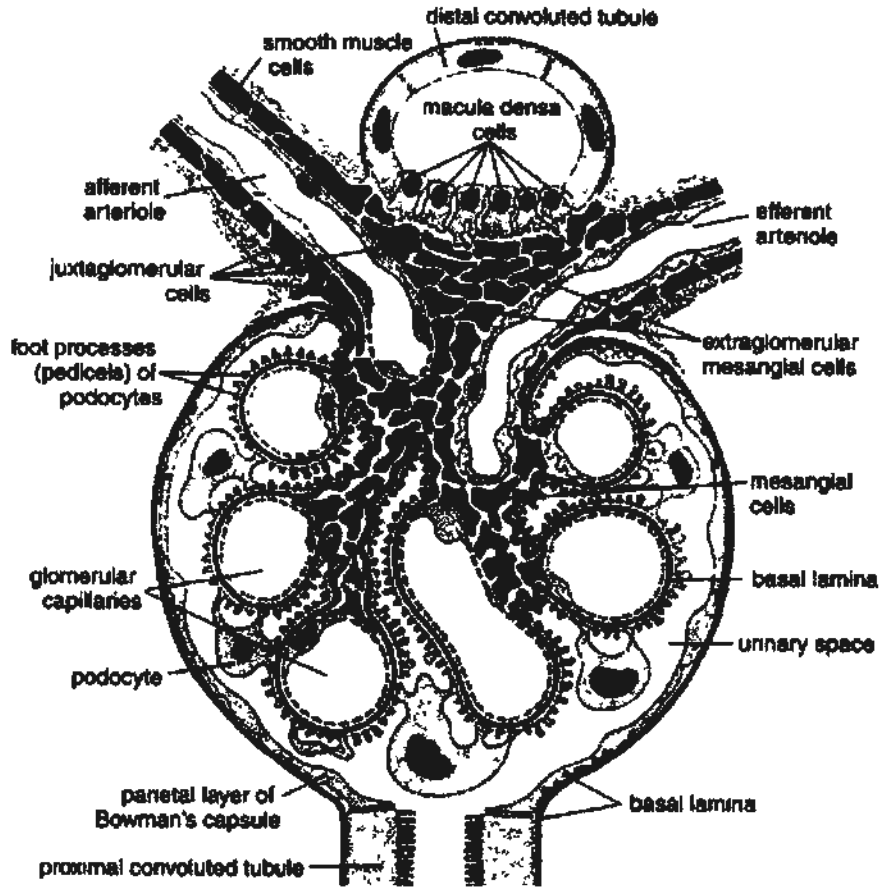


Figure 4 Renal corpuscle

The renal corpuscle consists of capillaries and a Bowman's capsule. The capillary tufts originate from the afferent arteriole and divide into several lobules, then rejoin and form the efferent arteriole. The central region of the capillary tuft is occupied by mesangium that consists of messagial cells and their surrounding matrix materials. The glomerular filtration barrier includes the fenestrated endothelium, GBM, and the podocyte slit diaphragm. At the urinary pole, the epithelial cells of Bowman's capsule form the proximal tubule.

http://anatomy.iupui.edu/courses/histo_D502/D502f04/lecture.f04/urinaryf04/urinaryf04.html

1.1.4.2.2. Diabetic kidney

1.1.4.2.2.1. Diabetic glomerulopathy

Light microscopy of early stage diabetic nephropathy shows glomerular hypertrophy (enlargement) characterized by GMB thickening and mesangial matrix expansion. The glomerular volume is enlarged by 70% compared to non-diabetic glomeruli [206]. With progression of the disease, there is overt glomerular damage which includes diffuse lesions, nodular lesions, and exudative lesions [207].

1.1.4.2.2.1.1. Diffuse lesion

Diffuse glomerulosclerosis [32] describes the widespread increase in eosinophilic, periodic acid-schiff (PAS) positive material in the mesangium. The thickening of the GBM can be found before the diagnosis of diabetes [208]. The diffuse GBM thickening and mesangial matrix expansion are accompanied by appearance of microalbuminuria. Bowman's capsule becomes fibrotic at advanced stages of diabetic glomerulopathy [207].

1.1.4.2.2.1.2. Nodular lesion

The nodular lesion was firstly described by Kimmelstiel and Wilson and is termed the Kimmelstiel-Wilson nodule (KWN) [209]. The presence of KWN is pathognomic of diabetes and indicates progressive diabetic kidney damage. The KWN is characterized by accumulation of homogeneous eosinophilic material in the mesangium to form round laminated nodules. This is in contrast to the diffuse mesangial expansion observed in diffuse glomerulosclerosis. The diameter of the nodule is on average about 40μm, and some nodules may be larger than 100μm. Large nodular lesions are usually acellular in the central region with lines of cells localized at the periphery [207]. Early and moderately nodular lesions with more cells in the nodules show increased expression of fibronectin, laminin, and types IV and V collagen. In late stage disease, there is a diminution in the mesangial matrix proteins except for type V collagen [210]. There are two populations of nodular lesions. The smaller, numerous nodules are more likely to arise from the expansion of mesangial materials under a background of diffuse lesions. On the other hand, the larger nodules may originate from microaneurysms [211, 212]. This change may coexist with mesangiolysis, the appearance of fibrillar materials, and consequently increased mesangial materials. The findings indicate that the factors relating to the pathogenesis

of microaneurysms may be involved in the formation of nodular lesions [211, 212].

1.1.4.2.2.1.3. Exudative lesions

The exudative lesion, such as the fibrin cap [213, 214], is due to accumulation of hyaline eosinophilic homogeneous materials (plasma fraction) between endothelial cells and the GBM of the capillary loops. The endothelial cells in the lesions often show vacuoles or protein droplets. The glomerular lobule containing exudative lesions usually shows adhesion with Bowman's capsule. The exudative lesion is frequent but not specific to diabetic nephropathy [211].

1.1.4.2.2.2. Vascular-interstitial changes in diabetic nephropathy

Glomerulosclerosis is often accompanied by tubular atrophy, interstitial fibrosis and arteriosclerosis. The tubular basement membrane adjacent to a sclerotic glomerulus is often thickened and laminated. The tubular epithelial cells are finely vacuolated and contain droplets. Occasionally protein casts are abundant in the tubular lumens. Vascular changes such as arteriosclerosis of muscular arteries and arteriolosclerosis of small arterioles are common findings in diabetic kidneys. Hyaline arteriolosclerosis is a frequent manifestation of early diabetic renal disease and more pronounced in diabetes than in other diseases. The differences from hyaline changes in hypertensive nephropathy where hyalinosis is confined to afferent glomerular arteriole, hyalinosis in the diabetic kidney is found in both afferent and efferent glomerular arterioles. Interstitial fibrosis is also common in diabetic nephropathy and may be accompanied by global glomerulosclerosis and chronic inflammatory infiltration [207]. Interstitial fibrosis is related to decline in the glomerular filtration rate (GFR) [215].

1.1.4.2.2.3. Pathogenesis of diabetic nephropathy

In the pathogenesis of renal disease, perturbations in metabolic, inflammatory and, hemodynamic pathways are frequently implicated, which often have genetic predisposition. Hyperglycemia is the most important pathological factor that distinguishes diabetic kidney disease from others forms of renal disease. In both the DCCT and UKPDS, intensive glycemic control was associated with reduced onset and progression of microalbuminuria in patients with T1DM and T2DM respectively [79, 89, 95, 216]. In the DCCT, HbA_{1c} level in the intensive and conventionally-treated group were 7.0% and 9.1% respectively. Intensively-treated

patients with normoalbuminria had 34% relative risk reduction in new onset of microalbuminuria and 44%, for overt proteinuria compared to the conventionally-treated group [95]. These clinical benefits persisted 8 years after the completion of DCCT [86, 216]. In the UKPDS, the HbA1c level in the intensively and conventionally-treated groups with 7.9% and 7% after 7 years of follow up with similar renal benefits as observed in T1DM [79]. Epidemiological analysis of the DCCT, UKPDS, and European Diabetes (EURODIAB) studies further showed continuous relationships between reduction in HbA_{1c} and risk of diabetic nephropathy with no apparent threshold value [217], thus emphasizing the critical importance of achieving optimal glycemic control to prevent diabetic nephropathy.

1.1.4.2.2.3.1. Glucose transporter and glucotoxicity

Approximately 20-25% of cardiac output goes to the kidney for excretory function. Glucose transporters promote glucose entry to the cells to provide energy and regulate glucose trafficking for various purposes [218]. In the kidney, there are several isoforms of glucose transporters which regulate glucose trafficking across the plasma membrane into cells. Several types of glucose transporter are expressed in glomeruli (Table 3) [218]. GLUT1 is the predominant isoform expressed in the glomerulus and is also found in mesangial cells, endothelial cells and podocytes [218]. Mesangial cells are crucial for maintenance of capillary structure and regulation of glomerular filtration. Under hyperglycemic conditions, the glomeruli show hypertrophy, diffuse basement membrane thickening, increased proliferation of mesangial cells and extracellular matrix expansion resulting in glomerulosclerosis. These expanded structural changes may account for the increased expression of GLUT1 under hyperglycemic condition [219]. Diabetic glomeruli are known to express increased vascular endothelial growth factor (VEGF) which can stimulate expression of TGF-β1. The latter in turn can upregulate the expression of GLUT1 to promote glucose uptake in mesangial cells for metabolism [219] [220].

High glucose exposure also induces increased GLUT1 expression in podocytes which regulate turnover of basement membrane and maintenance of glomerular filtration. The enhanced intracellular glucose concentration in podocytes may stimulate extracellular matrix (ECM) production and possible apoptosis in podocytes [218]. In contrast to retinal endothelial cells which do not show increased GLUT1 level in response to high glucose challege [221], GLUT1 can be detected in

glomerular endothelial cells although these results need further verification given the technical difficulty in isolating these cells in primary culture for detailed molecular study [222]. Considering the cytoarchitectural relation between glomerular endothelial cells and mesangial cells, enhanced glucose concentration of endothelial cells may contribute to the development of diabetic nephropathy by promoting ECM formation and activating profibrogenic cytokines in mesangial cells [218].

Table 3 Glucose transporter expression in whole glomeruli and in specific glomerular cell types

	Effect of diabetes	Mesangial cell	Podocyte	Endothelial cell
GLUT1	Increase	+	+	+
GLUT2	NA	NA	+	NA
GLUT4	Decrease/ Increase	+	+	NA
GLUT5	Increase	+	NA	NA
GLUT8	Increase	NA	+	NA
	NA	+	NA	NA

In diabetes, GLUT4 is decreased in mesangial cells and increased in podocytes.
GLUT: glucose transporter; NA: information not available.

1.1.4.2.2.3.2. Hemodynamic changes

Hemodynamic changes represent another important etiological factor in diabetic nephropathy. The coexistence of hypertension in diabetic patients markedly increases the frequency and severity of cardiac events, especially in the presence of microalbuminuria [223]. Untreated or inadequately controlled hypertension accelerates the decline of renal function in diabetic patients [224]. There is now strong evidence showing the independent effect of upregulation of the RAAS in the initiation and progression of diabetic nephropathy [225]. In both T1DM and T2DM, inhibition of the RAAS by angiotensin converting enzyme inhibitors or angiotensin II receptor blockers reduced progression of albuminuria, rate of decline in renal function and onset of cardio-renal complications [224].

Hyperglycemia is the major risk factor in the development of macro- and microvascular complications of T2DM in part due to the modifying effects of glucotoxicity on morphology and functions in endothelial and VSMC [132]. The predominant glucose transporter in vascular endothelial and vascular smooth muscle cells is GLUT1. Alteration in expression of GLUT1 may lead to increased intracellular glucose [226], excessive protein glycosylation and uncontrolled production of free radicals. The overall consequences are basement-membrane thickening, enhanced vascular permeability and occlusion followed by ischaemia and extracellular deposition with loss of structure and function of the vasculature [227]. In addition to its effects on large blood vessels, GLUT1 also alters the intra-glomerular hemodynamics through activation of inflammatory cytokines and growth factors such as VEGF, TGF-β1, and angiotensin II [228]. Activation of these cytokines may participate in the pathophysiological processes of vasodilatation, hyperfiltration, and mesangial matrix accumulation [228].

Another factor which may alter glomerular hemodynamic balance is ApoE. As described, ApoE ε4 is associated with hypercholesterolaemia and increased risk of cardiovascular complications [174, 186]. Although some researchers suggested a link between ApoE ε2 and diabetic nephropathy, others have reported a faster rate of progression in renal function in ApoE ε4 carriers compared to non-carriers [229, 230].

1.1.4.2.2.3.3. Lipotoxicity

Both cross-sectional and prospective studies have demonstrated the association of diabetic nephropathy with an adverse apolipoprotein profile characterized by

increased level of VLDL, LDL and small LDL particle size as well as reduced level and particle size of HDL [122]. As a major lipid transport protein, ApoE has been associated with the initiation and progression of diabetic nephropathy [231]. Epidemiological data have reported risk association of the ApoE ε2 allele with diabetic nephropathy [232], macroalbuminuria [233] and renal failure [234]. These correlations may result from the specific effects of the ApoE ε2 isoform on lipid metabolism. The genetic polymorphism of the ApoE ε2 isoform results in a change from Arg to Cys in residue 158 when compared with ApoE ε3 [174]. This amino acid change reduces the ability of ApoE ε2 to bind to its receptors, which contributes to the higher levels of TG and VLDL as well as decreased level of HDL in ApoE ε2 carriers [174]. These TG-rich lipoproteins can enhance glycosylated or oxidized LDL accumulation in mesangial cells [232] and macrophages [235], which in turn can activate various cytokines to promote accumulation of extracellular matrix and mesangial expansion. In this connection, TG and low HDL-C have been shown to be independent predictors for new onset of chronic kidney disease in T2DM [236]. Unlike other lipoproteins which are synthesized in liver and/or gut, ApoE is synthesized by many tissues, including kidney, brain, and lung and can influence a wide range of biological functions including platelet aggregation, lymphocyte activation, and foam cell formation which may also contribute to these risk associations [122].

In most association studies of ApoE polymorphisms and diabetic nephropathy, ApoE ε2 allele appears to be risk-conferring while ApoE ε4 was protective [229, 237, 238]. However, other researchers have reported accelerated rates of decline in renal function in ApoE ε4 carriers [229, 230]. Given the emerging role of lipotoxicity in diabetic nephropathy and the importance of genetic risk associated with lipid metabolism, further research is needed to clarify the risk association between ApoE genetic polymorphism and diabetic nephropathy.

1.2. Working hypothesis

Pancreatic amyloidosis, glomerulopathy and arteriolar hyalinosis are classical histopathological features of T2DM. In this thesis, I hypothesize that genetic variants encoding proteins implicated in intermediary metabolism and vascular biology may give rise to these histopathological changes.

In light of the close association between AD and T2DM, the importance of genetic

factors in AD and T2DM as well as the shared structural component of Aβ amyloid (ApoE) and pancreatic amyloid comprising of ApoE, I selected ApoE as a possible candidate gene in my study of islet amyloidosis.

Since amylin, insulin and various vasoactive hormones are substrates of IDE, this was also selected as a candidate gene for islet amyloidosis. In addition, I examined the risk association between IDE genetic variants and CVD risk in T2D and protein expression of some of its substrates.

Given multiple pathways involved in diabetic nephropathy including lipotoxicity, glucotoxicity and vasculopathy, I further examined the role of genetic variants of glucose transporter (GLUT1) as well as IDE and ApoE in renal morphology.

The following diagram summarises my overall understanding regarding the potential interrelationships amongst these vascular and metabolic pathways and histopathological changes.

Here, based on extensive review of the literature, I argue that genetic polymorphisms of ApoE can cause dyslipidemia and abnormal cellular repair which results in mesanagial activation, vasculopathy, and neuronal damage resulting in loss of structure and function of the pancreatic islets and renal tissues. Based on the known association of ApoE genetic polymorphisms with AD, genetic polymorphisms of ApoE also increase formation and reduce clearance of pancreatic amyloid. Apart from the possible effect of reducing the clearance of paneratic amyloid, genetic polymorphism of IDE may reduce the clearance of vasoactive and growth factors to increase CVD risk in T2DM. The loss of islet architecture due to direct toxic effects of amylin oliogmers and accumulation of amyloid can worsen hyperglycemia to induce renal dysfunction which can be further aggravated by the presence of genetic variants of GLUT1 (Figure 5).

1.3. Research objectives

Due to the invasive nature of the study of pathological changes in the diabetic pancreas and kidneys in human subjects, the examination of genetic-pathological correlations using autopsy specimens is a possible strategy to resolve some of the controversial issues such as relationships of ApoE polymorphisms and diabetic nephropathy and provide new insights into the mechanims of pancreatic amyloidosis.

In this thesis, I shall investigate the associations between genetic variants and

pancreatic/renal pathological changes in T2DM. Specifically, I shall test the following hypotheses:

(1) ApoE and IDE genetic polymorphisms are associated with islet amyloid.

(2) ApoE and GLUT1 polymorphisms are associated with diabetic glomerulopathy.

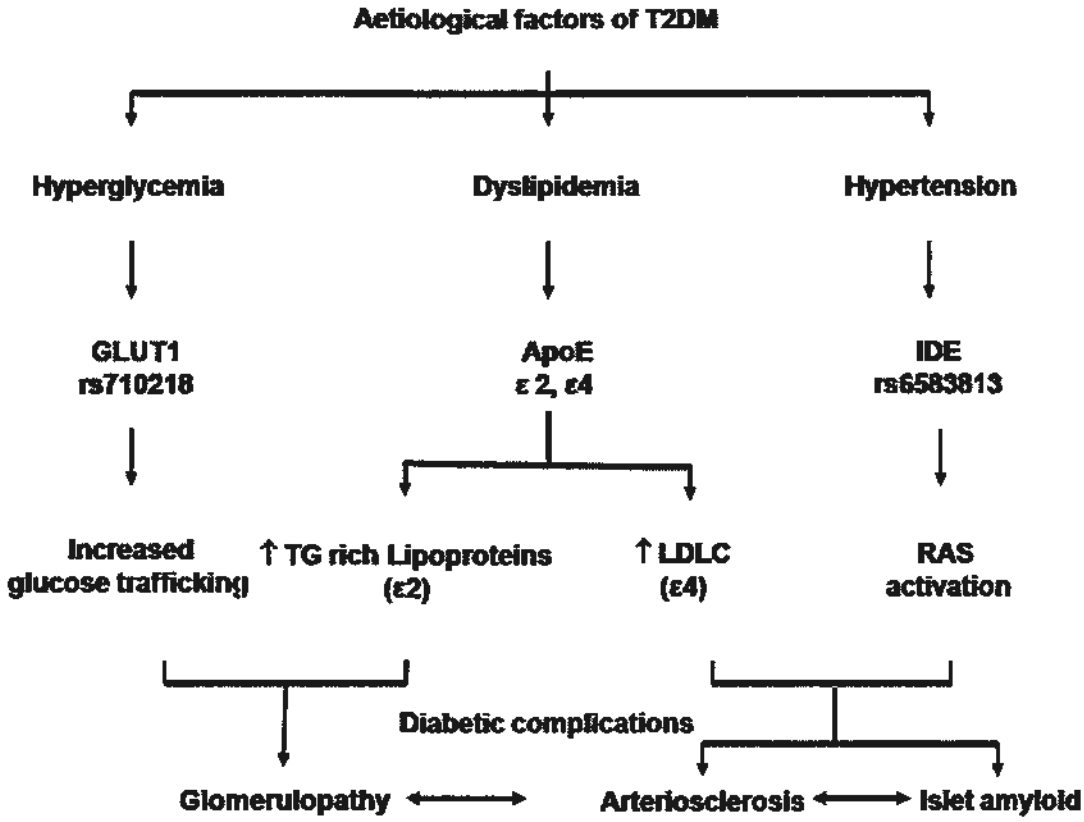


Figure 5 Hypothesis.

ApoE and IDE genetic polymorphisms may be associated with islet amyloid and vascular complications while ApoE and GLUT1 polymorphisms may be associated with renal pathological changes in T2DM.

CHAPTER 2 METHODOLOGY

2. METHODOLOGY

2.1. Research design

Since there is no quantifiable clinical feature that correlates with islet amyloid deposits and that pancreatic biopsy is not ethically acceptable, in this thesis, I used autopsy cases in all clinicopathological studies. Ethical approval for human tissue studies was in accordance to the institutional guidelines and granted by the Clinical Research Ethics Committee of The Chinese University of Hong Kong. Autopsy reports and clinical details were reviewed to extract relevant data of clinical characteristics and histopathological changes. Secondary diabetes and T1DM were excluded based on clinical and pathological information. In a consecutive study population of autopsy cases and based on clinical diagnosis listed in the autopsy report. T2DM and age- and sex-matched non-diabetic cases were selected according to predefined criteria [27].

I used both genotyping and histological examination to evaluate the genetic-pathological associations in the autopsy specimens of pancreas and kidney. DNA samples of diabetic and control cases were extracted from the nucleolus-rich spleen and/or liver tissues, followed by genotyping using PCR. In a subgroup of pancreatic and renal sections, the histological findings in the autopsy reports were ascertained by two histopathologists (Guan J and Zhao HL) with expertise in diabetic pathology.

I further used histological and immunohistochemical stains to detect in situ protein expression associated with pancreatic and renal structural changes. Stained sections were examined by light and immunofluorescence microscopy and quantified by the metamorph image system (MetaMorph 4.0 image acquisition program for Windows, 1999, Downingtown, PA).

Figure 6 summarises the overall research design.

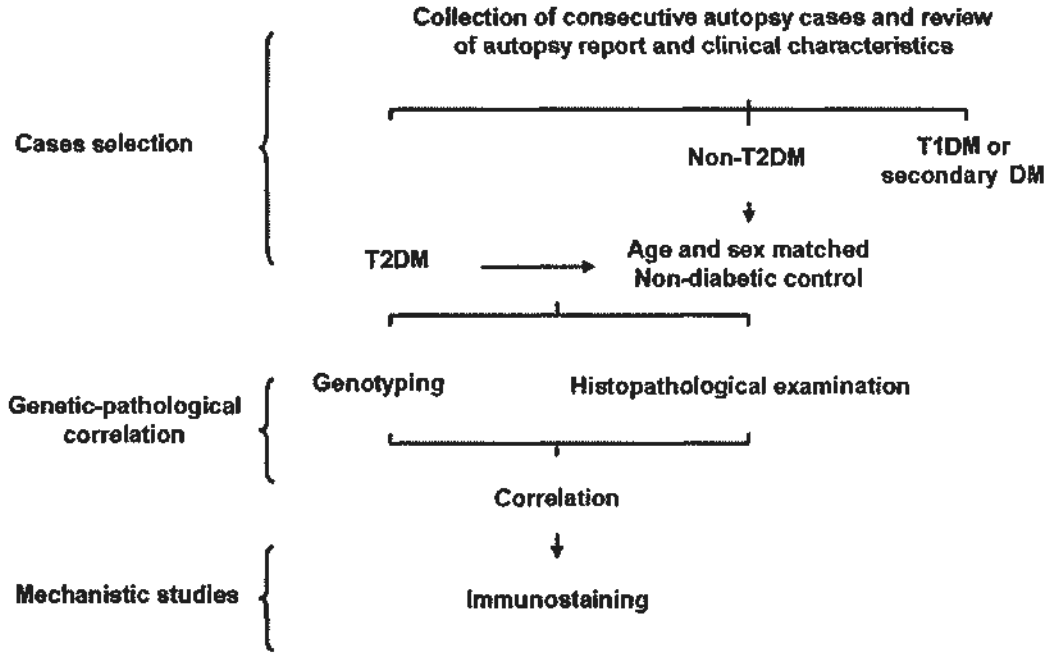


Figure 6 Summary of the overall research design.

A flow chart summarising the study design to examine the genetic-pathological and protein expression in autopsy samples of diabetic pancreas and kidneys.

2.2. Autopsy case selection

2.2.1 Study setting

In this thesis, all autopsy cases were archived at the Department of Anatomical and Cellular Pathology of the Prince of Wales Hospital (PWH) which is the teaching hospital of the Chinese University of Hong Kong. On average, 300-400 autopsy cases were performed on an annual basis at the PWH. About 10% of these cases had T2DM listed as one of the diagnoses in the autopsy reports. The CUHK Clinical Research Ethics Committee approved the use of these autopsy samples for genetic-pathological studies of T2DM. Since not all of the deaths at PWH were autopsied, we sought to minimize selection bias by including consecutive autopsy cases.

2.2.2 Diagnosis of T2DM

The diagnosis of T2DM was based on the clinical history of "T2DM" or "noninsulin dependent diabetes mellitus (NIDDM)" provided in the autopsy reports. I used autopsy cases archived during two periods (1984-1988 and 1994-1999) in this thesis. During these two periods totaling 11 years, 3693 autopsy cases were performed in the Department of Anatomical and Cellular Pathology in PWH. Autopsy reports in 1980s and 1990s were independently reviewed by two research staff (Guan J and Zhao HL) respectively. Among these cases, 328 cases had a clinical diagnosis of T2DM. Among the 3365 nondiabetic cases, 209 cases were selected and matched for age and sex (Table 4).

Table 4 Clinical characteristics of Type 2 diabetic and control cases

	T2DM (N=328)	Control (N=209)	*P* value
Age (years)	70.6±10.8	69.0±15.2	0.1752
Male	44.8 (147)	47.8 (100)	0.4922

Mean ± SD or % (*n*). Comparison was done by the Student *t*-test or the chi-square test.

2.2.3 Clinical assessments

Professor HL Zhao examined available clinical records of the T2DM and control cases and retrived available data on blood pressure, plasma glucose (PG), HbA_{1c} and renal function. All clinical data were collected using the last available results one

month before death to reflect the long term control of the disease. If results within one month of death were not available, the clinical data just before death were not included in this study. At the PWH, plasma creatinine was measured using the Jaffe method on a Dimension AR system (Dade Behring, Deerfield, IL, USA). The normal reference range was 0.5 – 0.9 mg/dL (44 – 80 μmol/L). Glomerular filtration function was estimated using the formula proposed by the Modification of Diet in Renal Disease (MDRD) Study Group (Glomerular filtration rate in mL/min/1.73m^2 = 170 × [plasma creatinine in mg/dL]$^{-0.999}$ × [age in year]$^{-0.176}$ × [0.762 if patient is female] × [plasma urea in mg/dL]$^{-0.170}$ × [plasma albumin in g/dL]$^{0.318}$) [239]. Hypertension was defined by a blood pressure ≥ 140/90 mmHg or treatment with antihypertensive medications.

Clinical details were not available for all autopsy cases. The numbers of cases with available clinical data are shown, as appropriate. All control cases were selected from samples archived during the 1984-1988 period. However, since clinical records before 1994 have been destroyed, clinical parameters in control cases were not available except for age and sex which were included in the autopsy reports.

2.2.4 Representativeness of study population for pancreatic changes

In the T2DM cases (n=328), pancreatic tissue sections were available for further investigation only in 117 cases. In the remaining 211 cases, either the tissue sections were missing or not suitable for examination. Except for a higher proportion of CVA in cases without pancreatic sections, both groups were largely comparable in terms of clinical and biochemical characteristics (Table 5). All control cases had pancreatic sections available for histological examination.

Table 5 Representativeness of 117 diabetic cases with pancreas sections

	Diabetic cases with pancreas sections	Diabetic cases without pancreas section	*P*-value
	N=117	N=211	
Age (years)	70.1±11.8	71.0±10.2	0.5008
Female	60.7 (71)	52.1 (110)	0.1358
	N=25	N=35	
BMI (kg/m^2)	24.0±5.5	22.5±3.8	0.2199
	N=57	N=114	
FPG (mmol/L)	12.4±6.1	11.1±4.9	0.1271
	N=12	N=28	
HbA_{1c} (%)	8.4±2.7	8.1±2.4	0.6809
	N=55	N=106	
Plasma creatinine (μmol/L)	156.0 (105.0-299.0)	149.0 (91.8-204.5)	0.1083
Plasma urea (mmol/L)	12.8 (7.7-21.4)	10.5 (6.2-18.4)	0.1301
Estimated GFR ($mL/min/1.73m^2$)	29.9 (14.9-50.0)	34.0 (20.8-57.4)	0.0565
	N=59	N=117	
Systolic blood pressure (mmHg)	146.2±37.6	148.3±39.1	0.7311
Diastolic blood pressure (mmHg)	78.2±18.2	79.2±22.1	0.7601
	N=115	N=211	
Hypertension	55.7 (64)	67.3 (142)	0.0372
	N=112	N=185	
Death due to cardiovascular disease	42.9 (48)	45.4 (84)	0.6684
	N=117	N=211	
CHD	53.0 (62)	63.5 (134)	0.0628
LVH	43.6 (51)	47.9 (101)	0.4568
CAS	40.2 (47)	47.9 (101)	0.1797
CVA	9.4 (11)	25.7 (54)	0.0004

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary

arteriosclerosis; CVA: cerebrovascular accident.

Data are presented as or mean±SD, medians (interquartile range) or % (n). They were compared by Student *t*-test, Mann–Whitney U test, Chi-square test or Fisher's exact test, as appropriate.

2.2.5 Representativeness of 51 re-examined diabetic cases for renal lesions study

In the diabetic cases, 51 cases with renal tissue sections available were recruited consecutively for detailed microscopic examination to validate the histological findings in the autopsy reports. There was no significant difference in clinical characteristics between the two groups (Table 6). All of the control cases had renal sections available for histological examinations.

Table 6 Representativeness of 51 T2DM cases in which renal lesions were re-examined

	Case with detailed microscopic examination	Case without detailed microscopic examination	*P*-value
	N=51	N=277	
Age (years)	69.2±13.2	70.9±10.3	0.3914
Female	52.9 (27)	55.6 (154)	0.7261
	N=11	N=49	
BMI (kg/m^2)	24.6±7.4	22.8±3.7	0.4534
	N=26	N=145	
FPG (mmol/L)	13.4±7.6	11.2±4.8	0.1799
	N=7	N=33	
HbA_{1c} (%)	8.7±2.9	8.1±2.4	0.5301
	N=24	N=137	
Plasma creatinine (μmol/L)	183.5 (106.8-287.3)	149.0 (96.5-236.5)	0.2877
Plasma urea (mmol/L)	12.2 (6.6-24.4)	11.1 (6.9-18.5)	0.5877
Estimated GFR ($mL/min/1.73m^2$)	29.0 (14.9-53.8)	33.9 (19.3-53.9)	0.2942
	N=51	N=252	
Diabetic nephropathy	70.6 (36)	65.5 (165)	0.4811
	N=27	N=149	
Systolic blood pressure (mmHg)	144.0±34.8	148.2±39.2	0.5992
Diastolic blood pressure (mmHg)	77.4±16.8	79.1±21.6	0.7033
	N=51	N=275	
Hypertension	54.9 (28)	64.7 (178)	0.1815
	N=51	N=246	
Death due to cardiovascular disease	43.1 (22)	44.7 (110)	0.8365
	N=51	N=277	
CHD	52.9 (27)	61.0 (169)	0.2802

LVH	49.0 (25)	45.8 (127)	0.6764
CAS	54.9 (28)	43.3 (120)	0.1267
CVA	11.8 (6)	21.4 (59)	0.1141

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident. Diagnosis of DN was given when the cases had histological changes of diabetic kidney disease [38].

Data are presented as or mean±SD, medians (interquartile range) or % (n). They were compared by Student t-test, Mann–Whitney U test, Chi-square test or Fisher's exact test, as appropriate.

2.3. Pathological assessment

2.3.1. Tissue processing

At least half of the postmortem examinations were performed within 24 hours after the patient's death. Representative specimens were taken from all selected cases during postmortem examinations. Tissue was fixed in 10% buffered formalin, dehydrated in gradient ethanol, lucidified in xylene and embedded in paraffin wax. Sections (4-6 μm) were cut on microtome and mounted on glass slides. Detailed protocol for tissue processing is included in **Appendix I-1** under the subheading of **tissue processing**.

2.3.2. Histopathological assessment

2.3.2.1 Hematoxylin and Eosin staining

Hematoxylin and Eosin (H&E) staining is the most popular staining method in diagnostic pathology. As a routine in diagnostic pathology, most of the diagnosis was made following tissue fixation, dehydration, embedding and H&E staining. Compared to other staining methods, H&E staining is inexpensive, convenient and provides most of the pathological information. In this thesis, pathological changes in the pancreas and kidney were described using H&E stained sections.

For H&E staining, tissue sections were deparaffinized and rehydrated, followed by H&E staining. After dehydration and lucidification, slides were mounted with histomount (**Appendix I-2**).

2.3.2.2 Thioflavin T staining

Thioflavin T is a dye used to visualize plaques composed of amyloid proteins. When it binds to amyloid fibrils, it can be selectively excited at 442 nm, resulting in a fluorescence signal at 482 nm [240]. This characteristic can be observed when amyloid fibrils are present. However, thioflavin T does not label precursor monomers, small oligmers or high beta sheet content [241]. In this study, we used thioflavin T to identify fibrillar amyloid deposits in pancreatic islets.

To identify islet amyloid deposits, sections (6μm) were deparaffinized and rehydrated, followed by staining with thioflavin T (10uM) and DAPI, as shown in **Appendix I-3.**

2.3.2.3 Definition of pathological changes

Table 7 and 8 list the criteria for various histopathological diagnoses of renal and pancreatic changes in T2DM and control cases [205-207]. All the pathological changes were assessed by pathologists who were blind to diabetic status and genotypes. Diagnosis of DN was given when the cases had histological changes of diabetic kidney disease [38]. Death due to renal disease or CVD was defined by the same diagnosis listed as the primary cause of death in the autopsy report.

Table 7 Diagnosis of different pathological changes in kidneys [205-207]

Diagnosis	Definition
Glomerulopathy	
GGS	Sclerotic lesions involving most if not all of the glomerular tuft (>50%) and were not segments (Figure 7, panel A)
NGS	Single or multiple nodules in the glomeruli. These are round, homogenous and eosinophilic masses located in the centrolobular areas. With time, the nodules become laminated and acellular, in which case, only a rim of peripheral mesangial nuclei is visible. (Figure 7, panel B)
DGS	Enlarged glomeruli with expanded mesangial areas and diffusely thickened basement membranes giving finger-like appearance (Figure 7, panel C)
Glomerular hypertrophy	Increase in the size of glomeruli (Figure 7, panel D)
Tubulointerstitial lesion	
Hyaline arteriolosclerosis	Thickening of the arteriolar wall due to deposition of glassy, homogeneous materials (Figure 8, panel A)
Arteriosclerosis	Vascular changes characterized by thickening of arterial walls with loss of elasticity (Figure 8, panel B)
Tubular lesion	Tubular atrophy with thickened basement membranes and occasional protein casts in the lumen (Figure 8, Panel C)
Interstitial fibrosis	Excessive fibrosis in the connective tissue within the interstitium (Figure 8, panel D)
Near normal structure	No apparent pathological change apart from those related to age (Figure 7 and Figure 8, panel A and B)

GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis

Table 8 Diagnosis of different pathological changes in pancreas

Tissue	Diagnosis	Definition
Pancreas	**Islet amyloidosis**	Thioflavin T reactive deposites within the pancreatic islets (Figure 9, panel A)
	Vascular and Interstitial lesions	
	Hyaline arteriolosclerosis	Similar features as described in the renal section (Figure 9, panel B)
	Arteriosclerosis	Similar features as described in the renal section (Figure 9, panel C)
	Interstitial fibrosis	Similar features as described in the renal section (Figure 9, panel D)
	Interstitial fat infiltration	Fat accumulation in exocrine acini and endocrine islets (Figure 9, panel E)
	Near normal structure	Similar features as described in the renal section (Figure 9, panel F)

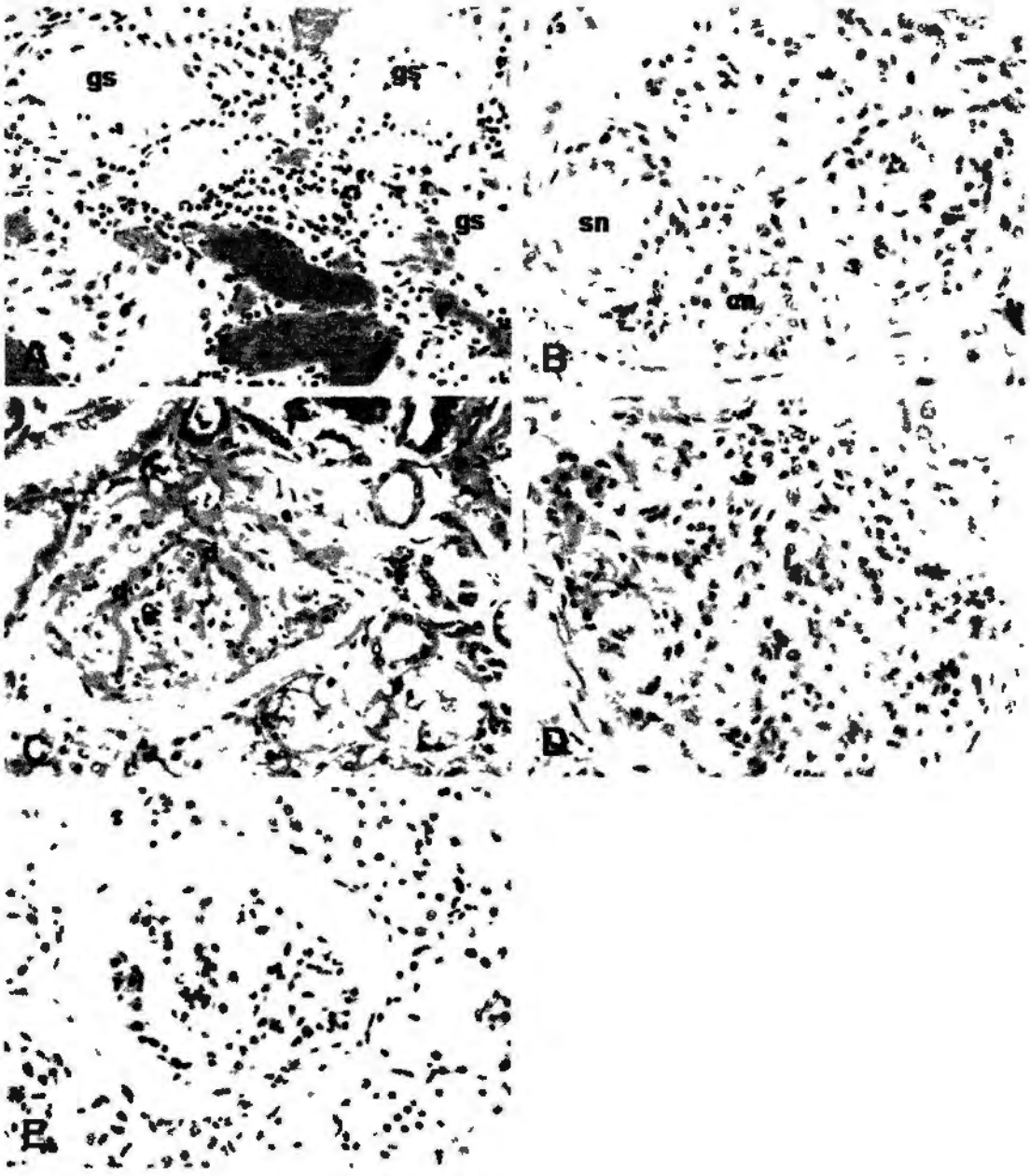


Figure 7 Glomerular changes in T2DM

(A) Global glomerulosclerosis (gs). (B) Nodular glomerulosclerosis with sclerotic nodules (sn) and cellular nodules (cn). (C) Diffuse glomerular sclerosis (d). (D) Glomerular hypertrophy. (E) Near normal glomerulus. (H&E stain, magnification ×400.)

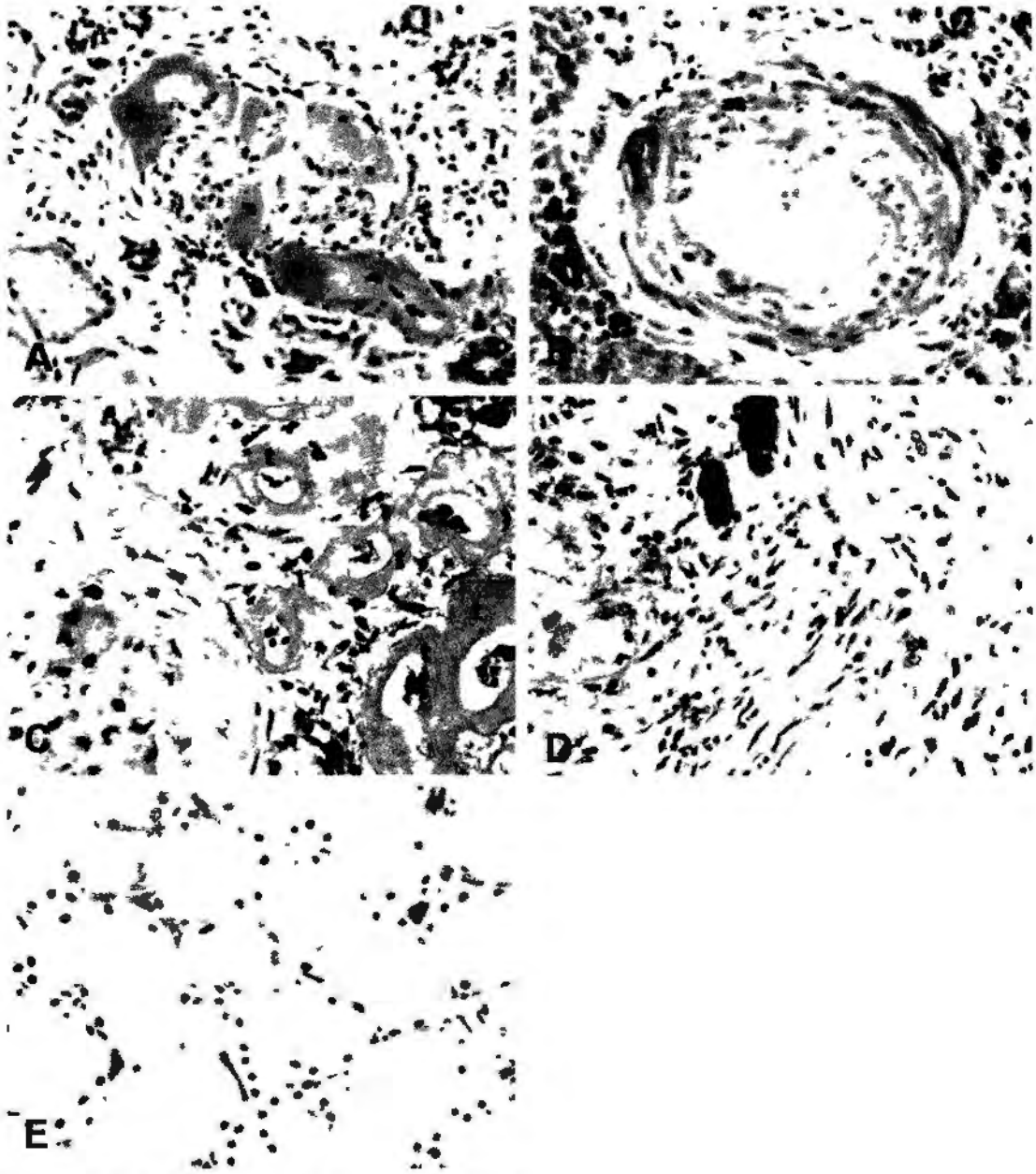


Figure 8 Tubulointerstitial changes in T2DM.

(A) Arteriolar hyalinosis (h). (B) Arteriosclerosis (as). (C) Tubular atrophy with basement membrane thickening (t). (D) Interstitial fibrosis (f). (E) Near normal tubulointerstitium. (H&E stain, magnification ×400.)

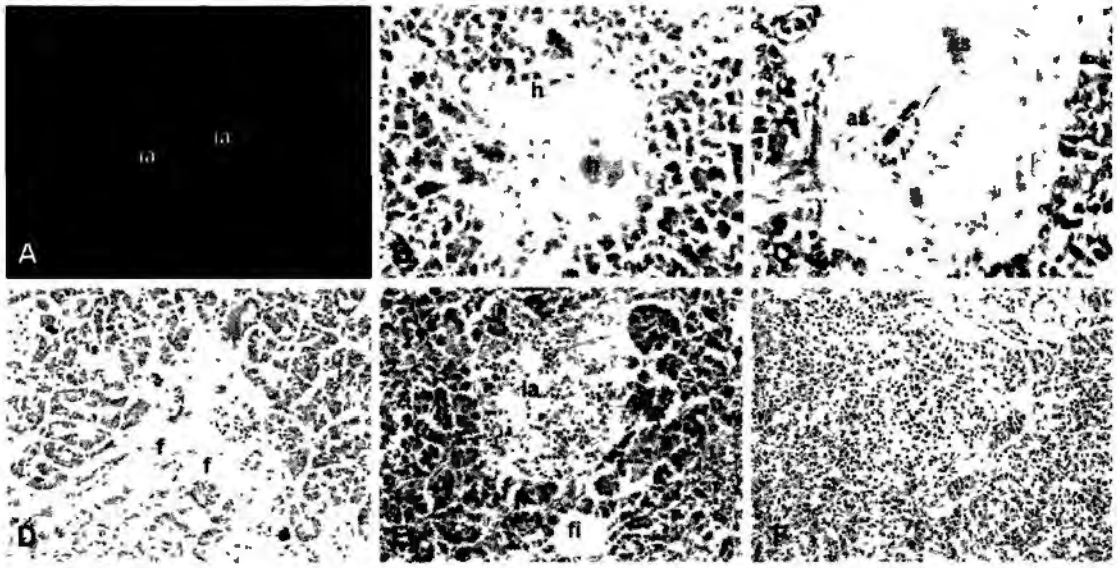


Figure 9 Histopathological spectrum of pancreatic changes in T2DM.

(A) Islet amyloidosis. The pancreatic section were stained with thioflavin T (Green) and anti-insulin antibody (Red). (B) Arteriolar hyalinosis (h). (C) Arteriosclerosis (as). (D) Interstitial fibrosis (f). (E) Fat infiltrates (fi). (F) Near normal structure. (panel A: Immunofluorescence microscopy, magnification ×200. panel B and C: H&E stain, magnification ×400. panel D, E and F: H&E stain, magnification ×200.)

2.3.3. Immunostaining and image analysis

2.3.3.1 Immunostaining

Immunohistochemical and immunofluorescence staining have been widely used in diagnostic pathology and basic research to understand the in situ distribution and localization of specific molecular markers and expression of proteins in different tissues. This technique is sensitive, specific and easy to apply in routinely processed materials including postmortem samples.

We used either DAB chrome or fluorescence to visualize antibody-antigen reactions. The DAB chrome is included in the DAKO EnVision system (DAKO, K4006, CA, USA) for light microscopy. The secondary antibody in this system is conjugated with horseradish peroxidase (HRP) labeled polymer. The polymer does not contain avidin or biotin. Consequently, non-specific background is eliminated or reduced. This 2-step procedure offers greater sensitivity and convenience in routine histological work (Figure 10). Fluorescence microscopy was performed with the Invitrogen secondary antibodies conjugated with fluorescein isothiocyanate (FITC, green) or Alexa Fluor (red). Compared with traditional single labeling, immunofluorescence can be performed with double labeling using antibodies derived from different animals mixed and incubated as a cocktail. This procedure is more time efficient and can provide information on protein interactions within the same tissue section (Figure 11). The procedures of these immunostaining methods are listed in **Appendix I-4** and **I-5**.

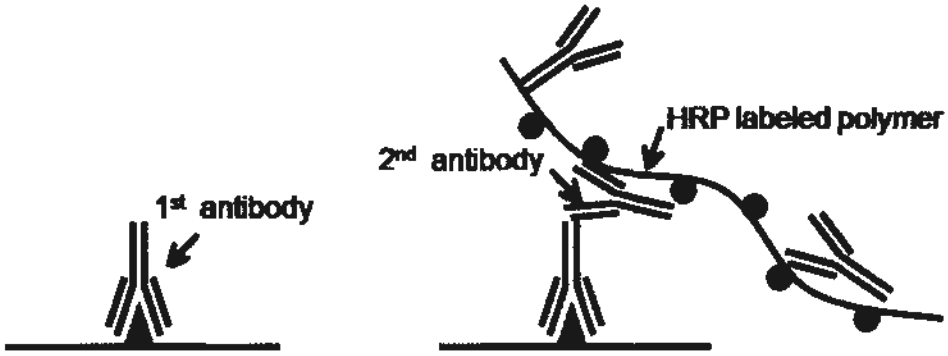


Figure 10 DAKO EnVision system

The tissue sections are firstly incubated with the 1st antibody, followed by incubation with 2nd antibody which is conjugated with HRP labeled polymer. After DAB colour reaction, protein expression can be observed under a light microscope.

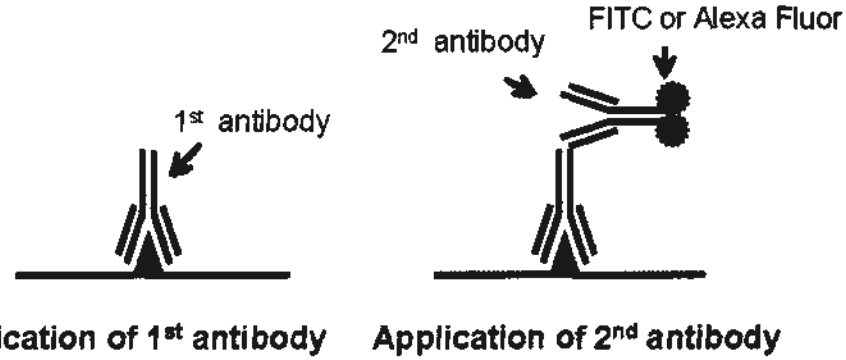


Figure 11 Immunofluorescence staining

The tissue sections are firstly incubated with the 1st antibody, followed by incubation with 2nd antibody which is conjugated with FITC or Alexa Fluor. Then protein expression can be observed under a fluorescence microscope.

2.3.3.2 Image analysis

After immunostaining, we used image analysis software to extract target information from images, and turned them into quantitative data. Compared with semi-quantitation by computer-coded scoring, image analysis using computer program provides more precise and objective data. All immunostaining and image analysis work were assessed with reviewers being blind to diabetic status and genotypes. The procedure of operation is listed in **Appendix I-6**. Microscopic images were obtained using a Digital Spot Camera with the same settings. For each case, at least 10 islets or glomeruli were randomly captured at an objective magnification of 20 × or 40 × respectively and analysed using MetaMorph software (MetaMorph 4.0 image acquisition program for Windows, 1999, Downingtown, PA). ApoE expression was indicated by the proportion of ApoE-positive area in glomeruli to the total glomeruli area examined. The islet area was the pixels of islet area. Beta to alpha cell ratio refers to the insulin positive area to glucagon positive area ratio (Figure 12). The expression of insulin, amylin, glucagon, and IDE was indicated by areas stained positively for these proteins in each islet to the total islet area examined. Frequency of islet amyloid was defined by the number of thioflavin T positive islets to total islet number examined. Severity of islet amyloid was indicated by the thioflavin T positive area in islets to the total thioflavin-positive islet area examined.

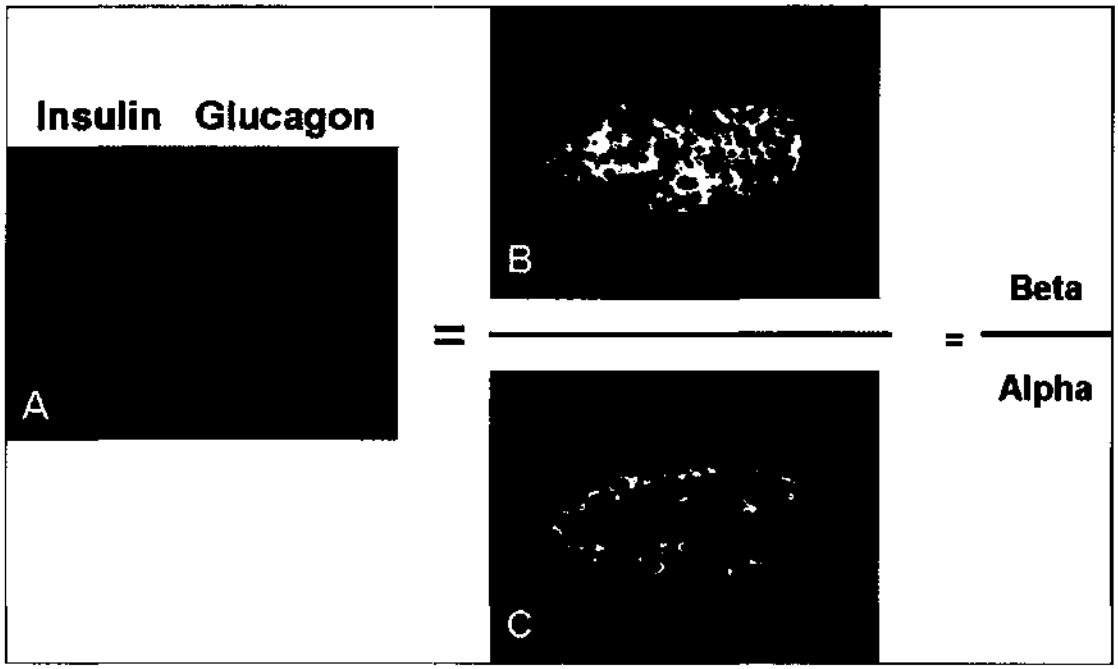


Figure 12 Image analysis procedure used to estimate the beta to alpha cell area ratio in pancreatic islets.

The islet was stained with mouse-anti insulin antibody (red) and rabbit-anti glucagon (green) antibody (panel A). Reactivity was analysed using the metamorph analysis system. When we calculated the pixels of insulin, the insulin positive area (Red, panel A) would appear yellow (panel B) in the window of the software and the yellow area could be calculated. The same procedure was repeated to calculate the glucagon positive area (green, panel A). The number of pixels of insulin positive area (yellow, panel B) and glucagon positive (green, panel C) area were obtained by using the software to estimate the beta to alpha cell ratio as reflected by pixels of red area to that of green area.

2.4. SNP detection

2.4.1. DNA extraction

Total genomic DNA samples were extracted from paraffin blocks. Pitfalls for the DNA samples isolated from paraffin blocks include degradation of target DNA during tissue removal and fixation and DNA fragmentation during formalin fixation [242]. To improve the DNA quality in archived paraffin blocks, I used the white blood cell-concentrated spleen tissues and a modified DNA extraction protocol [243]. Tissue sections of 4–6 μm were cut from formalin-fixed, paraffin-embedded spleen blocks and collected into an autoclaved microtube (1.5 mL). Genomic DNA was extracted as previously described [243]. After incubation with an 800μL lysis buffer at 65°C overnight, 700μL freshly prepared phenol: chloroform (1:1) mixture was added to the supernatant. This step was repeated, followed by further extraction and purification using chloroform: isoamyl alcohol (24:1) mixture. The upper aqueous supernatant was pipetted to a fresh microtube, mixed with 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes 100% ethanol by vortexing followed by incubation at −20°C for at least 30 min. The precipitated DNA was collected by centrifugation at 9590g at 4°C for 20 min. The extracted DNA sample was dissolved in a Tris–HCl EDTA buffer (pH 8.0) after thorough drying at room temperature. DNA quality was examined by gel electrophoresis and assessed by the ratio of absorbance at 260–280 nm. A260/280 ratio of 1.7 to 1.9 indicates highly pure genomic DNA samples (**appendix II-1**).

2.4.2. PCR amplification

During the formalin fixation [242], the length of the DNA fragment averages 100-500bp. Seven pairs of primers which lead to 100-300bp PCR products were used, with denaturation at 95°C for 30 s, annealing for 1 min and extension at 72◦C for 1 min for 40 cycles. The primers and the annealing temperatures are shown in Table 9. The protocol was described in **Appendix II-2**.

Table 9 Primers and annealing temperatures of PCR in different target genes

Gene	SNPs	Primers	Product size	Annealing temperature
Apo E	rs429358 (T/C), rs7412 (T/C)	P1: 5'TCGCGGGCCCCGGCCTGGTACA3', P2: 5'CAACTGAGCCCGGTGGCGG3'	263bp	68°C
IDE	rs6583813 (T/C)	P1: 5'CAAACAATACGATACCAAAGA3', P2:5'TTTATACTGAGCATTTTCCA3'	117bp	49°C
GLUT1	rs710218 (A/T)	P1: 5'- CCTTCCCTCAATCCATCT -3' P2: 5'-GAGCAGCAACAATACCAA-3'	170bp	50°C

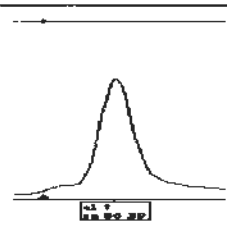
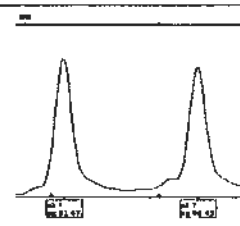
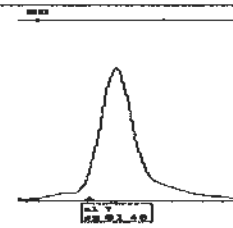
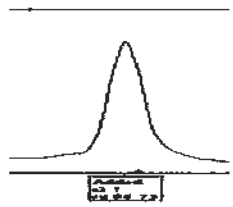
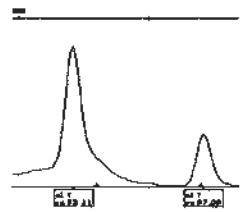
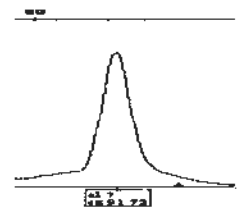
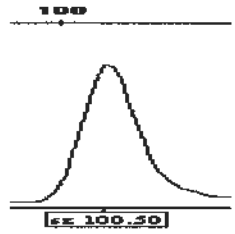
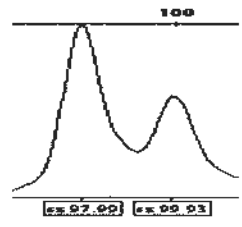
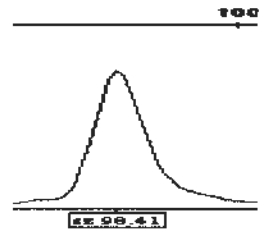
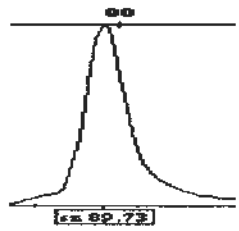
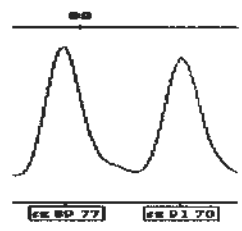
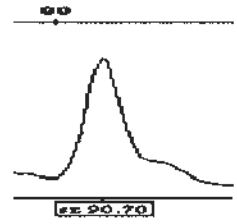
2.4.3. Genotyping

Amongst various genotyping techniques, DNA sequencing is considered the gold standard method but is expensive. Other techniques such as restriction fragment length polymorphism can be time-consuming while Taqman is also costly. Compared with these methods, the ligase detection reaction (LDR) is a popular method which is reliable, efficient and low cost. In this study, following a PCR amplification of the regions of interest, each polymorphism was detected using a thermostable ligase that joined pairs of adjacent oligonucleotides complementary to the sequences of interest. Ligation occurred only when the sequence at the junction between the paired oligonucleotides was exactly complementary to the template sequence. Thus, LDR can readily discriminate different genotypes [244-247]. In this study, LDR was performed using four sets of probes (Table 10). PCR product, ligase and probe mixture reacted in 35 cycles of amplification consisting of denaturation at 94˚C for 30 s and annealing and extension at 60˚C for 2 min. The LDR product was then sequenced to confirm the results (ABI PRISM 3100 DNA Sequencer) (**appendix II-3**). The sequencing maps are shown in table 11

Table 10 LDR probes of different SNPs for candidate genes

Gene	SNPs		Probes	Product size
Apo E	rs429358		p-CACGTCCTCCATGTCCGCGCTTTTTTTTTTTTTTTTTTTT-FAM	
		T	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCAGGCGGCCGCA	87bp
		C	TTTTTTTTTTTTTTTTTTTTTTCTGCACCAGGCGGCCGCG	82bp
	rs7412		p-CTTCTGCAGGTCATCGGCATCGTTTTTTTTTTTTTTTTTTTTT-FAM	
		T	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCCTGGTACACTGCCAGGCa	97bp
		C	TTTTTTTTTTTTTTTTTTTTTTTTTTGCCTGGTACACTGCCAGGCg	92bp
IDE	rs6583813		P-GCTCTTTGGTATCGTATTGTTTGTTTTTTTTTTTTTTTTTTTTT-FAM	
		T	TTTTTTTTTTTTTTTTTTTTTTTTTTGTTGTTGTTGTCGTTGTCGGCGGCA	100bp
		C	TTTTTTTTTTTTTTTTTTTTTTTTGTTGTTGTTGTCGTTGTCGGCGGCG	98bp
GLUT1	rs710218		P-GCAGAACTTGTACCACAGGGGAAGGTTTTTTTTTTTTTTTTTTT-FAM	
		A	TTTTTTTTTTTTTTTTTTTTTAGAGGGTTAAGAATCTGTACAGGT	90bp
		T	TTTTTTTTTTTTTTTTTTTTTTAGAGGGTTAAGAATCTGTACAGGA	92bp

Table 11 Sequencing maps of LDR products

Gene	SNPs	Genotypes		
Apo E	rs429358	 TT	 TC	 CC
	rs7412	 TT	 TC	 CC
IDE	rs6583813	 TT	 TC	 CC
GLUT1	rs710218	 AA	 AT	 TT

2.5. Statistical analysis

2.5.1. Sample size estimation

Our group has reported that 40% of diabetic autopsy cases had islet amyloid (defined by positive Congo Red staining in pancreatic islet) which was associated with a reduced β/α cell ratio [27] and that 32.3% of cases had classic diabetic glomerulopathy [248]. According to published data, the frequency of ApoE ε2 carriers (ε2/ε2 and ε2/ε3) was 17.8% [249] and that of the AA genotype of GLUT1 was 44.4% [250]. Assuming that the presence of any risk allele will increase the risk of diabetic nephropathy by 2 fold, I estimated that 98 (ApoE ε2) and 70 (GLUT1) T2DM autopsy cases would give more than 80% power with an error rate of 0.05. The percentage of ApoE ε4 carriers (ε3/ε4 and ε4/ε4) was 12.4% [249] and the frequency of IDE rs6583813 TT genotype was 44.4% [251]. A sample size of 105 (ApoE ε4) and 45 (IDE) T2DM cases will give 80% power with an error rate of 0.05. Assuming that the presence of any of the genetic variants will increase the risk of diabetes by 2 fold, a sample size of 300 cases and 200 control subjects will have a 80% power with an error rate of 0.05.

2.5.2. Data analysis

Observed distributions of genotypes were analysed for deviation from the Hardy–Weinberg equilibrium by chi-square tests with one degree of freedom. Allele frequency refers to the number of occurrences of the test allele in the group divided by the total number of alleles. Data are presented as mean±SD (normal distributed continuous variables), medians (interquartile range) (skewed continuous variables) or % (n) (categorical variables). Means were compared using the Student's *t*-test and one-way analysis of variance (ANOVA). Bonferroni adjustment was used in post-hoc test for multiple comparisons. Categorical variables were compared using the chi-square test, Fisher's exact test or linear trend test. The odds ratio (OR) and corresponding 95% confidence interval (CI) were calculated using logistic regression. A two-tailed *P*-value <0.05 was considered significant. Calculations were performed using SPSS (Statistics Package for the Social Sciences 10.0.7 for Windows, 2000, SPSS Inc., Chicago, IL, USA).

CHAPTER 3 RESULTS

3. RESULTS

3.1. Demographic data

3.1.1. Demographic data of whole study population

The clinicopathological characteristics of the 328 diabetic and 209 age and sex-matched nondiabetic cases are shown in Table 11. Compared with control subjects, the diabetic cases had higher frequencies of phenotypes related to renal disease (diabetic nephropathy, death due to renal disease) and vasculopathy (hypertension, CHD, LVH, CAS, death due to CVD and history of CVA).

Table 12 Clinicopathological characteristics of type 2 diabetic and non-diabetic cases

	T2DM	Control	*P*-value
	N=328	N=209	
Age (years)	70.7±10.8	69.0±15.2	0.1752
Female	55.2 (181)	52.2 (109)	0.4922
	N=326	N=209	
Hypertension	63.2 (206)	13.4 (28)	<0.0001
	N=328	N=209	
CHD	59.8 (196)	13.4 (28)	<0.0001
LVH	46.3 (152)	5.3 (11)	<0.0001
CAS	45.1 (148)	12.0 (25)	<0.0001
CVA	19.9 (65)	8.6 (18)	0.0004
	N=303	N=209	
Diabetic nephropathy	66.3 (201)	0.0 (0)	<0.0001
	N=297	N=209	
Death due to renal disease	8.8 (26)	1.0 (2)	0.0002
	N=297	N=209	
Death due to cardiovascular disease	44.4 (132)	19.1 (40)	<0.0001

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident. Data are mean±SD or % (*n*) and were compared by the Student *t*-test, chi-square test or Fisher's exact test.

3.1.2. Renal pathological changes in T2DM and non-diabetic cases

In the 328 T2DM cases, 303 had records of diabetic nephropathy. Among the 303 cases, we consecutively selected a subgroup of 51 T2DM cases and reviewed their renal tissue sections for detailed pathological changes. Table 13 compares the renal pathological changes between controls and a subgroup of T2DM cases with repeat evaluation by Guan J and Zhao HL. Except for tubular lesions, T2DM cases were more likely to have glomerular, vascular and interstitial pathological changes than control cases. Overall, 98% of this subgroup of T2DM cases had some abnormal histopathological changes.

Table 13 Renal pathological characteristics of type 2 diabetic and non-diabetic cases

	T2DM	Control	*P*-value
Renal pathology	N=51	N=209	
Glomerular lesion	82.4 (42)	49.8 (104)	<0.0001
GGS	72.5 (37)	44.5 (93)	0.0003
NGS	29.4 (15)	0.0 (0)	<0.0001
DGS	5.9 (3)	0.0 (0)	0.0072
Glomerular hypertrophy	35.3 (18)	13.4 (28)	0.0002
Vascular lesions	96.1 (49)	57.9 (121)	<0.0001
Hyaline arteriolosclerosis	80.4 (41)	8.6 (18)	<0.0001
Arteriosclerosis	78.4 (40)	56.9 (119)	0.0047
Tubulointerstitial lesions	58.8 (30)	44.5 (93)	0.0662
Tubular lesion	35.3 (18)	28.2 (59)	0.3218
Interstitial fibrosis	51.0 (26)	35.4 (74)	0.0404
Vascular-tubulointerstitial lesions	98.0 (50)	66.0 (138)	<0.0001
Near normal structure	2.0 (1)	27.8 (58)	0.0001

GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis. Data are % (*n*) and were compared by Chi-square test or Fisher's exact test.

3.1.3. Pancreatic pathological changes in type 2 diabetic and non-diabetic controls

Pancreatic pathological characteristics of T2DM cases and control are shown in Table 14. Diabetic pancreas showed higher percentage of pathological changes than nondiabetic pancreas. Nearly 96.6% of the T2DM cases had vascular interstitial lesions compared to 67.5% in the control cases. Overall, 30.8% of T2DM cases and only 1.9% of control cases had islet amyloid.

Table 14 Pancreatic pathological characteristics of type 2 diabetic and non-diabetic control subjects

	T2DM	Control	*P*-value
Pancreas lesions	N=117	N=209	
Vascular lesions	76.9 (90)	22.0 (46)	<0.0001
Hyaline arteriolosclerosis	70.1 (82)	7.2 (15)	<0.0001
Arteriosclerosis	54.7 (64)	19.6 (41)	<0.0001
Interstitial lesions	89.7 (105)	60.8 (127)	<0.0001
Interstitial fibrosis	59.0 (69)	21.1 (44)	<0.0001
Interstitial fat infiltration	79.5 (93)	53.6 (112)	<0.0001
Vascular interstitial lesions	96.6 (113)	67.5 (141)	<0.0001
Islet amyloid	30.8 (36)	1.9 (4)	<0.0001
Near normal structure	3.4 (4)	24.9 (52)	<0.0001

Data are % (*n*) and were compared by Chi-square test or Fisher's exact test.

3.1.4. Summary of the study population

Figure 13 summarises the cases selection for the DN study population and islet amyloid study population including the number of cases successfully genotyped, and number of cases with detailed examination of pathological changes.

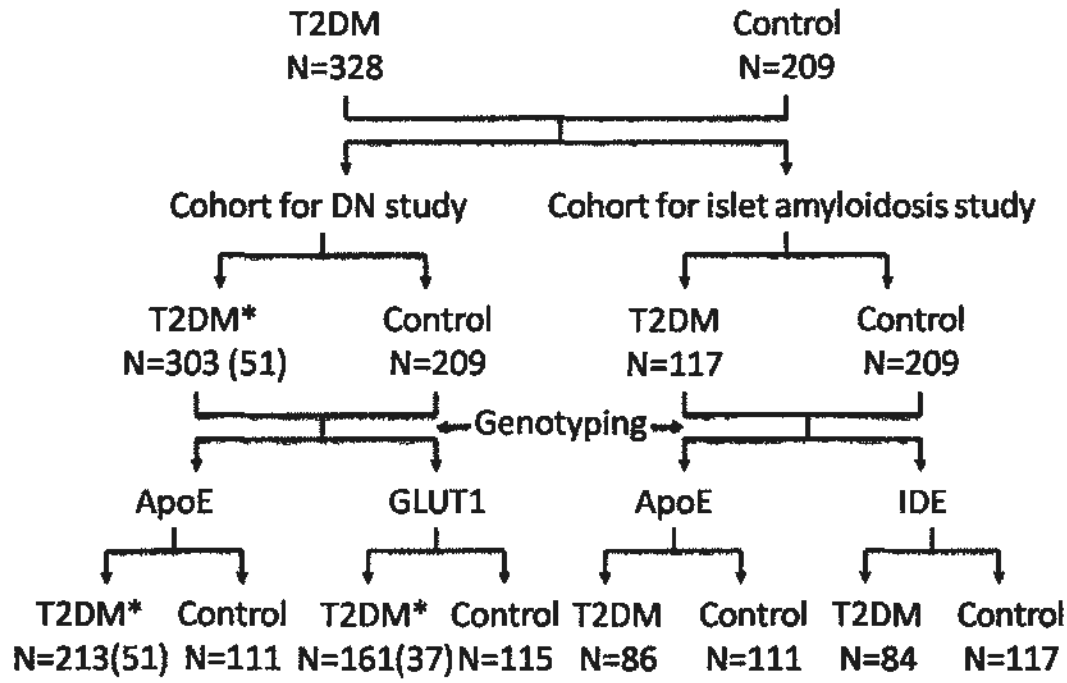


Figure 13 Summary of study population

In the study population for the DN study, 51 cases had detailed record of renal pathological changes *Data were cases with genotype data. Number in bracket referred to number of cases with detailed records of renal pathological changes. In the study population for the islet amyloidosis study, all T2DM cases had detailed records of pancreatic pathological changes. All control cases had detailed records of renal and pancreatic pathological changes.

3.2. Islet amyloidosis

3.2.1. Prevalence of islet amyloidosis

There were more cases with islet amyloidosis in the T2DM [30.8% (36/117)] than control group [1.9% (4/209), OR=22.8, 95%CI=7.9-66.0, $P<0.0001$] (Table 14).

3.2.2. Clinical and pathological characteristics associated with islet amyloidosis

In the T2DM cases, the clinical characteristics were largely similar between cases with and those without islet amyloid except for a higher GFR and lower plasma creatinine in the amyloid group. Diabetic cases with islet amyloidosis had a higher percentage of pancreatic vascular interstitial lesions than those without amyloidosis (Table 15). In H&E stained sections, pancreatic tissue with islet amyloidosis showed extensive vascular changes. The lesions included extensive hyaline arteriolosclerosis in small arterioles and atherosclerotic lesions in larger pancreatic arteries with pronounced thickening of the arterial wall. In some instances, lumenal atresia was found in arterioles. In contrast, the arteriosclerosis-associated changes in the pancreas free from amyloidosis were relatively mild (Figure 14). Interstitial lesions were also found to be more frequent in pancreas with islet amyloidosis compared to those without. Islets affected by amyloid deposits often showed intra-islet and peri-islet fibrosis (Figure 15) and to a lesser extent fat infiltration (Figure 14). Possibly due to the small number of cases with amyloidosis in the control group, no meaningful difference was detected (Table 15).

Table 15 Clinicopathological characteristics of T2DM and control cases in relation to the presence of islet amyloidosis

	T2DM			Control		
	With islet amyloidosis	Without amyloidosis	*P*-value	With islet amyloidosis	Without amyloidosis	*P*-value
	N=36	N=81		N=4	N=205	
Age (years)	69.8±10.4	70.3±12.4	0.8356	78.3±13.5	68.8±15.2	0.2217
Female	52.8 (19)	64.2 (52)	0.2431	50.0 (2)	52.2 (107)	1.0000
	N=8	N=17				
BMI (kg/m^2)	22.9±2.5	24.5±6.5	0.5171	-	-	-
	N=19	N=38				
FPG (mmol/L)	10.6±3.9	13.4±6.8	0.1083	-	-	-
	N=20	N=39				
Systolic blood pressure (mmHg)	154.6±30.2	141.9±40.5	0.2238	-	-	-
Diastolic blood pressure (mmHg)	81.4±15.5	76.6±19.5	0.3495	-	-	-
	N=19	N=36				
Plasma creatinine (μmol/L)	124.0 (88.0-164.0)	207.5 (115.0-329.8)	0.0209	-	-	-
Plasma urea (mmol/L)	11.7 (8.1-17.9)	14.0 (7.3-22.0)	0.3527	-	-	-
GFR ($mL/min/1.73m^2$)	38.0 (27.8-57.6)	21.0 (11.6-36.7)	0.0139	-	-	-
	N=36	N=79				
Hypertension	66.7 (24)	50.6 (40)	0.1085	25.0 (1)	13.2 (27)	0.4400
	N=36	N=81				
CHD	55.6 (20)	51.9 (42)	0.7110	25.0 (1)	13.2 (27)	0.4400
LVH	50.0 (18)	40.7 (33)	0.3512	0.0 (0)	5.4 (11)	1.0000
CAS	33.3 (12)	43.2 (35)	0.3145	25.0 (1)	11.7 (24)	0.4016
CVA	11.1 (4)	8.6 (7)	0.7353	0.0 (0)	8.8 (18)	1.0000

	N=35	N=80				
Diabetic nephropathy	71.4 (25)	78.8 (63)	0.3940	-	-	-
	N=34	N=78				
Death due to renal disease	8.8 (3)	10.3 (8)	1.0000	0.0 (0)	1.0 (2)	1.0000
Death due to cardiovascular disease	38.2 (13)	44.9 (35)	0.5140	25.0 (1)	19.0 (39)	0.5754
Renal pathology	N=36	N=81		N=4	N=205	
Glomerular lesion	66.7 (4)	83.3 (35)	0.3119	50 (2)	49.8 (102)	1.0000
GGS	66.7 (4)	71.4 (30)	1.0000	50 (2)	44.4 (91)	1.0000
NGS	16.7 (1)	28.6 (12)	1.0000	0 (0)	0.0 (0)	-
DGS	0.0 (0)	4.8 (2)	1.0000	0 (0)	0.0 (0)	-
Glomerular hypertrophy	50.0 (3)	35.7 (15)	0.6583	0 (0)	13.7 (28)	1.0000
Vascular lesions	100.0 (6)	95.2 (40)	1.0000	50 (2)	58.0 (119)	1.0000
Hyaline arteriolosclerosis	66.7 (4)	83.3 (35)	0.3119	25 (1)	8.3 (17)	0.3044
Arteriosclerosis	100.0 (6)	76.2 (32)	0.3203	25 (1)	57.6 (118)	0.3169
Tubulointerstitial lesions	16.7 (1)	61.9 (26)	0.0733	50 (2)	44.4 (91)	1.0000
Tubular lesion	16.7 (1)	40.5 (17)	0.3883	0 (0)	28.8 (59)	0.5789
Interstitial fibrosis	16.7 (1)	52.4 (22)	0.1908	50 (2)	35.1 (72)	0.6158
Vascular-tubulointerstitial lesions	100.0 (6)	97.6 (41)	1.0000	75 (3)	65.9 (135)	1.0000
Near normal structure	0.0 (0)	2.4 (1)	1.0000	25 (1)	27.8 (57)	1.0000
Pancreas lesions	N=36	N=81		N=4	N=205	
Vascular lesions	88.9 (32)	71.6 (58)	0.0406	0.0 (0)	22.4 (46)	0.5780
Hyaline arteriolosclerosis	83.3 (30)	64.2 (52)	0.0369	0.0 (0)	7.3 (15)	1.0000
Arteriosclerosis	72.2 (26)	46.9 (38)	0.0111	0.0 (0)	20.0 (41)	1.0000
Interstitial lesions	94.4 (34)	87.7 (71)	0.3387	50.0 (2)	61.0 (125)	0.6461
Interstitial fibrosis	75.0 (27)	51.9 (42)	0.0188	25.0 (1)	21.0 (43)	1.0000
Interstitial fat	83.3 (30)	77.8 (63)	0.4922	25.0 (1)	54.1 (111)	0.3391

	infiltration						
Vascular lesions	interstitial	100.0 (36)	95.1 (77)	0.3104	50.0 (2)	67.8 (139)	0.5974
Near normal structure		0.0 (0)	4.9 (4)	0.3104	0.0 (0)	25.4 (52)	0.5740

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD, medians (interquartile range) or % (n). They were compared by Student t-test, Mann–Whitney U test, Chi-square test or Fisher's exact test, as appropriate.

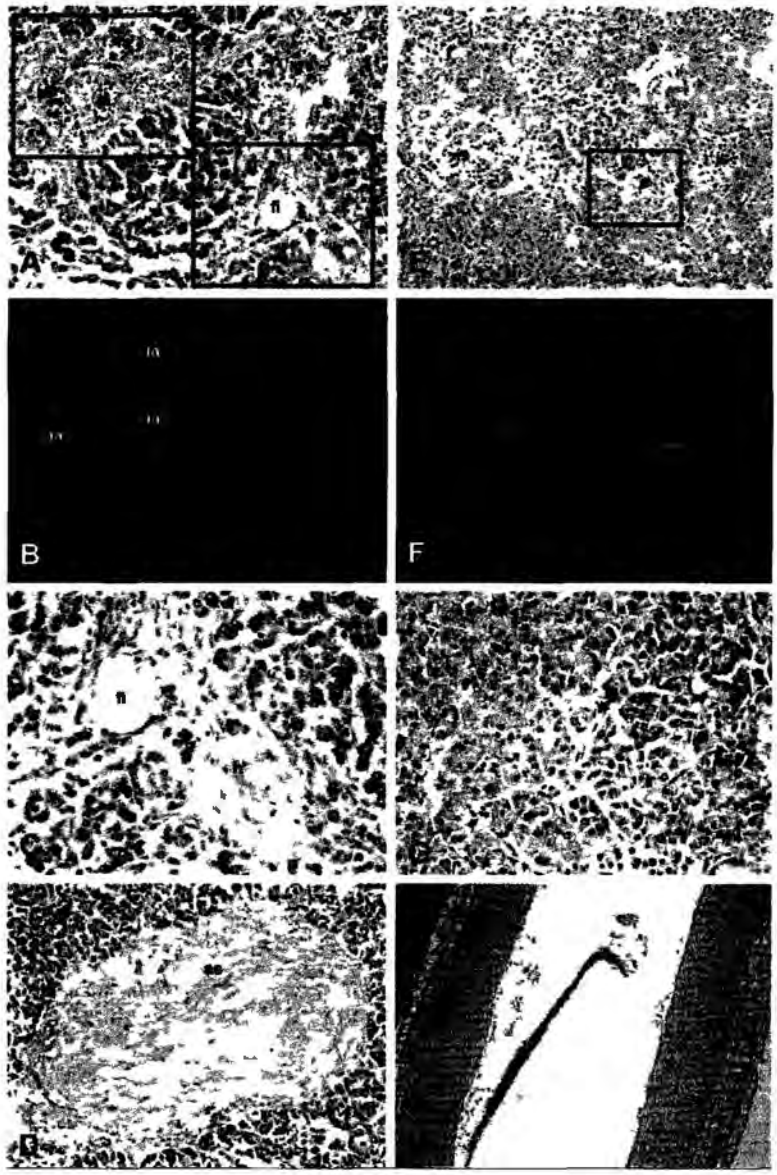


Figure 14 Concomitant vascular lesions with islet amyloidosis in T2DM.

Serial pancreatic sections with (panels A, B, C and D) and without (panels E, F, G and H) islet amyloidosis were stained with H&E and thioflavin T (green) as well as anti-insulin antibody (red). Panels B and F were the same islet(s) stained with thioflavin T (green) and insulin (red) shown in panel A and E respectively. Panels C and G were the high magnification of part of panel A and E respectively. Islet amyloidosis (ia) (panels A and B) was accompanied by hyaline arteriolosclerosis (h), fat infiltration (fi) (panels A and C) and arteriosclerosis (as) (panel F). Pancreas without islet amyloidosis was unlikely to have hyaline arteriolosclerosis, fat infiltration and arteriosclerosis (panels E, F, G and H). (Panels A, C, D, E, G and H: H&E stain, panel B and F: Immunofluorescence stain; panel H: magnification ×100, panels A, D, E and F: magnification ×200, panels B, C and G: magnification ×400.)

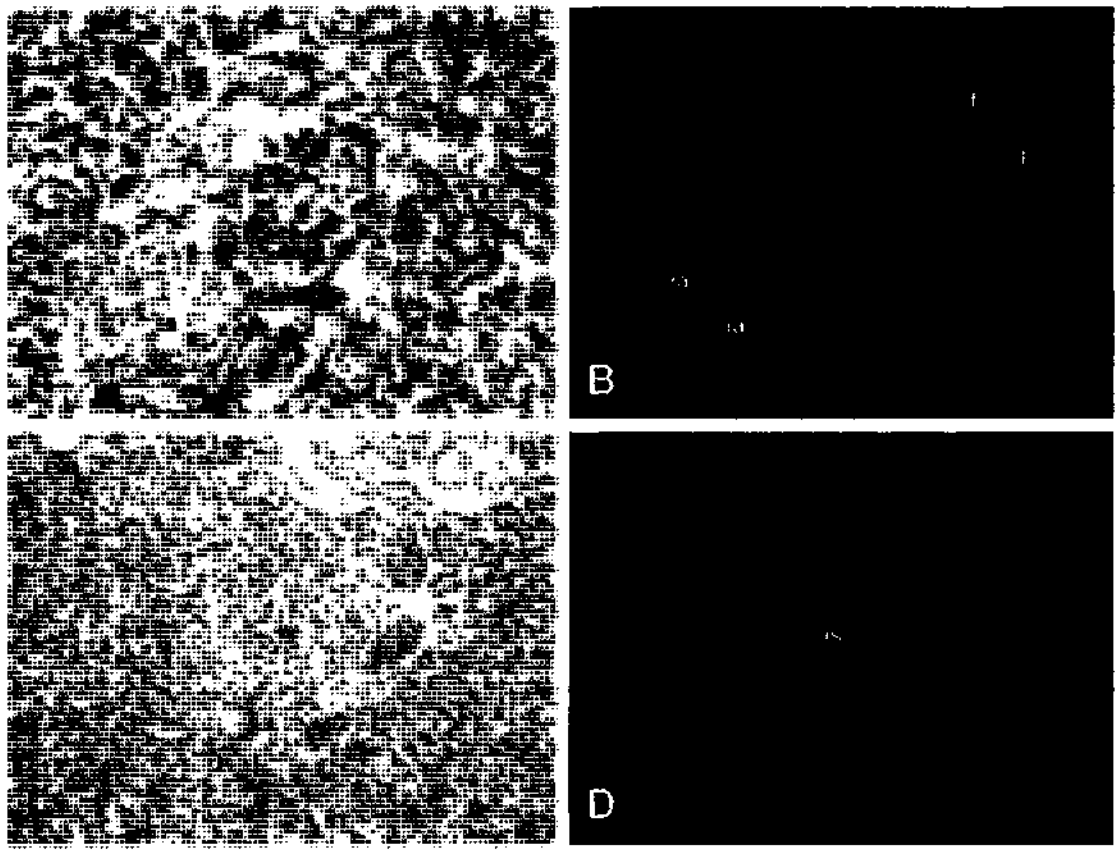


Figure 15 Interstitial fibrosis in pancreas with or without islet amyloidosis.

Serial pancreatic sections with (panels A and B) and without (panels C and D) islet amyloidosis were stained with H&E and thioflavin T (green) as well as anti-insulin antibody (red). Amyloidosis in islets was often accompanied by fibrosis, or even surrounded by fibrotic tissue. Pancreases without islet amyloidosis have relatively minor vascular-interstitial lesions compared to those with islet amyloidosis. (Panels A and C: H&E stain, panels B and D: Immunofluorescence stain; magnification ×200.)

3.2.3. Association between islet amyloidosis and pancreatic beta to alpha cell ratio

In a subgroup analysis, I selected age- and sex- matched T2DM cases with islet amyloidosis (n=14), T2DM cases without amyloidosis (n=10) and control cases (n=6) to calculate the pancreastic beta to alpha cell ratio. Both islet beta- and alpha-cells were decreased in amyloid containing islets. After adjustment for multiple comparisons and using non-diabetic, non-amyloid containing cases for reference (4.6±3.8), the beta to alpha cell ratio was lower in T2DM cases with islet amyloidosis (1.6±0.6, P=0.006) but not in those without amyloidosis (2.0±1.0, P=0.032) (Figure 16).

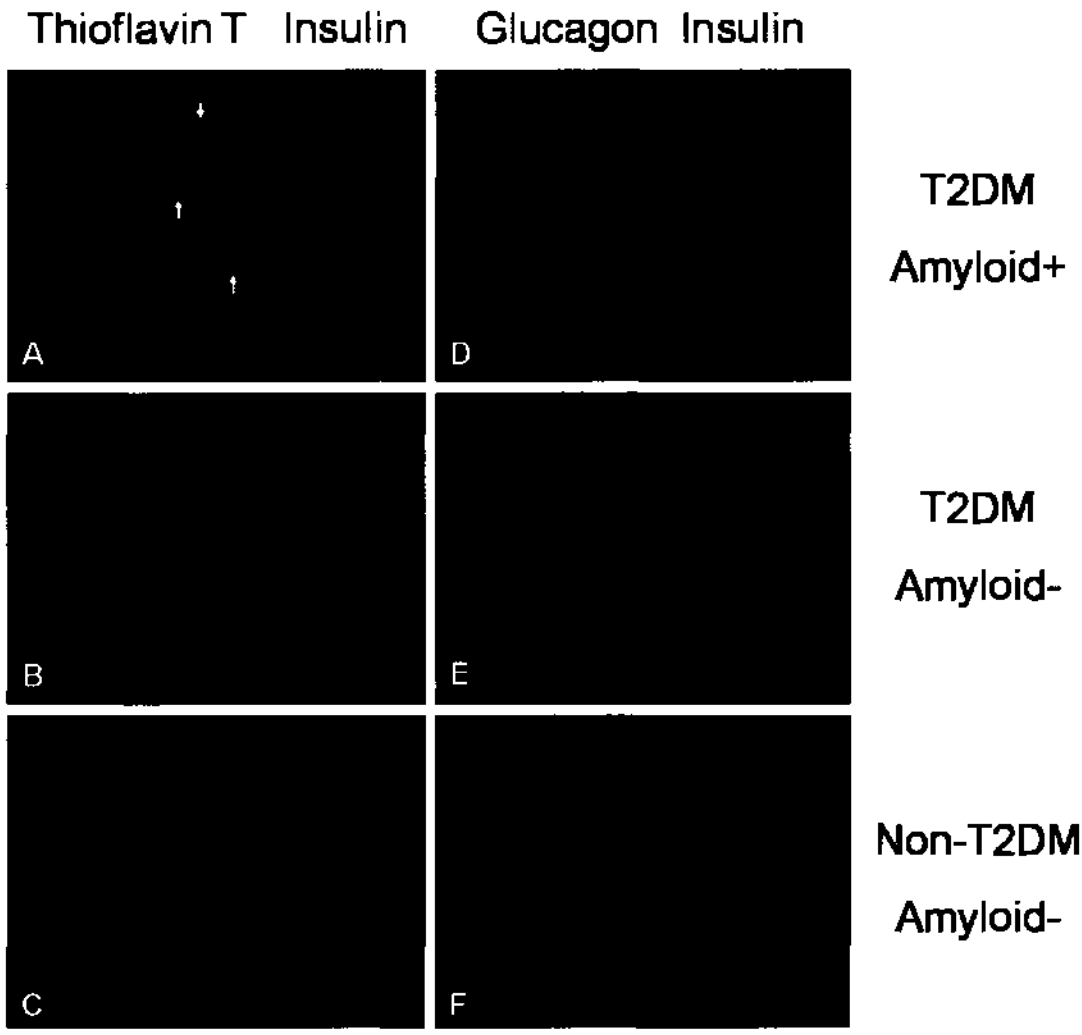


Figure 16 Association between islet amyloidosis and pancreatic beta to alpha cell ratio.

Immunofluorescence staining was performed in diabetic pancreatic sections with islet amyloidosis (A and D), without amyloidosis (B and E) and non-diabetic pancreatic sections (C and F). The sections were stained with Thioflavin T (green) and anti-insulin antibody (red) (A, B, C) as well as anti-insulin antibody (red) and anti-glucagon antibody (green), respectively. Islet cells were replaced by thioflavin T-reactive amyloid deposits (green) (A). Diabetic islets (A, B) had more irregularity than nondiabetic islets (C). Islet amyloid deposition (D) was associated with diminished beta and alpha cells when compared with amyloid-free islets in diabetic (E) or nondiabetic pancreas (F). (Immunofluorescence microscopy, magnification ×400.)

3.3. Diabetic glomerulopathy

3.3.1 Clinicopathological characteristics of T2DM cases with or without DN

Based on autopsy reports, over 60% of T2DM cases had diabetic nephropathy defined by presence of diabetes-related renal changes. These changes were validated in a subgroup in which detailed examination was performed by 2 pathologists (GJ and ZHL) with experience in diabetic pathologies. Compared with cases free from DN, DN patients showed lower level of calculated GFR, higher plasma creatinie and urea as well as higher frequency of vascular disorders (CHD, LVH and CVA) (Table 16). In the subgroup analysis, cases with DN had higher frequency of NGS (Figure 17) and hyaline arteriosclerosis (Figure 18) than those without DN.

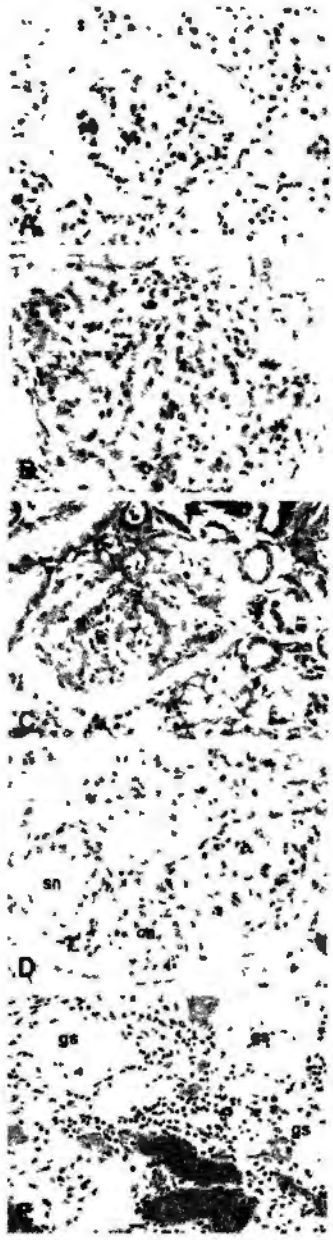
Table 16 Clinicopathological characteristics of type 2 diabetic cases with or without diabetic nephropathy

	With diabetic nephropathy	without diabetic nephropathy	*P*-value
	N=201	N=102	
Age (years)	71.0±10.5	69.7±11.3	0.3217
Female	56.7 (114)	53.9 (55)	0.6434
	N=50	N=9	
BMI (kg/m^2)	23.2±4.8	23.5±3.2	0.8362
	N=142	N=25	
FPG (mmol/L)	11.3±5.5	12.7±3.7	0.2322
	N=144	N=26	
Systolic blood pressure (mmHg)	148.1±39.1	140.8±36.4	0.3784
Diastolic blood pressure (mmHg)	79.1±21.1	77.2±20.4	0.6662
	N=133	N=24	
Plasma creatinine (μmol/L)	156.0 (109.0-252.0)	90.5 (70.0-134.8)	0.0002
Plasma urea (mmol/L)	12.4 (7.3-21.1)	7.5 (5.2-11.1)	0.0003
GFR ($mL/min/1.73m^2$)	29.8 (16.0-45.2)	60.5 (34.7-70.7)	0.0001
	N=200	N=102	
Hypertension	64.0 (128)	63.7 (65)	0.9625
	N=201	N=102	
CHD	66.7 (134)	50.0 (51)	0.0049
LVH	59.2 (119)	22.5 (23)	0.0000
CAS	50.7 (102)	39.2 (40)	0.0573
CVA	23.5 (47)	11.8 (12)	0.0150
	N=191	N=86	
Death due to renal cause	9.4 (18)	7.0 (6)	0.5029
Death due to Cardiovascular disease	48.7 (93)	37.2 (32)	0.0756
Detailed renal pathology in subgroup analysis	N=36	N=15	

Glomerular lesion	86.1 (31)	73.3 (11)	0.4206
GGS	75.0 (27)	66.7 (10)	0.7316
NGS	41.7 (15)	0.0 (0)	0.0021
DGS	5.6 (2)	6.7 (1)	1.0000
Glomerular hypertrophy	36.1 (13)	33.3 (5)	0.8500
Vascular lesions	97.2 (35)	93.3 (14)	0.5059
Hyaline arteriolosclerosis	91.7 (33)	53.3 (8)	0.0039
Arteriosclerosis	77.8 (28)	80.0 (12)	1.0000
Tubulointerstitial lesions	63.9 (23)	46.7 (7)	0.2548
Tubular lesion	33.3 (12)	40.0 (6)	0.6499
Interstitial fibrosis	55.6 (20)	40.0 (6)	0.3113
Vascular-tubulointerstitial lesions	97.2 (35)	100.0 (15)	1.0000
Near normal structure	2.8 (1)	0.0 (0)	1.0000
Pancreas lesions	N=88	N=27	
Vascular lesions	76.1 (67)	77.8 (21)	0.8603
Hyaline arteriolosclerosis	69.3 (61)	70.4 (19)	0.9172
Arteriosclerosis	55.7 (49)	51.9 (14)	0.7265
Interstitial lesions	88.6 (78)	92.6 (25)	0.7290
Interstitial fibrosis	53.4 (47)	74.1 (20)	0.0568
Interstitial fat infiltration	77.3 (68)	85.2 (23)	0.3761
Islet amyloidosis	28.4 (25)	37.0 (10)	0.3940
Vascular interstitial lesions	96.6 (85)	96.3 (26)	1.0000
Near normal structure	3.4 (3)	3.7 (1)	1.0000

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD, medians (interquartile range) or % (n). They were compared by Student t-test, Mann–Whitney U test, Chi-square test or Fisher's exact test, as appropriate.



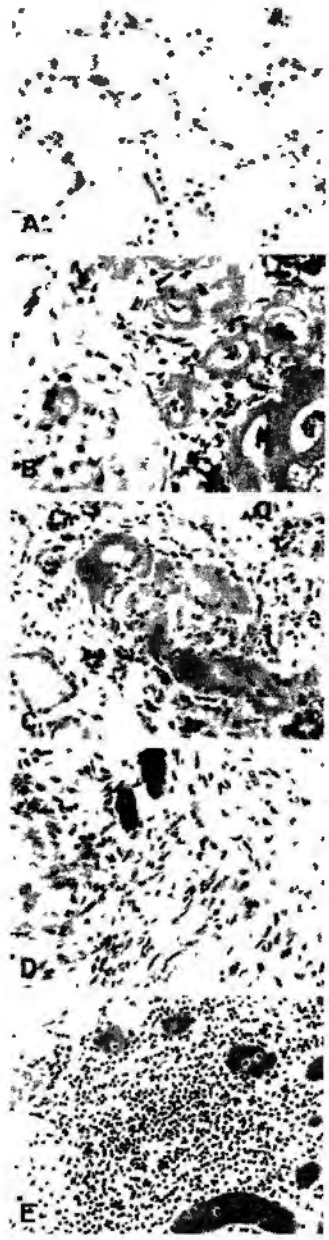


Figure 17 **Figure 18**

Figure 17 Histopathological spectrum of glomerular changes in T2DM.

(A) Normal glomerulus. (B) Glomerular hypertrophy. (C) Diffuse glomerular sclerosis (d). (D) Nodular glomerulosclerosis with sclerotic nodules (sn) and cellular nodules (cn). (E) Global glomerulosclerosis (gs) and chronic inflammatory infiltrates. (HE stain, magnification ×400.)

Figure 18 Histopathological spectrum of tubulointerstitial changes in T2DM.

(A) Normal tubulointerstitium. (B) Tubular atrophy with basement membrane thickening (t). (C) Arteriolar hyalinosis (h). (D) Interstitial fibrosis (f). (E) Chronic inflammatory infiltration (i). (HE stain, magnification ×400.)

3.4. Association with ApoE polymorphism

3.4.1 ApoE polymorphisms and expression in diabetic nephropathy

3.4.1.1 ApoE genotype and allele frequencies

ApoE gene polymorphism (rs429358 and rs7412) were successfully genotyped in 213 diabetic and 111 control cases respectively. The ApoE rs429358 genotypes were in Hardy-Weinberg equilibrium in both cases (*P*=0.7897) and control (*P*=0.2098). For ApoE rs7412, the genotypes were in Hardy-Weinberg equilibrium for the control group (*P*=0.3696) but deviated in the T2DM cases (*P*=0.0067). In the 51 cases re-examined for renal lesions, the genotypes were in Hardy-Weinberg equilibrium for both SNPs (*P*=0.8287 in ApoE rs429358, *P*=0.1104 in ApoE rs7412).

Table 17 shows the frequency of different genotypes and alleles of ApoE polymorphism. Since the frequency of ε3/ε3 [66.1% (141/213) in diabetic cases and 79.3% (88/111) in control groups] was higher than that of other genotypes, I used the predominant ε3/ε3 genotype as the referent group. The frequency of the ε2 allele (*P*=0.0071) and ε2 carriers (ε2/ε2, ε2/ε3) (*P*=0.0369, crude OR=2.1, 95%CI=1.0-4.2; P=0.0309, adjusted OR=2.2, 95%CI=1.1-4.4 after adjustment by age and sex) was higher in the T2DM than in the control cases. The non ε3/ε3 carriers (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε4, and ε4/ε4) were more likely to be diabetic with an OR of 1.9 (P=0.0141, crude OR=2.0, 95%CI=1.1-3.4; P=0.0161, adjusted OR=1.9, 95%CI=1.1-3.3) compared with control cases.

Table 17 ApoE allele and genotype frequencies in type 2 diabetic cases and controls

	T2DM (N=213)	Control (N=111)
Allele		
ε2	12.9 (55)*	6.3 (14)
ε3	79.8 (338)	88.3 (196)
ε4	7.8 (33)	5.4 (12)
Genotype		
ε2/ε2	3.8 (8)	0.9 (1)
ε2/ε3	15.0 (32)	9.9 (11)
ε2/ε4	3.3 (7)	0.9 (1)
ε3/ε3	66.2 (141)	79.3 (88)
ε3/ε4	11.3 (24)	8.1 (9)
ε4/ε4	0.5 (1)	0.9 (1)
Carrier		
ε2 carrier (ε2/ε2, ε2/ε3)	19.4 (40)**	10.9 (12)
ε3 carrier (ε3/ε3)	68.4 (141)	80.0 (88)
ε4 carrier (ε3/ε4, ε4/ε4)	12.1 (25)	9.1 (10)

Data are % (*n*); *P=0.007 *vs.* ε3 allele group, *P=0.0369 *vs.* ε3 carrier, calculated by Chi square test.

3.4.1.2. Clinicopathological characteristics of ApoE carriers

3.4.1.2.1. Clinicopathological characteristics of ApoE carriers in T2DM

The majority of cases were ε3 allele carriers in the control and T2DM cases. Previous studies indicate associations of ApoE ε2 and ε4 genotypes with different diseases including dyslipidemia [252], CVD [253], AD [254] and renal disease [231]. For comparison purpose, we categorized these cases into three groups: ε2 carriers (ε2/ε2, ε2/ε3), ε3 carriers (ε3/ε3) and ε4 carriers (ε3/ε4, ε4/ε4). Table 18 shows the clinicopathological characteristics of cases with each of these three ApoE carrier groups. Compared with ε3 carriers, ε2 allele carriers were more likely to have renal glomerular hypertrophy (P=0.0421) with an OR of 4.33 (95%CI 1.2–16.3, P = 0.0421). After further adjustment for risk factors (age, sex, hypertension and death cause due to renal disease), the adjusted odds ratio of ε2 and ε4 carriers were 5.42 (95% CI = 1.1–26.8, P = 0.0382) and 22.50 (95% CI = 1.1–454.9, P = 0.0424) (Table 19). Other clinicopathological parameters were not significantly different among these three groups.

Table 18 Clinicopathological characteristics of ApoE allele carriers in T2DM

	ε2 (ε2/ε2, ε2/ε3)	ε3 (ε3/ε3)	ε4 (ε3/ε4, ε4/ε4)
	N=40	N=141	N=25
Age (year)	68.9±11.6	70.4±11.1	72.1±10.5
Female	37.5 (15)*	56.0 (79)	64.0 (16)
	N=16	N=35	N=7
BMI (kg/m2)	23.4±4.7	23.1±4.6	23.0±4.3
	N=30	N=113	N=22
FPG (mmol/L)	10.7±6.2	11.6±5.1	12.3±5.6
	N=33	N=114	N=23
Systolic blood pressure (mmHg)	135.6±36.9	151.2±37.5	149.5±41.7
Diastolic blood pressure (mmHg)	75.5±16.2	80.2±20.9	80.7±23.8
	N=32	N=105	N=19
Plasma creatinine (μmol/L)	141.0	158.0	124.0
	(96.8-237.8)	(103.0-263.0)	(80.0-151.0)
Plasma urea (mmol/L)	11.0 (6.2-17.2)	12.0 (7.0-20.7)	9.9 (6.2-14.7)
GFR (ml/min/1.73m^2)	36.1 (20.6-56.8)	29.0 (15.5-48.6)	38.0 (31.6-56.0)
	N=40	N=139	N=25
Hypertension	57.5 (23)	64.7 (90)	60.0 (15)
	N=40	N=141	N=25
CHD	65.0 (26.0)	71.6 (101.0)	64.0 (16.0)
LVH	57.5 (23.0)	63.8 (90.0)	80.0 (20.0)
CAS	50.0 (20.0)	55.3 (78.0)	52.0 (13.0)
CVA	15.0 (6.0)	26.4 (37.0)	28.0 (7.0)
	N=38	N=137	N=23
Diabetic nephropathy	73.7 (28)	77.4 (106)	91.3 (21)
	N=39	N=139	N=25
Death due to renal diseases	5.1 (2)	6.5 (9)	4.0 (1)
Death due to Cardiovascular disease	46.2 (18.0)	55.4 (77.0)	32.0 (8.0)
Detailed renal pathology in	N=14	N=34	N=3

subgroup analysis			
Glomerular lesion	71.4 (10)	76.5 (26)	66.7 (2)
GGS	64.3 (9)	76.5 (26)	66.7 (2)
NGS	21.4 (3)	32.4 (11)	33.3 (1)
DGS	14.3 (2)	2.9 (1)	0.0 (0)
Glomerular hypertrophy	57.1 (8)**	23.5 (8)	66.7 (2)
Vascular lesions	85.7 (12)	100.0 (34)	100.0 (3)
Hyaline arteriolosclerosis	78.6 (11)	79.4 (27)	100.0 (3)
Arteriosclerosis	78.6 (11)	79.4 (27)	66.7 (2)
Tubulointerstitial lesion	50.0 (7)	58.8 (20)	100.0 (3)
Tubular lesion	35.7 (5)	32.4 (11)	66.7 (2)
Interstitial fibrosis	50.0 (7)	50.0 (17)	66.7 (2)
Vascular-tubulointerstitial lesions	92.9 (13)	100.0 (34)	100.0 (3)
Near normal structure	7.1 (1)	0.0 (0)	0.0 (0)

*P=0.0384 versus ε3 carrier group, compared with pearson's Chi-square test; **$P = 0.0421$ versus ε3 carrier group, compared with Fisher's exact test, a two-tailed P-value <0.025 was considered significant. CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as mean±SD, medians (interquartile range) or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Medians among three groups were compared using Kruskal-Wallis H test. Medians between two groups were compared using Mann–Whitney U test, a two-tailed P-value <0.025 was considered significant. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant.

Table 19 Associations of ApoE alleles and genotypes on renal glomerular hypertrophy in T2DM

	Crude OR (95%CI)	P	Adjusted* OR (95%CI)	P
ε2 carrier (ε2/ε2, ε2/ε3)	4.33 (1.15-16.26)	0.0421	5.42 (1.10-26.80)	0.0382
ε3 carrier (ε3/ε3)	1	1	1	1
ε4 carrier (ε3/ε4)	6.5 (0.52-81.42)	0.1718	22.50 (1.11-454.90)	0.0424
Non ε3/ε3 carrier	4.64 (1.33-16.19)	0.0160	6.72 (1.58-28.59)	0.0100

* Adjusted by age, sex, hypertension and death due to renal disease; OR: odds ratio.

3.4.1.2.2. Clinicopathological characteristics of ApoE carriers in control cases

Table 20 shows the clinicopathological characteristics of control cases in relation to the three different ApoE carrier groups. ApoE ε4 allele carriers had higher frequency of renal tubulointerstitial changes (P=0.0423) and tubular changes (P=0.0022) than ε3 allele carriers. After further adjustment for risk factors (age, sex, hypertension and death cause due to renal disease), the ε4 carriers had an adjusted odds ratio of 10.1 (95%CI=1.7–60.3, P=0.0114) for these renal changes (Table 21). Other clinicopathological parameters were not significantly different among these three groups.

Table 20 Clinicopathological characteristics of ApoE allele carriers in control cases

	ε2 (ε2/ε2, ε2/ε3)	ε3 (ε3/ε3)	ε4 (ε3/ε4, ε4/ε4)
	N=12	N=88	N=10
Age (year)	63.2±17.9	67.3±16.7	76.0±15.7
Female	75.0 (9.0)	45.5(40.0)	60.0(6.0)
Hypertension	16.7 (2.0)	14.8 (13.0)	20.0 (2.0)
CHD	8.3 (1.0)	10.2 (9.0)	10.0 (1.0)
LVH	8.3 (1.0)	4.5 (4.0)	0.0 (0.0)
CAS	8.3 (1.0)	9.1 (8.0)	20.0 (2.0)
CVA	0.0 (0.0)	10.2 (9.0)	10.0 (1.0)
Death due to renal diseases	0.0 (0.0)	1.1 (1.0)	0.0 (0.0)
Death due to Cardiovascular disease	8.3 (1.0)	14.8 (13.0)	20.0 (2.0)
Detailed renal pathology in subgroup analysis	N=12	N=88	N=10
Glomerular lesion	16.7 (2.0)	52.3 (46.0)	60.0 (6.0)
GGS	16.7 (2.0)	46.6 (41.0)	40.0 (4.0)
DGS	0 (0)	0 (0)	0 (0)
Glomerular hypertrophy	8.3 (1.0)	13.6 (12.0)	40.0 (4.0)
Vascular lesions	41.7 (5.0)	59.1 (52.0)	80.0 (8.0)
Hyaline arteriolosclerosis	16.7 (2.0)	9.1 (8.0)	20.0 (2.0)
Arteriosclerosis	41.7 (5.0)	59.1 (52.0)	80.0 (8.0)
Tubulointerstitial lesion	25.0 (3.0)	43.2 (38.0)	80.0 (8.0)*
Tubular lesion	8.3 (1.0)	28.4 (25.0)	80.0 (8.0)**
Interstitial fibrosis	25.0 (3.0)	34.1 (30.0)	60.0 (6.0)
Vascular-tubulointerstitial lesions	50.0 (6.0)	67.0 (59.0)	90.0 (9.0)
Near normal structure	50.0 (6.0)	26.1 (23.0)	10.0 (1.0)

* P=0.0423 and **P=0.0022 versus ε3 carrier group, compared with Fisher's exact test, a two-tailed P-value <0.025 was considered significant. CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD or % (n). Means were compared using the one-way ANOVA.

Dunnett t adjustment was used in post-hoc test for multiple comparisons. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed *P*-value <0.025 was considered significant.

Table 21 Associations of ApoE ε4 carriers on renal tubular changes in control cases

	Crude OR (95%CI)	P	Adjusted* OR (95%CI)	P
Tubulointerstitial lesions	5.3 (1.1-26.2)	0.0427	4.9 (0.8-30.4)	0.0878
Tubular lesion	10.1 (2.0-50.8)	0.0051	10.1 (1.7-60.3)	0.0114

* Adjusted by age, sex, hypertension and death due to renal disease; OR: odds ratio. Referent group: ApoE ε3 carriers group.

3.4.1.2.3. Clinicopathological characteristics of ApoE carriers in the entire study population

Table 22 shows the clinicopathological characteristics of different ApoE carriers in the entire study population. Compared to the ApoE ε3 carriers, ε2 (P=0.0336) and ε4 (P=0.0192) carriers (Table 22) showed higher percentage of renal glomerular hypertrophy with adjusted odds ratio of 3.4 (95%CI=1.2-9.5, P=0.0212) and 5.2 (95%CI=1.4-18.9, P=0.0124) respectively (Table 22). ApoE ε4 carriers were more likely to have renal tubulointerstitial (P=0.0110) and tubular lesions (P=0.0012) (Table 22) compared to ApoE ε3 carriers with adjusted odds ratio of 6.4 (95%CI=1.2-33.7, P=0.0280) and 7.6 (95%CI=1.9-30.9, P=0.0048) (Table 23). Other clinicopathologecal parameters were not found significantly different.

Table 22 Clinicopathological characteristics of ApoE allele carriers in the entire study population

	ε2 (ε2/ε2, ε2/ε3)	ε3 (ε3/ε3)	ε4 (ε3/ε4, ε4/ε4)
	N=52	N=229	N=35
Age (year)	67.6±13.4	69.2±13.6	73.2±12.1
Female	46.2(24.0)	52.0 (119.0)	62.9(22.0)
	N=52	N=227	N=35
Hypertension	48.1 (25.0)	45.4 (103.0)	48.6 (17.0)
	N=52	N=229	N=35
CHD	51.9 (27.0)	48.0 (110.0)	48.6 (17.0)
LVH	46.2 (24.0)	41.0 (94.0)	57.1 (20.0)
CAS	40.4 (21.0)	37.6 (86.0)	42.9 (15.0)
CVA	11.5 (6.0)	20.2 (46.0)	22.9 (8.0)
	N=38	N=137	N=23
Diabetic nephropathy	73.7 (28.0)	77.4 (106.0)	91.3 (21.0)
	N=51	N=227	N=35
Death due to renal diseases	3.9 (2.0)	4.4 (10.0)	2.9 (1.0)
Death due to Cardiovascular disease	37.3 (19.0)	39.6 (90.0)	28.6 (10.0)
Detailed renal pathology in subgroup analysis	N=26	N=122	N=13

Glomerular lesion	50.0 (13.0)	61.5 (75.0)	61.5 (8.0)
GGS	42.3 (11.0)	54.9 (67.0)	46.2 (6.0)
NGS	11.5 (3.0)	9.0 (11.0)	7.7 (1.0)
DGS	7.7 (2.0)	0.8 (1.0)	0.0 (0.0)
Glomerular hypertrophy	34.6 (9.0)*	16.4 (20.0)	46.2 (6.0)**
Vascular lesions	65.4 (17.0)	70.5 (86.0)	84.6 (11.0)
Hyaline arteriolosclerosis	50.0 (13.0)	28.7 (35.0)	38.5 (5.0)
Arteriosclerosis	61.5 (16.0)	64.8 (79.0)	76.9 (10.0)
Tubulointerstitial lesion	38.5 (10.0)	47.5 (58.0)	84.6 (11.0)†
Tubular lesion	23.1 (6.0)	29.5 (36.0)	76.9 (10.0) ‡
Interstitial fibrosis	38.5 (10.0)	38.5 (47.0)	61.5 (8.0)
Vascular-tubulointerstitial lesions	73.1 (19.0)	76.2 (93.0)	92.3 (12.0)
Near normal structure	26.9 (7.0)	18.9 (23.0)	7.7 (1.0)

*P=0.0336 and †P=0.0110 versus ε3 carrier group, compared with pearson's Chi-square test; **P=0.0192 and ‡P=0.0012 versus ε3 carrier group, compared with Fisher's exact test, a two-tailed P-value <0.025 was considered significant. CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis. Data are presented as or mean±SD or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant.

Table 23 Associations of ApoE carriers with renal changes in the entire study population

	Crude OR (95%CI)	P	Adjusted* OR (95%CI)	P
ApoE ε2 carrier				
Renal glomerular hypertrophy	2.7 (1.1-6.9)	0.0382	3.4 (1.2-9.5)	0.0212
ApoE ε4 carrier				
Renal glomerular hypertrophy	4.4 (1.3-14.4)	0.0152	5.2 (1.4-18.9)	0.0124
Renal tubulointerstitial lesions	6.1 (1.3-28.5)	0.0224	6.4 (1.2-33.7)	0.0280
Renal tubular lesion	8.0 (2.1-30.6)	0.0025	7.6 (1.9-30.9)	0.0048

* Adjusted by age, sex, hypertension and death due to renal disease; OR: odds ratio. Referent group: ApoE ε3 carriers group.

3.4.1.3 ApoE genotype and protein expression in diabetic glomerulosclerosis

In the T2DM group, carriers of ε2/ε3 (N = 9), ε3/ε3 (N = 10) and ε3/ε4 (N = 9) genotypes matched for age, sex and serum creatinine were selected for examination of renal ApoE protein expression using immunohistochemical staining. Within the glomeruli, ApoE immunoreactivity was mainly found in mesangial cells (Figure 19), although they were also expressed in the peripheral region in most of nodular lesions. Compared to ε3/ε3 (1.2±0.7%) or ε3/ε4 (1.0±0.6%) genotype carriers, those with ε2/ε3 genotypes (3.0±1.2%) displayed more intense ApoE staining in the glomerular mesangial regions and nodular lesions (P=0.0003 versus ε3/ε3 genotype; P=0.0001 versus ε3/ε4 genotype) (Figure 19A). In contrast, ε3/ε4 cases had diffuse ApoE expression in glomerular capillaries (Figure 19C). ApoE expression was also localized in the basal part of renal tubular epithelial cells (Figure 20). There was a tendancy for ApoE ε2/ε3 genotypes to be associated with increased expression of ApoE in these renal tubulointerstitial lesions albeit short of significance (5.4±8.0% in ε2/ε3 genotypes, 0.5±0.1% in ε3/ε3 genotypes, 2.5±4.8% in ε3/ε4 genotypes, P=0.1103 versus ε3/ε3 genotype, P=0.3791 versus ε3/ε4 genotype).

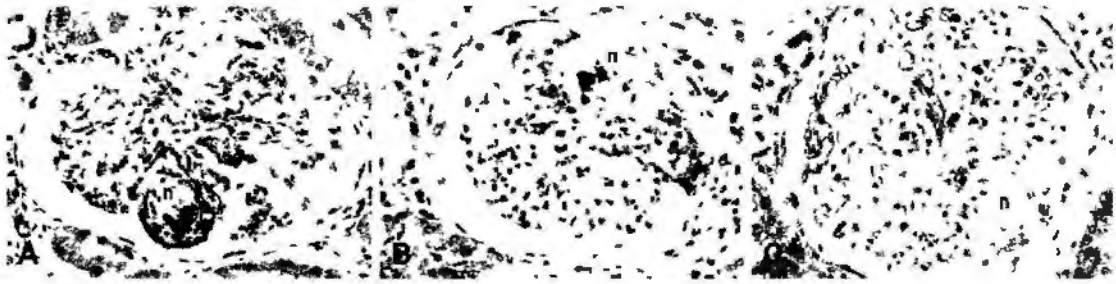


Figure 19 ApoE protein expression in diabetic nodular glomerulosclerosis.
Immunostaining with a monoclonal ApoE antibody (brown) was performed on kidney tissue sections from carriers with ε2/ε3 (A), ε3/ε3 (B) or ε3/ε4 (C) genotypes. Carriers of ε2 allele showed abundant ApoE protein expression in mesangial cells and excessive deposition in nodular lesions (n), whereas ε4 carriers revealed immunoreactivity in glomerular capillaries. (Immunohistochemical stain, magnification ×400.)

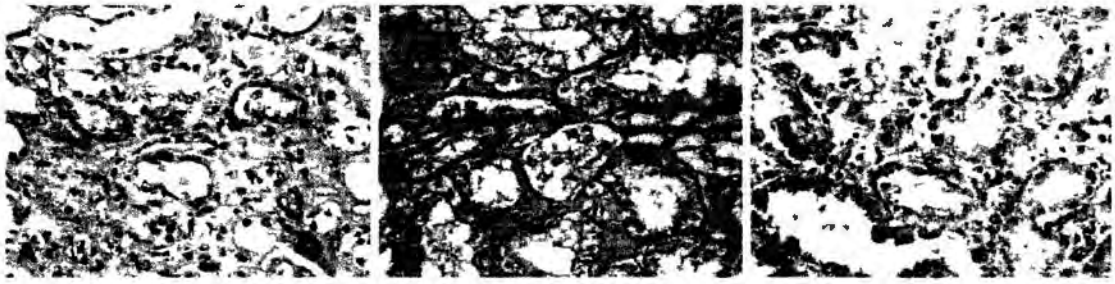


Figure 20 ApoE protein expression in diabetic renal tubules.
Immunostaining with a monoclonal ApoE antibody was performed on kidney tissue sections from carriers with ε2/ε3 (A), ε3/ε3 (B) or ε3/ε4 (C) genotypes. There was a tendancy for more ApoE to be expressed especially in the basal part of renal tubular epithelial cells although this did not reach statistical significance amongst the three groups. (Immunohistochemical stain, magnification ×400.)

3.4.2 ApoE polymorphisms and islet amyloidosis

3.4.2.1 ApoE genotype and allele frequencies

In 117 T2DM cases and 209 control cases with available pancreatic tissues, 86 T2DM and 111 control cases were genotypied successfully. Both ApoE genotypes were in Hardy-Weinberg equilibrium in T2DM cases (P=0.5670 for rs429358, P=0.1254 for rs7412) and controls (P=0.2098 for rs429358, P=0.3696 for rs7412). The majority of cases were ε3 allele carriers in the control and T2DM cases. For comparison purposes, we categorized these cases into groups based on their predominant allele. Thus, in the T2DM group, 65.5% (n=55) had the ε3/ε3 genotype and were categorized as ε3 carriers, 25.0% (n=21) had either ε2/ε2 or ε2/ε3 genotypes (ε2 carrier) and 9.5% (n=8) had ε3/ε4 or ε4/ε4 genotypes (ε4 carrier). There was an increased frequency of the ε2 allele and the ε2/ε3 genotype in the diabetic group compared to the control group (P=0.0292). The frequency of ε2 carriers (ε2/ε2, ε2/ε3) was also higher in diabetic cases than controls (25.0% (21/84) in the diabetic group versus 10.9% (12/110) in the control group, P=0.0085) (table 24). There were increased frequencies of ε2 allele (P=0.0024), ε2/ε3 genotype (P=0.0292, crude OR=2.47, 95%CI=1.08-5.67; P=0.0242, adjusted OR=2.6, 95%CI=1.1-6.2) and ε2 carriers (ε2/ε2, ε2/ε3) (P=0.0085, crude OR=2.8, 95%CI=1.3-6.1; P=0.0075, adjusted OR=3.0, 95%CI=1.3-6.6) in the T2DM group than the control cases. Analysed as a group, non ε3/ε3 carriers (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε4, and ε4/ε4) was associated with T2DM with an OR of 2.1 (P=0.0168, crude OR=2.2, 95%CI=1.14-4.07; P=0.0200, adjusted OR=2.1, 95%CI=1.1-4.0) compared with control cases.

Table 24 ApoE allele and genotype frequencies in type 2 diabetic cases and controls

	T2DM	Control
Allele	N=172	N=222
ε2	15.7 (27)*	6.3 (14)
ε3	78.5 (135)	88.3 (196)
ε4	5.8 (10)	5.4 (12)
Genotype	N=86	N=111
ε2/ε2	4.7 (4)	0.9 (1)
ε2/ε3	19.8 (17)**	9.9 (11)
ε2/ε4	2.3 (2)	0.9 (1)
ε3/ε3	64.0 (55)	79.3 (88)
ε3/ε4	9.3 (8)	8.1 (9)
ε4/ε4	0.0 (0)	0.9 (1)
Carrier	N=84	N=110
ε2 carrier (ε2/ε2, ε2/ε3)	25.0 (21)†	10.9 (12)
ε3 carrier (ε3/ε3)	65.5 (55)	80.0 (88)
ε4 carrier (ε3/ε4, ε4/ε4)	9.5 (8)	9.1 (10)

Data are % (*n*); *P=0.0024 *vs.* ε3 allele group, **P=0.0292 *vs.* ε3/ε3 genotype, †P=0.0085 *vs.* ε3 carrier, calculated by Chi square test.

3.4.2.2 Correlations of islet amyloidosis with ApoE polymorphisms

Consistent with our prior report [27], the percentage of cases with islet amyloidosis was higher in the T2DM group (30.8% (36/117)) than the control group (1.9% (4/209), OR=22.8, 95%CI=7.9-66.0, $P<0.0001$) (Table 14). In both the T2DM (Table 25) and control group, there was no difference in clinical and metabolic profile amongst different ApoE genotypes. In the T2DM cases, compared to cases with ε3 carrier status, cases with ε4 carriers status were more likely to have islet amyloidosis [62.5% (5/8) vs. 23.6% (13/55), $P=0.0365$] with an odds ratio of 5.4 (95%CI=1.1-25.6, $P=0.0345$) which increased to 7.0 (95%CI=1.3-38.0, $P=0.0232$) after adjustment for age, sex and hypertension (Table 26). Due to infrequent islet amyloidosis in control cases, we did not analyse the association of ApoE carriers and islet amyloidosis in control cases and the entire study population.

Table 25 Clinicopathological characteristics of ApoE carriers with T2DM

	ε2 (ε2/ε2, ε2/ε3)	ε3 (ε3/ε3)	ε4 (ε3/ε4, ε4/ε4)
	N=21	N=55	N=8
Age(year)	67.1±11.8	70.6±12.7	71.4±10.5
Female	61.9 (13)	40.0 (22)	25.0 (2)
	N=21	N=53	N=8
Hypertension	47.6 (10)	69.8 (37)	62.5 (5)
	N=7	N=16	N=2
BMI (kg/m^2)	23.7±5.9	24.1±5.9	24.1±0.8
	N=14	N=36	N=6
FPG (mmol/L)	10.7±7.3	13.2±5.8	11.5±3.6
	N=16	N=36	N=6
Systolic blood pressure (mmHg)	128.8±37.2*	157.1±33.8	136.3±41.4
Diastolic blood pressure (mmHg)	74.3±14.0	81.0±19.0	78.0±19.7
	N=15	N=34	N=5
Plasma creatinine (μmol/L)	146.0 (99.0-185.0)	233.5 (112.3-333.3)	112.0 (93.0-152.5)
Plasma urea (mmol/L)	12.8 (7.8-23.1)	13.2 (7.2-21.8)	9.9 (7.1-12.7)
GFR ($mL/min/1.73m^2$)	32.6 (27.8-54.5)	16.3 (13.3-40.9)	36.4 (35.0-47.0)
	N=21	N=53	N=8
Death due to renal disease	9.5 (2)	5.7 (3)	12.5 (1)
Death due to cardiovascular disease	47.6 (10)	52.8 (28)	25 (2)
	N=21	N=55	N=8
CHD	66.7 (14)	58.2 (32)	62.5 (5)
LVH	57.1 (12)	50.9 (28)	62.5 (5)
CAS	57.1 (12)	45.5 (25)	50 (4)
CVA	9.5 (2)	12.7 (7)	12.5 (1)

Pancreas lesions	N=21	N=55	N=8
Vascular lesions	76.2 (16)	80.0 (44)	75 (6)
Hyaline arteriolosclerosis	76.2 (16)	74.5 (41)	62.5 (5)
Arteriosclerosis	71.4 (15)	49.1 (27)	62.5 (5)
Interstitial lesions	81.0 (17)	90.9 (50)	87.5 (7)
Interstitial fibrosis	71.4 (15)	63.6 (35)	50 (4)
Interstitial fat infiltration	66.7 (14)	81.8 (45)	87.5 (7)
Islet amyloidosis	33.3 (7)	23.6 (13)	62.5 (5)**
Vascular interstitial lesions	95.2 (20)	94.5 (52)	100 (8)
Near normal structure	4.8 (1)	5.5 (3)	0 (0)

* P=0.0441 vs. ε3 carrier, caculated by Dunnett t test; **P=0.0345 vs. ε3 carrier, caculated by Fisher's exact test; a two-tailed P-value <0.025 was considered significant. CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident.

Data are presented as or mean±SD, medians (interquartile range) or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Medians among three groups were compared using Kruskal-Wallis H test. Medians between two groups were compared using Mann–Whitney U test, a two-tailed P-value <0.025 was considered significant. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant.

Table 26 Associations of ApoE carriers with islet amyloidosis

	Crude OR (95%CI)	P	Adjusted* OR (95%CI)	P
ε2 carrier (ε2/ε2, ε2/ε3)	1.6 (0.5-4.9)	0.3929	2.4 (0.6-9.0)	0.1884
ε3 carrier (ε3/ε3)	1	1	1	1
ε4 carrier (ε3/ε4, ε4/ε4)	5.4 (1.1-25.6)	0.0345	7.0 (1.3-38.0)	0.0232

* Adjusted by age, sex, hypertension; OR: odds Ratio.

3.4.2.3 Association of vascular lesions and islet amyloidosis

T2DM cases with islet amyloidosis showed significantly higher percentage of pancreatic vascular interstitial lesions than cases free from amyloidosis [88.9% (32) vs. 71.6% (58), P=0.0406 for vascular lesion; 83.3% (30) vs. 64.2% (52), P=0.0369 for hyaline arteriolesclerosis; 72.2% (26) vs. 46.9% (38), P=0.0111 for arteriosclerosis and 75.0% (27) vs. 51.9% (42), P=0.0188 for pancreatic interstitial fibrosis) (Table 14).

3.4.2.4 Immunolocalization of ApoE and amyloid P in islet amyloid deposits and arteriosclerotic lesions

Immunofluorescence staining were performed on diabetic pancreatic cases with islet amyloidosis (N=6), without amyloidosis (N=6) and non-diabetic pancreatic controls (N=6). As one of the common components in extracellular amyloid deposits, ApoE immunoreactivity corresponded to the thioflavin T staining and amylin-reactive fibrillar amyloid deposition (Figure 21). Amyloid P is also a chaperone protein in all histological types of amyloid deposits [255] (Figure 22). Immunofluorescence microscopy revealed presence of both ApoE and amyloid P proteins in arteriolar wall with hyaline changes (Figure 23). Light microscopy of immunohistochemical staining confirmed immunolocalization of amyloid P in islet amyloid deposits, arteriosclerotic lesions and fibrotic tissues (Figure 24). These findings confirmed that these proteins are common constituents of arteriosclerosis and amyloidosis in T2DM.

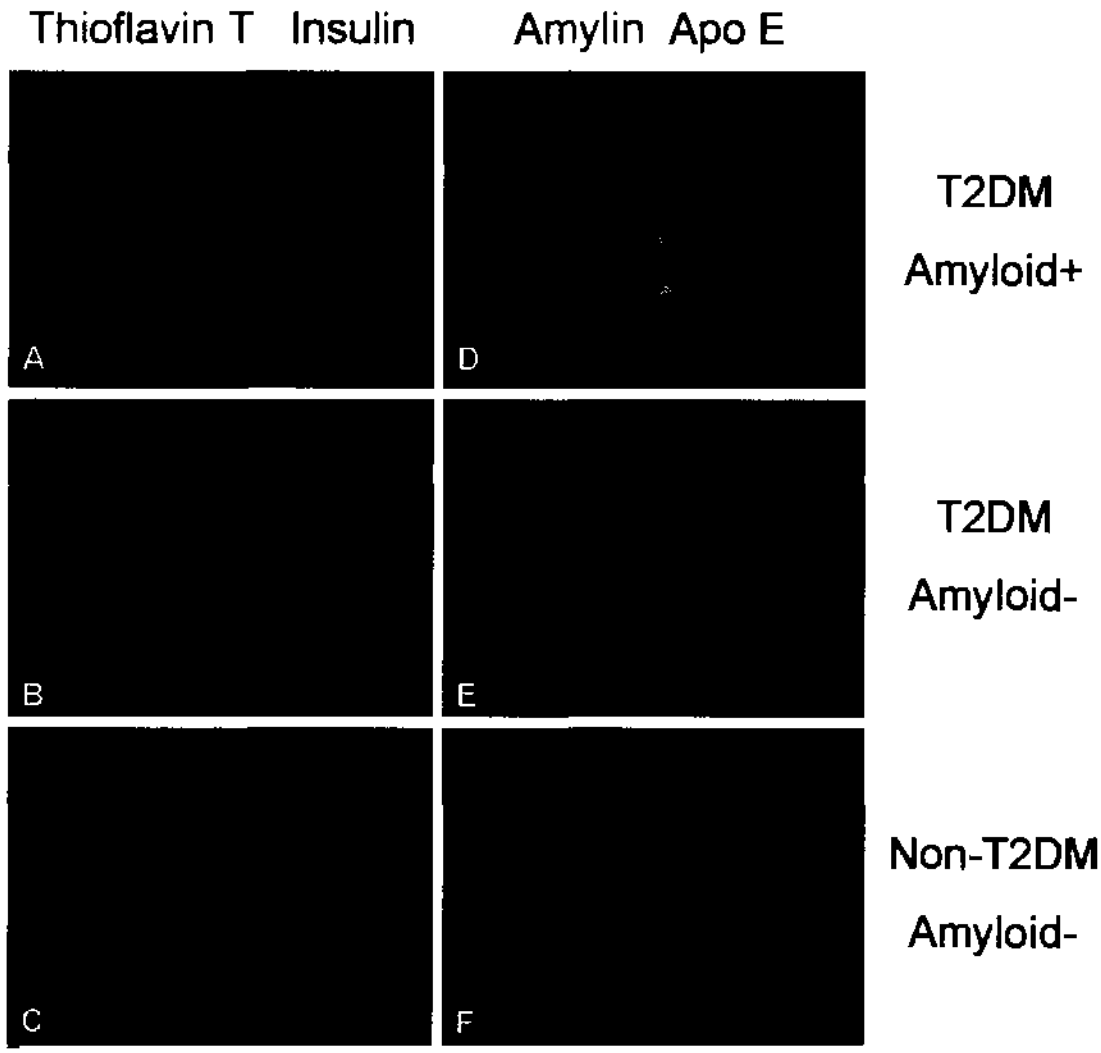


Figure 21 Co-localization of Apo E protein and islet amyloidosis.

Immunofluorescence staining were performed on diabetic pancreatic sections with islet amyloidosis (A and D), without amyloidosis (B and E) and non-diabetic pancreatic sections (C and F). The sections were stained with Thioflavin T (green) and monoclonal anti-insulin antibody (red) (A, B and C) as well as polyclonal anti-amylin antibody (green) and monoclonal anti-ApoE antibody (red) (D, E, F), respectively. ApoE immunoreactivity was co-localized with amylin-reactive extracellular amyloid deposits, as demonstrated in yellow (D), whereas there was no Apo E expression in the diabetic (E) or non-diabetic islet (F) without amyloidosis. (Immunofluorescence stain, magnification ×400.)

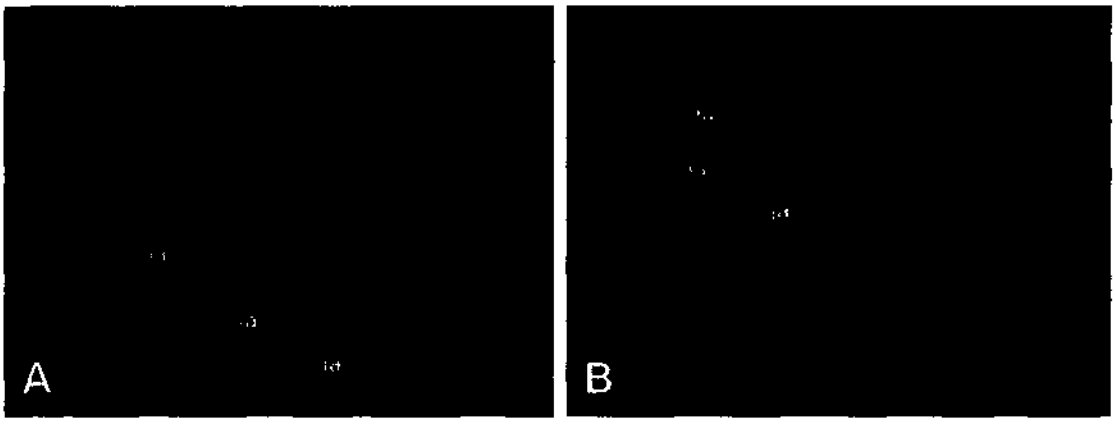


Figure 22 Co-localization of Amyloid P and islet amyloidosis in T2DM.

Serial sections of a representive islet with amyloidosis were stained with thioflavin T (panel A) and amyloid P (panel B). Panel A showed the extracellular amyloid fibril deposition. Amyloid P protein was also localized with the extracellular amyloid fibril area. (Immunofluorescence stain, magnification ×200.)

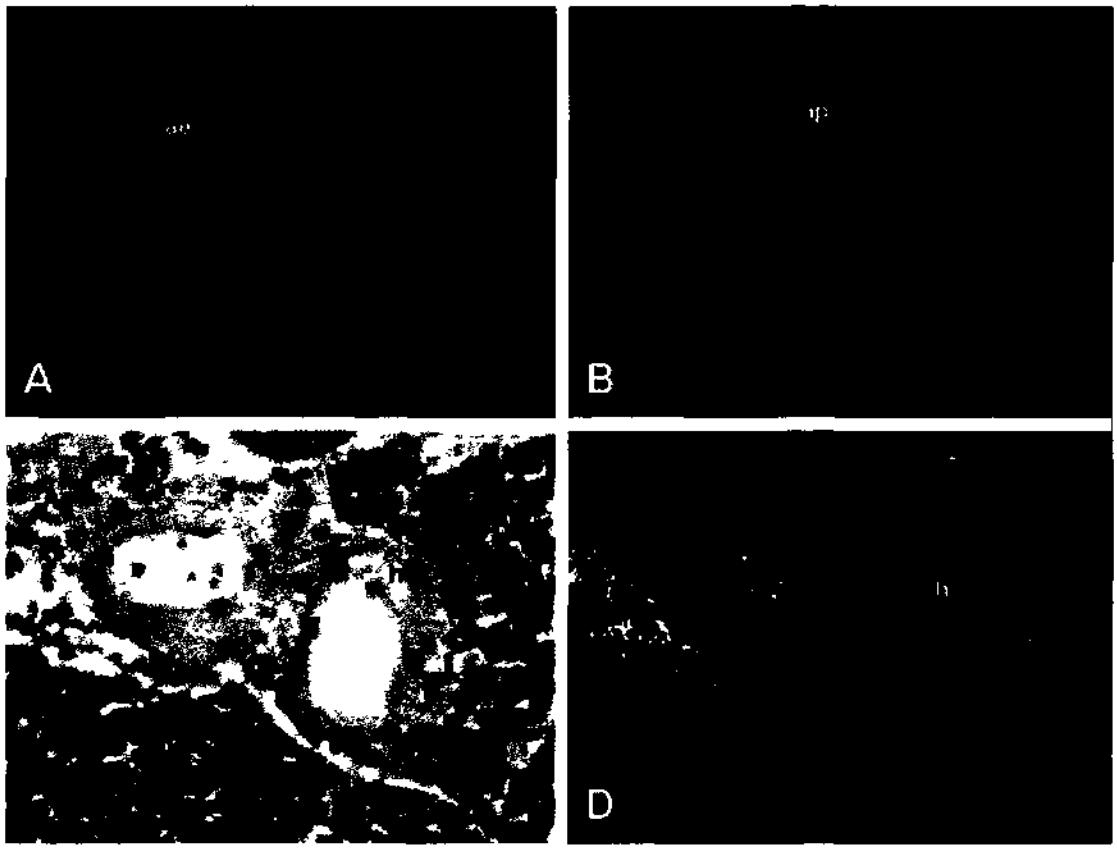


Figure 23 Co-expression of ApoE and Amyloid P protein in arteriosclerosis in T2DM. Serial pancreatic sections were stained with monoclonal anti-ApoE antibody (red, panel A), polyclonal anti-Amyloid P antibody (Red, panel B) and H&E (panel C). Panel D is negative control. As the common components of extra-cellular islet amyloidosis, ApoE (ae) and Amyloid P (ap) preoteins were also co-localized in arteriosclerotic lesions (h). (A and B: Immunofluorescence stain, C: H&E stain; magnification ×400.)

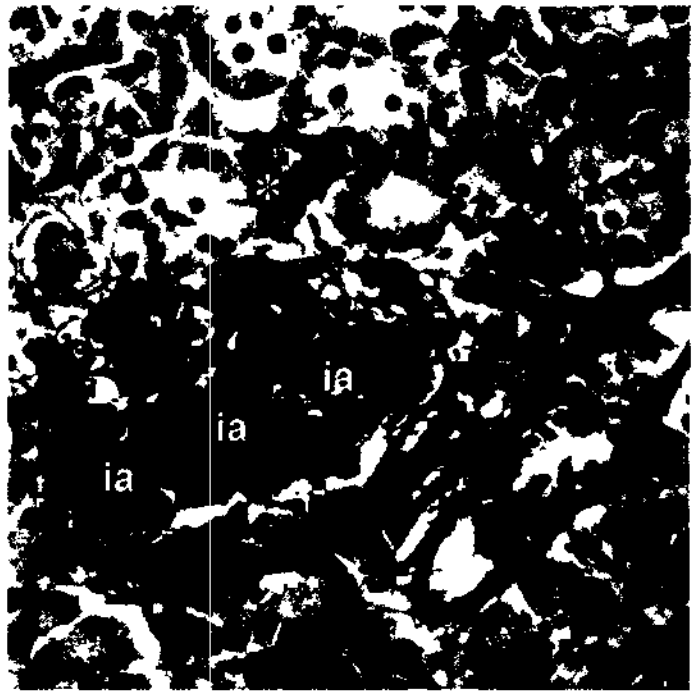


Figure 24 Immunolocalization of amyloid P protein in islet amyloidosis and arteriosclerosis in diabetic pancreas.

Pancreatic tissue section was stained with polyclonal anti-Amyloid P antibody. Light microscopy demonstrates presence of amyloid P component in islet amyloid deposits (ia), arteriosclerotic lesion (*) and fibrotic tissues (arrows) adjacent to amyloidosis. Immunohistochemical stain, original magnification ×400.

3.5. Association with IDE polymorphism

3.5.1. IDE genotype frequency

IDE genetic polymorphisms were successfully genotyped in 84 diabetic and 117 control cases. The genotypic distributions of rs6583813 did not deviate from Hardy-Weinberg equilibrium in either the T2DM cases (P=0.0718) or in the control group (P=0.9898). T2DM cases tended to have higher percentage of the C allele than control cases (Table 27), although the difference was not significant.

Table 27 IDE genotype frequencies in type 2 diabetic cases and controls

	T2DM	Control
Allele	N=168	N=234
T	66.7 (112)	72.2 (169)
C	33.3 (56)	27.8 (65)
Genotype	N=84	N=117
TT	48.8 (41)	52.1 (61)
CT	35.7 (30)	40.2 (47)
CC	15.5 (13)	7.7 (9)

% (number)

3.5.2. Correlation of IDE polymorphism with cardiovascular disease

3.5.2.1. Correlation of IDE polymorphism with cardiovascular disease in T2DM

Table 28 shows the clinicopathological characteristics of different IDE genotypes under additive model. There was a graded and significant increase in cardiovascular conditions (hypertension, CHD, CVA and death due to cardiovascular disease) across the genotypes. Compared to the predominant TT genotype, C carriers (CC and CT genotype, dominant model) had higher percentages of hypertension, CHD, CAS, CVA and death due to cardiovascular disease in T2DM (Table 29).

Logistic regression analysis showed that the risk of hypertension, CVA and death due to CVD in C carriers was increased by up to 2-fold compared to TT genotype carriers after adjusting for confounding factors (Table 30). The cardiovascular conditions in relation to different IDE genotype are shown in table 30.

Table 28 Clinicopathological characteristics of IDE genotypes in T2DM

	TT	CT	CC
	N=41	N=30	N=13
Age (year)	68.4±13.4	72.1±12.9	71.1±8.1
Female	61.0 (25)	63.3 (19)	30.8 (4)
	N=7	N=7	N=3
BMI (kg/m^2)	23.3±5.6	23.7±3.0	22.6±3.1
	N=18	N=19	N=8
Fasting plasma glucose (mmol/L)	11.0±4.5	11.2±6.7	16.2±9.2
	N=18	N=20	N=8
Systolic blood pressure (mmHg)	145.4±41.9	152.4±43.9	144.5±27.0
Diastolic blood pressure (mmHg)	79.9±17.2	79.4±24.3	78.9±14.1
	N=15	N=19	N=8
Plasma creatinine (μmol/L)	146.0 (115.0-243.0)	185.0 (106.0-250.0)	198.0 (83.5-325.3)
Plasma urea (mmol/L)	12.0 (7.7-21.4)	12.4 (7.0-15.8)	13.8 (8.1-19.5)
GFR ($ml/min/1.73m^2$)	32.6 (15.6-49.5)	28.2 (14.9-38.0)	25.5 (14.6-63.8)
	N=39	N=30	N=13
Hypertension §	43.6 (17)	63.3 (19)	76.9 (10)
	N=41	N=30	N=13
CHD §	39.0 (16)	66.7 (20) *	76.9 (10)**
LVH	39.0 (16)	50.0 (15)	53.8 (7)
CAS	31.7 (13)	60.0 (18)	46.2 (6)
CVA §	2.4 (1)	20.0 (6)‡	23.1 (3)‡‡
	N=40	N=30	N=13
Diabetic nephropathy	80.0 (32)	73.3 (22)	92.3 (12)
	N=39	N=30	N=13
Death due to renal disease	7.7 (3)	3.3 (1)	7.7 (1)
Death due to	25.6 (10)	53.3 (16) †	69.2 (9)††

Cardiovascular disease §			
Renal pathology	N=18	N=18	N=4
Glomerular lesion	83.3 (15)	77.8 (14)	100.0 (4)
GGS	72.2 (13)	72.2 (13)	75.0 (3)
NGS	22.2 (4)	22.2 (4)	25.0 (1)
DGS	5.6 (1)	5.6 (1)	0.0 (0)
Glomerular hypertrophy	38.9 (7)	33.3 (6)	75.0 (3)
Vascular lesions	100.0 (18)	88.9 (16)	100.0 (4)
Hyaline arteriolosclerosis	83.3 (15)	72.2 (13)	100.0 (4)
Arteriosclerosis	83.3 (15)	77.8 (14)	50.0 (2)
Tubulointerstitial lesions	61.1 (11)	55.6 (10)	50.0 (2)
Tubular lesion	44.4 (8)	38.9 (7)	50.0 (2)
Interstitial fibrosis	50.0 (9)	50.0 (9)	50.0 (2)
Vascular-tubulointerstitial lesions	100.0 (18)	94.4 (17)	100.0 (4)
Near normal structure	0.0 (0)	5.6 (1)	0.0 (0)
Pancreas lesions	N=41	N=30	N=13
Vascular lesions	80.5 (33)	70.0 (21)	69.2 (9)
Hyaline arteriolosclerosis	75.6 (31)	66.7 (20)	61.5 (8)
Arteriosclerosis	51.2 (21)	46.7 (14)	61.5 (8)
Interstitial lesions	90.2 (37)	83.3 (25)	92.3 (12)
Interstitial fibrosis	58.5 (24)	46.7 (14)	46.2 (6)
Interstitial fat infiltration	82.9 (34)	76.7 (23)	76.9 (10)
Islet amyloidosis	31.7 (13)	20.0 (6)	30.8 (4)
Vascular interstitial lesions	97.6 (40)	90.0 (27)	100.0 (13)
Near normal structure	2.4 (1)	10.0 (3)	0.0 (0)

*P=0.0214, †P=0.0186 and **P=0.0172 vs. TT genotype, compared using pearson's Chi-square

test; ‡P=0.0367, ††P=0.0077 and ‡‡P=0.0393 vs. TT genotype, compared using Fisher's exact test; CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD, medians (interquartile range) or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Medians among three groups were compared using Kruskal-Wallis H test. Medians between two groups were compared using Mann–Whitney U test, a two-tailed *P*-value <0.025 was considered significant. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant. §P values for trend<0.0500, linear-by-linear test was used to compare categorical variables for trend.

Table 29 Clinicopathological characteristics of IDE C carriers in T2DM

	TT	C carrier (CT, CC)	P value
	N=41	N=43	
Age (year)	68.4±13.4	71.8±11.6	0.2136
Female	61.0 (25)	53.5 (23)	0.4882
	N=7	N=10	
BMI (kg/m^2)	23.3±5.6	23.3±2.9	0.9794
	N=18	N=27	
Fasting plasma glucose (mmol/L)	11.0±4.5	12.7±7.7	0.3933
	N=18	N=28	
Systolic blood pressure (mmHg)	145.4±41.9	150.1±39.5	0.7024
Diastolic blood pressure (mmHg)	79.9±17.2	79.3±21.6	0.9092
	N=15	N=27	
Plasma creatinine (μmol/L)	146.0 (115.0-243.0)	185.0 (106.0-299.0)	0.6937
Plasma urea (mmol/L)	12.0 (7.7-21.4)	12.8 (7.2-15.8)	0.9372
GFR ($ml/min/1.73m^2$)	32.6 (15.6-49.5)	28.2 (14.9-38.0)	0.5905
	N=39	N=43	
Hypertension	43.6 (17)	67.4 (29)	0.0297
	N=41	N=43	
CHD	39.0 (16)	69.8 (30)	0.0047
LVH	39.0 (16)	51.2 (22)	0.2639
CAS	31.7 (13)	55.8 (24)	0.0261
CVA	2.4 (1)	20.9 (9)	0.0149
	N=40	N=43	
Diabetic nephropathy	80.0 (32)	79.1 (34)	0.9164
	N=39	N=43	
Death due to renal disease	7.7 (3)	4.7 (2)	0.6648

Death due to Cardiovascular disease	25.6 (10)	58.1 (25)	0.0030
Renal pathology	N=18	N=22	
Glomerular lesion	83.3 (15)	81.8 (18)	1.0000
GGS	72.2 (13)	72.7 (16)	1.0000
NGS	22.2 (4)	22.7 (5)	1.0000
DGS	5.6 (1)	4.5 (1)	1.0000
Glomerular hypertrophy	38.9 (7)	40.9 (9)	0.8968
Vascular lesions	100.0 (18)	90.9 (20)	0.4923
Hyaline arteriolosclerosis	83.3 (15)	77.3 (17)	0.7089
Arteriosclerosis	83.3 (15)	72.7 (16)	0.4761
Tubulointerstitial lesions	61.1 (11)	54.5 (12)	0.6760
Tubular lesion	44.4 (8)	40.9 (9)	0.8220
Interstitial fibrosis	50.0 (9)	50.0 (11)	1.0000
Vascular-tubulointerstitial lesions	100.0 (18)	95.5 (21)	1.0000
Near normal structure	0.0 (0)	4.5 (1)	1.0000
Pancreas lesions	N=41	N=43	
Vascular lesions	80.5 (33)	69.8 (30)	0.2567
Hyaline arteriolosclerosis	75.6 (31)	65.1 (28)	0.2931
Arteriosclerosis	51.2 (21)	51.2 (22)	0.9959
Interstitial lesions	90.2 (37)	86.0 (37)	0.7387
Interstitial fibrosis	58.5 (24)	46.5 (20)	0.2700
Interstitial fat infiltration	82.9 (34)	76.7 (33)	0.4808
Islet amyloidosis	31.7 (13)	23.3 (10)	0.3852*
Vascular interstitial lesions	97.6 (40)	93.0 (40)	0.6162
Near normal structure	2.4 (1)	7.0 (3)	0.6162

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis. * Power=0.2159.

Data are presented as or mean±SD, medians (interquartile range) or % (n). They were compared by Student t-test, Mann–Whitney U test, Chi-square test or Fisher's exact

test, as appropriate.

Table 30 Associations of IDE genotypes with vascular disorders in T2DM

	C carriers				CT genotype				CC genotype			
	Crude OR (95%CI)	P	Adjusted OR (95%CI)	P	Crude OR (95%CI)	P	Adjusted OR (95%CI)	P	Crude OR (95%CI)	P	Adjusted OR (95%CI)	P
Hypertension	2.7 (1.1-6.6)	0.0315	2.8 (1.1-7.0)*	0.0332	2.2 (0.8-5.9)	0.1061	2.1 (0.8-5.7)*	0.1530	4.3 (1.0-18.2)	0.0462	6.7 (1.4-33.2)*	0.0193
CHD	3.6 (1.5-8.9)	0.0054	2.8 (0.9-8.5)†	0.0691	3.1 (1.2-8.4)	0.0234	2.9 (0.8-10.5) †	0.0974	5.2 (1.2-21.9)	0.0242	2.5 (0.5-13.6) †	0.2822
CAS	2.7 (1.1-6.6)	0.0278	2.0 (0.7-5.4) †	0.1766	3.2 (1.2-8.6)	0.0194	2.7 (0.9-8.2) †	0.0802	1.8 (0.5-6.6)	0.3454	0.9 (0.2-4.0) †	0.8972
Death due to CVD	4.0 (1.6-10.3)	0.0037	2.9 (1.0-8.4) †	0.0479	3.3 (1.2-9.1)	0.0207	2.8 (0.8-9.6) †	0.1020	6.5 (1.6-25.9)	0.0077	3.4 (0.8-15.5) †	0.1092
CVA	10.6 (1.3-87.9)	0.0288	21.7 (1.1-414.5) †	0.0412	10.0 (1.1-88.2)	0.0381	18.4 (0.9-372.4) †	0.0578	12.0 (1.1-128.0)	0.0396	9.3 (0.6-149.7) †	0.1157

* Adjusted by age and sex; † Adjusted by age, sex and hypertension; OR: odds Ratio. Referential group: TT genotype

3.5.2.2. Correlation of IDE polymorphism with renal vascular changes in control cases

The nondiabetic control cases showed no differences in clinical, metabolic profile, pancreatic changes and cardiovascular disorders amongst different IDE genotypes. Compared to the TT genotype, the CT genotype in control cases had higher percentage of renal vascular-tubulointerstitial lesions and lower percentage of near-normal renal structure renal. C carriers were more likely to have renal vascular lesions (Table 31, additive and dominant models). After adjustment for age, sex, hypertension and death due to renal disease, P values remined significant for renal vascular lesions in CT genotype (P=0.0078, OR=3.9, 95%CI=1.4-10.7) and C carriers (P=0.0348, OR=2.7, 95%CI=1.1-6.7) as well as for arteriosclerosis in CT genotype carriers (P=0.0168, OR=3.4, 95%CI=1.2-9.7) (Table 32).

Table 31 Clinicopathological characteristics of IDE genotype in control cases

	TT	CT	CC
	N=61	N=47	N=9
Age (year)	65.9±17.2	68.2±15.8	68.9±10.8
Female	49.2 (30)	46.8 (22)	55.6 (5)
Hypertension	14.8 (9)	12.8 (6)	0.0 (0)
CHD	11.5 (7)	10.6 (5)	11.1 (1)
LVH	3.3 (2)	4.3 (2)	0.0 (0)
CAS	9.8 (6)	10.6 (5)	11.1 (1)
Death due to renal disease	0.0 (0)	2.1 (1)	0.0 (0)
Death due to Cardiovascular disease	14.8 (9)	12.8 (6)	0.0 (0)
CVA	6.6 (4)	17.0 (8)	0.0 (0)
Renal pathology	N=61	N=47	N=9
Glomerular lesion	39.3 (24)	55.3 (26)	22.2 (2)
GGS	32.8 (20)	51.1 (24)*	11.1 (1)
DGS	0 (0)	0 (0)	0 (0)
Glomerular hypertrophy	14.8 (9)	10.6 (5)	11.1 (1)
Vascular lesions #	44.3 (27)	72.3 (34)**	33.3 (3)
Hyaline arteriolosclerosis	8.2 (5)	17.0 (8)	0.0 (0)
Arteriosclerosis #	44.3 (27)	70.2 (33)†	33.3 (3)

Tubulointerstitial lesions	37.7 (23)	55.3 (26)	22.2 (2)
Tubular lesion	27.9 (17)	29.8 (14)	22.2 (2)
Interstitial fibrosis	31.1 (19)	44.7 (21)	11.1 (1)
Vascular-tubulointerstitial lesions	55.7 (34)	76.6 (36)††	33.3 (3)
Near normal structure	41.0 (25)	19.1 (9)‡	66.7 (6)
Pancreas lesions	N=61	N=47	N=9
Vascular lesions	16.4 (10)	19.1 (9)	22.2 (2)
Hyaline arteriolosclerosis	4.9 (3)	10.6 (5)	11.1 (1)
Arteriosclerosis	13.1 (8)	17.0 (8)	11.1 (1)
Interstitial lesions	59.0 (36)	53.2 (25)	44.4 (4)
Interstitial fibrosis	19.7 (12)	17.0 (8)	22.2 (2)
Interstitial fat infiltration	50.8 (31)	48.9 (23)	22.2 (2)
Islet amyloidosis §	0.0 (0)	0.0 (0)	11.1 (1)
Vascular interstitial lesions	63.9 (39)	63.8 (30)	55.6 (5)
Near normal structure	27.9 (17)	29.8 (14)	22.2 (2)

*P=0.0553, **P=0.0035, †P=0.0071, ††P=0.0244, ‡P=0.0154 vs. TT genotype, compared with pearson's Chi-square test. CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant. §P values for trend<0.0500, linear-by-linear test was used to compare categorical variables for trend. #P<0.0500 in C allele carriers (CC/CT) vs. TT genotype. They were compared by Chi-square test or Fisher's exact test, as appropriate.

Table 32 Associations of IDE genotypes with renal changes in control cases

	C carrier				CT genotype			
	Crude OR (95%CI)	P	Adjusted* OR (95%CI)	P	Crude OR (95%CI)	P	Adjusted* OR (95%CI)	P
Vascular lesions	2.5 (1.2-5.2)	0.0189	2.7 (1.1-6.7)	0.0348	3.3 (1.5-7.4)	0.0041	3.9 (1.4-10.7)	0.0078
Arteriosclerosis	2.3 (1.1-4.8)	0.0312	2.4 (1.0-5.9)	0.0613	3.0 (1.3-6.6)	0.0080	3.4 (1.2-9.7)	0.0168
Vascular-tubulointerstitial lesions	1.8 (0.9-3.9)	0.1226	1.6 (0.6-4.1)	0.3250	2.6 (1.1-6.0)	0.0264	2.7 (0.9-7.8)	0.0688
Near normal structure	0.5 (0.2-1.2)	0.1078	0.6 (0.2-1.7)	0.3386	0.3 (0.1-0.8)	0.0176	0.3 (0.1-1.0)	0.0481

* Adjusted by age, sex, hypertension and death due to renal disease; OR: odds ratio. Referential group: TT genotype

3.5.2.3. Correlation of IDE polymorphism with renal vascular changes in the entire study population

In the entire study population, the IDE CC genotype showed a higher percentage of CHD while the CT genotype had higher percentages of CVA, renal vascular tubule-interstitial lesions and a lower percentage of kidneys with near-normal structures than TT genotype carriers. IDE C carriers were more likely to have CHD, death due to cardiovascular disease, CVA and renal vascular lesions than TT genotype carriers (Table 33, additive and predominant model). After adjustment for confounding variables, renal vascular lesions (P=0.0434, OR=2.5, 95%CI=1.0-5.8) and CVA (P=0.0099, OR=4.2, 95%CI=1.4-12.3) were correlated with CT genotype and C carriers (P=0.0132, OR=3.8, 95%CI=1.3-11.1) (Table 34).

Table 33 Clinicopathological characteristics of IDE genotype in the entire study population

	TT	CT	CC
	N=102	N=77	N=22
Age (year)	66.9±15.8	69.7±14.7	70.2±9.1
Female	53.9 (55)	53.2 (41)	40.9 (9)
	N=100	N=77	N=22
Hypertension	26.0 (26)	32.5 (25)	45.5 (10)
	N=102	N=77	N=22
CHD§#	22.5 (23)	32.5 (25)	50.0 (11)*
LVH	17.6 (18)	22.1 (17)	31.8 (7)
CAS	18.6 (19)	29.9 (23)	31.8 (7)
CVA§#	4.9 (5)	18.2 (14)**	13.6 (3)
	N=40	N=30	N=13
Diabetic nephropathy	80.0 (32)	73.3 (22)	92.3 (12)
	N=100	N=77	N=22
Death due to renal disease	3.0 (3)	2.6 (2)	4.5 (1)
Death due to Cardiovascular disease§#	19.0 (19)	28.6 (22)	40.9 (9)
Renal pathology	N=79	N=65	N=13
Glomerular lesion	49.4 (39)	61.5 (40)	46.2 (6)
GGS	41.8 (33)	56.9 (37)	30.8 (4)

NGS	5.1 (4)	6.2 (4)	7.7 (1)
DGS	1.3 (1)	1.5 (1)	0.0 (0)
Glomerular hypertrophy	20.3 (16)	16.9 (11)	30.8 (4)
Vascular lesions #	57.0 (45)	76.9 (50)†	53.8 (7)
Hyaline arteriolosclerosis	25.3 (20)	32.3 (21)	30.8 (4)
Arteriosclerosis	53.2 (42)	72.3 (47)††	38.5 (5)
Tubulointerstitial lesions	43.0 (34)	55.4 (36)	30.8 (4)
Tubular lesion	31.6 (25)	32.3 (21)	30.8 (4)
Interstitial fibrosis	35.4 (28)	46.2 (30)	23.1 (3)
Vascular-tubulointerstitial lesions	65.8 (52)	81.5 (53)‡	53.8 (7)
Near normal structure	31.6 (25)	15.4 (10)‡‡	46.2 (6)
Pancreas lesions	N=102	N=77	N=22
Vascular lesions	42.2 (43)	39.0 (30)	50.0 (11)
Hyaline arteriolosclerosis	33.3 (34)	32.5 (25)	40.9 (9)
Arteriosclerosis	28.4 (29)	28.6 (22)	40.9 (9)
Interstitial lesions	71.6 (73)	64.9 (50)	72.7 (16)
Interstitial fibrosis	35.3 (36)	28.6 (22)	36.4 (8)
Interstitial fat infiltration	63.7 (65)	59.7 (46)	54.5 (12)
Islet amyloidosis	12.7 (13)	7.8 (6)	22.7 (5)
Vascular interstitial lesions	77.5 (79)	74.0 (57)	81.8 (18)
Near normal structure	17.6 (18)	22.1 (17)	9.1 (2)

*P=0.0089, **P=0.0043, †P=0.0119, ††P=0.0186, ‡P=0.0347, ‡‡P=0.0236 vs. TT genotype, compared with pearson's Chi-square test. CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant. §P values for trend<0.0500, linear-by-linear test was used to compare categorical variables for trend. # P<0.0500 in C allele carriers (CC/CT) vs. TT genotype. They were compared by Chi-square test or Fisher's exact test, as appropriate.

Table 34 Associations of IDE genotypes with renal changes in the entire study population

	C carrier				CT genotype				CC genotype			
	Crude		Adjusted		Crude		Adjusted	P	Crude		Adjusted	
	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)		OR (95%CI)	P	OR (95%CI)	P
CHD	2.0 (1.1-3.6)	0.0328	1.8* (0.9-3.8)	0.1003	1.7 (0.8-3.2)	0.1397	1.6* (0.7-3.5)	0.2368	3.4 (1.3-8.9)	0.0114	2.9* (1.0-8.6)	0.0569
Death due to cardiovascular disease	1.9 (1.0-3.7)	0.0470	1.6* (0..8-3.4)	0.2185	1.7 (0.8-3.4)	0.1367	1.5* (0.7-3.3)	0.3424	3.0 (1.1-7.9)	0.0314	2.1* (0.7-6.3)	0.1995
CVA	4.0 (1.4-11.4)	0.0087	3.8* (1.3-11.1)	0.0132	4.3 (1.5-12.6)	0.0074	4.2* (1.4-12.3)	0.0099	3.1 (0.7-13.9)	0.1471	3.1* (0.6-16.0)	0.1738
Renal pathology												
Vascular lesions	2.1 (1.0-4.0)	0.0356	1.8** (0.8-4.0)	0.1389	2.5 (1.2-5.2)	0.0130	2.5** (1.0-5.8)	0.0434	0.9 (0.3-2.9)	0.8337	0.6** (0.2-2.4)	0.4907
Arteriosclerosis	1.8 (0.9-3.4)	0.0856	1.5** (0.7-3.2)	0.2716	2.3 (1.1-4.6)	0.0197	2.2** (1.0-5.0)	0.0614	0.6 (0.2-1.8)	0.3303	0.4** (0.1-1.4)	0.1522
Vascular-tubulointerstitial lesions	1.7 (0.9-3.5)	0.1259	1.4** (0.6-3.3)	0.4697	2.3 (1.1-5.0)	0.0371	2.2** (0.8-5.8)	0.1214	0.6 (0.2-2.0)	0.4072	0.3** (0.1-1.4)	0.1375
Near normal structure	0.6	0.1145	0.7**	0.4394	0.4	0.0262	0.4**	0.0775	1.9	0.3100	3.5**	0.0823

	(0.3-1.2)	(0.3-1.7)	(0.2-0.9)	(0.1-1.1)	(0.6-6.1)	(0.9-14.2)

* Adjusted by age, sex, and hypertension; ** Adjusted by age, sex, hypertension and death due to renal disease; OR: odds ratio. Referential group: TT genotype.

3.5.3. Relationship of IDE genotype and IDE substrate expression in pancreas

In the T2DM group, carriers of TT (N = 6) and CT/CC (N = 6) genotypes matched for age and sex were selected for examination of the protein expression of IDE and its substrates using immunohistochemical staining. In agreement with previous studies [256], extensive IDE expression was found in the pancreas. IDE immunoreactivity was mainly localized in islet endocrine cells and to a lesser extent in exocrine acinar cells. As shown in figure 25, IDE expression in diabetic islets was found to be similar between TT and CT/CC carriers (13.8±7.6 in the TT group vs. 13.6±5.9 in the CT/CC carriers, P=0.9244) (Figure 25, panel A, B and C).

Immunofluorescence microscopy also showed similar immunoreactivity of the IDE substrates (amylin, insulin, and glucagon) in pancreatic islets (Figure 25). Compared with TT genotype patients, C carriers had similar expression of amylin (8.7%±4.3% in TT group vs. 8.0%±4.2% in C carriers, P=0.7247), insulin (23.2%±3.5% in TT group vs. 24.2%±6.2% in C carriers, P=0.7130), glucagon (15.8±4.8 in TT group vs. 17.0±7.0 in C carriers, P=0.7205) and IGF-1 (Figure 26).

3.5.4. Angiotensin-converting enzyme (ACE) expression in different IDE genotypes

ACE protein immunoreactivity was found extensively in diabetic glomeruli (Figure 27, panel A, E and I), tubules (Figure 27, panel B, F and J) and blood vessels (Figure 27, panel C, D, G, H, K and L). There was a tendency for C carriers to show higher ACE expression in renal glomeruli (1.2%±0.3% in C carriers vs. 0.3%±0.2% in TT group, P=0.0744) and blood vessels (1.0%±0.5% in C carriers vs. 0.5%±0.3% in TT group, P=0.1329) than TT genotype carriers, although the P values didn't reach significance. In the renal tubules, ACE was also numberically higher in C carriers (52.1%±21.7%) than TT group (34.6%±19.4%, P=0.2041).

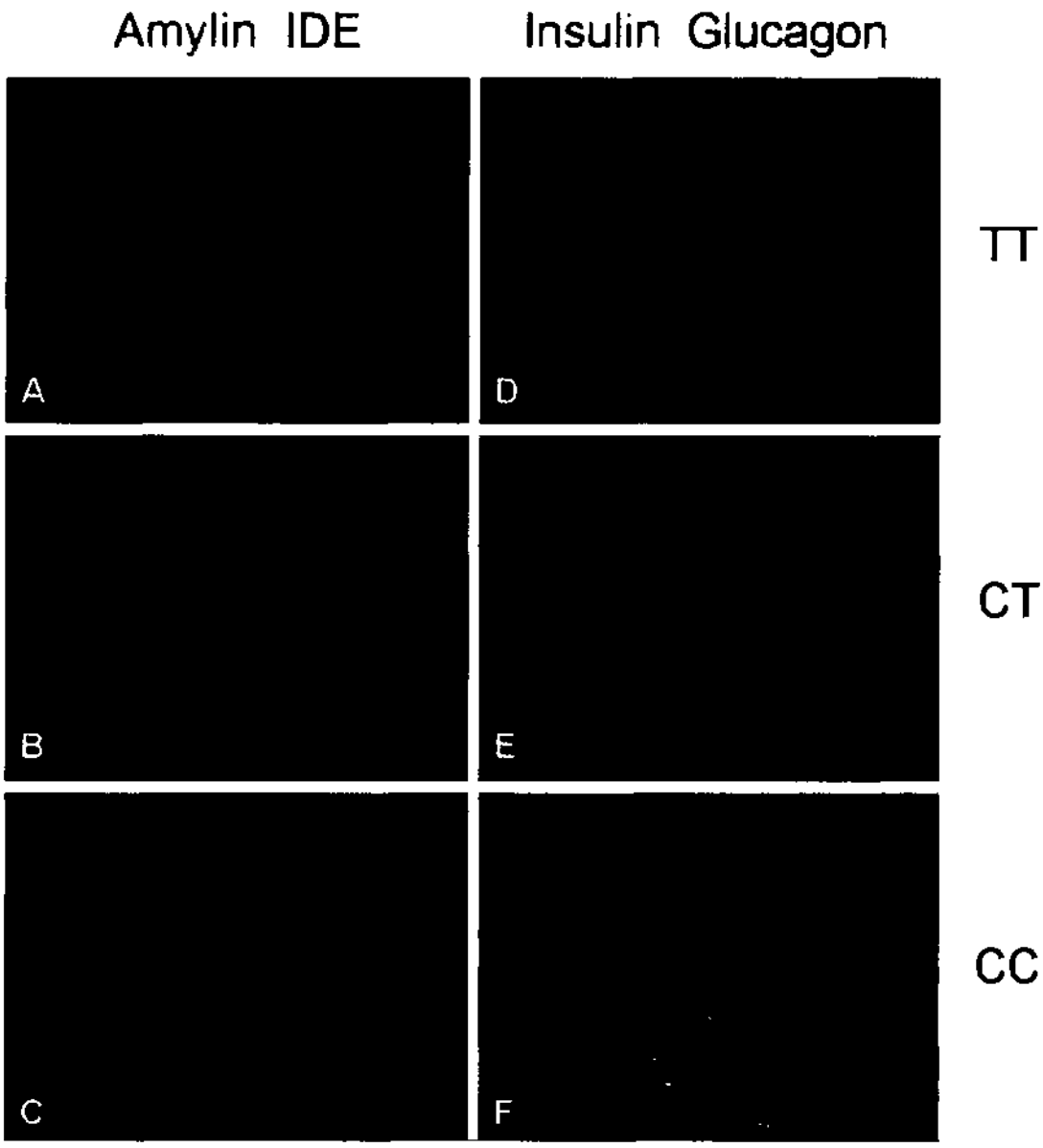


Figure 25 IDE and expression of its substrates in diabetic pancreas in different IDE genotypes.

Diabetic pancrestic sections were stained with rabbit-anti IDE (green), mouse anti-amylin (red), mouse-anti insulin (green) and rabbit-anti glucagon (red) antibodies. No significant differences were found in expression of IDE, amylin, insulin and glucagon proteins in diabetic pancreatic sections among different IDE genotype groups. (Immunofluorescence stain, magnification ×400.)

Figure 26 IDE and IGF-1 proteins expression in diabetic pancreas among different IDE genotypes.

Immunofluorescence staining were performed in diabetic pancreatic sections with rabbit anti-IDE (red) and goat-anti IGF-1 (green) antibodies among TT (panel A), CT (panel B) and CC (panel C) genotype carriers. (Immunofluorescence stain. magnification ×400.)

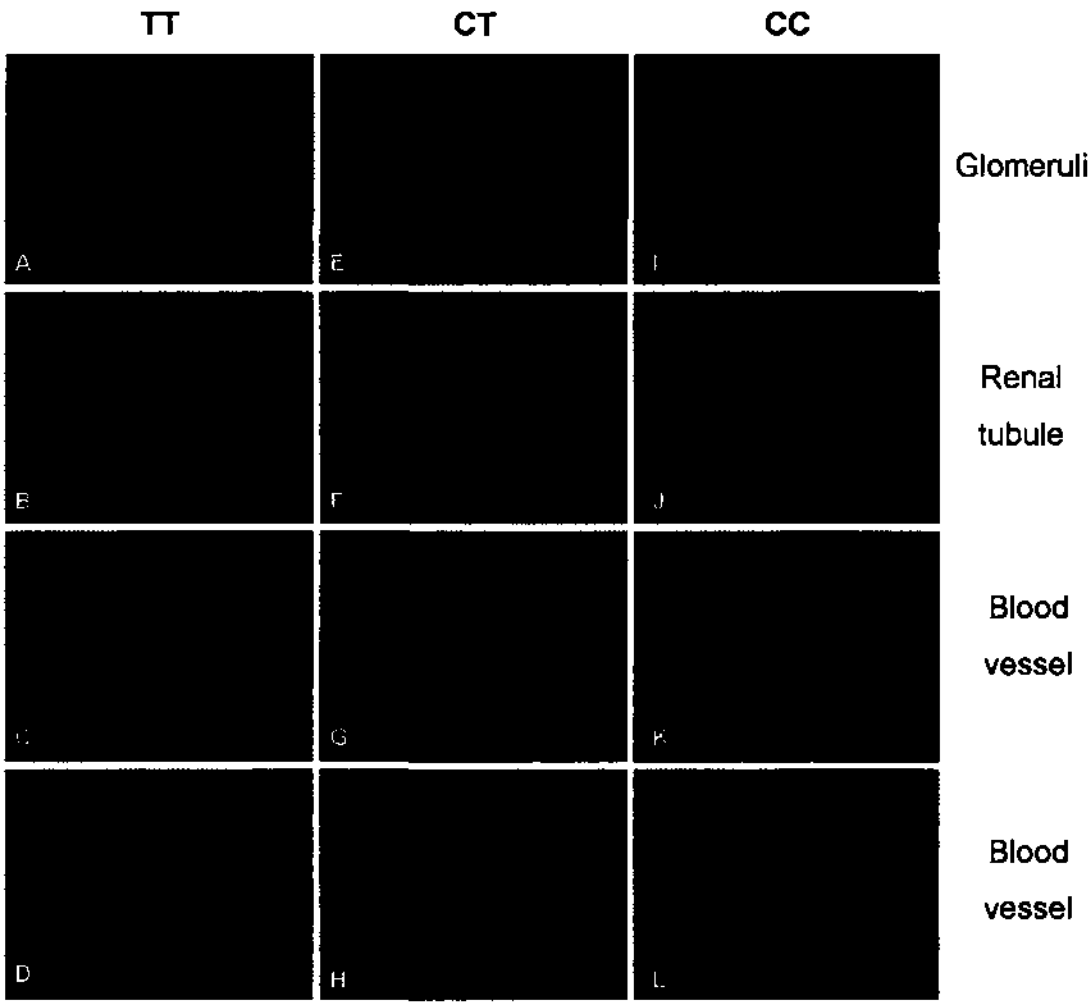


Figure 27 ACE protein expression in diabetic renal tissues among different IDE genotypes.

Kidney tissue sections were stained with a rabbit anti-ACE antibody (green). ACE protein was expressed extensively in renal glomeruli (panels A, E, and I), tubules (panel B, F, and J) and blood vessels (C, D, G, H, K, and L). Panels D, H, and L are the highlighted areas in the panels C, G and K respectively. The expression of ACE was numerically higher in C carriers than TT genotypes in renal glomeruli, tubule and blood vessels. (Immunofluorescence stain. A, B, D-F, H-J and L: magnification ×400, panel C, G and K: magnification ×100.)

3.6. Association with GLUT1 polymorphism

3.6.1. GLUT1 genotype frequency

GLUT1 polymorphisms were successfully genotyped in 161 T2DM and 115 control cases. The genotypes were in Hardy-Weinberg equilibrium in both the T2DM cases (P=0.3629) and control cases (P=0.0572). The distribution of GLUT1 genotypes was 49.1% (79/161) for AA, 39.8% (64/161) for AT and 11.2% (18/161) for TT in T2DM cases which were similar to the control group (Table 35). In these three genotypes, the predominant AA genotype was used as reference group.

Table 35 Distribution of GLUT1 genotypes in type 2 diabetic cases and controls

Genotype	T2DM	Control
Allele	N=322	N=230
A	68.9 (222)	72.2 (166)
T	31.1 (100)	27.8 (64)
Genotype	N=161	N=115
AA	49.1 (79)	55.7 (64)
AT	39.8 (64)	33.0 (38)
TT	11.2 (18)	11.3 (13)

% (number)

3.6.2. Correlation of GLUT 1 polymorphism and renal glomerulopathy

3.6.2.1. Correlation of GLUT1 polymorphism and renal glomerupathy in T2DM

GLUT1 genotype exhibited a dose-dependent association with hypertension: 69.2% (54/79) for AA, 59.4% (38/64) for AT, and 33.3% (6/18) for TT genotypes (P=0.0073). This dose-dependent association was also evident for glomerular hypertrophy: 59.1% (13/22) for AA, 40.0% (10/40) for AT and 0.0% (0/5) for TT genotypes (P=0.0190). The linear trend test showed significant P values. Compared with the referent AA genotype, the GLUT1 TT genotype was less likely to develop hypertension (*P*=0.0046) and renal glomerular hypertrophy (*P*=0.0407) (Table 36, recessive model). Logistic regression analysis showed reduced odds ratios of hypertension in T allele carriers (OR=0.5, 95%CI=0.3-1.0, *P*=0.0444) and TT genotype carriers (OR=0.2, 95%CI=0.1-0.7, *P*=0.0069) after adjustment for age and sex (Table 37).

Table 36 Clinicopathological characteristics of GLUT1 rs710218 genotype in T2DM

	AA	AT	TT
	N=79	N=64	N=18
Age (year)	70.8±10.7	71.1±11.2	72.3±11.5
Female	50.6 (40)	56.3 (36)	66.7 (12)
	N=18	N=16	N=6
BMI (kg/m^2)	22.8±3.6	22.0±2.6	23.2±5.4
	N=62	N=53	N=10
Fasting plasma glucose (mmol/L)	11.9±6.5	11.7±4.5	8.3±4.0
	N=64	N=53	N=10
Systolic blood pressure (mmHg)	150.0±41.0	150.3±39.2	127.8±29.3
Diastolic blood pressure (mmHg)	80.5±21.6	81.6±23.4	68.2±11.7
	N=59	N=48	N=9
Plasma creatinine (umol/L)	149.0 (105.0-243.0)	148.5 (90.3-224.5)	153.0 (106.0-234.5)
Plasma urea (mmol/L)	10.9 (7.2-18.7)	12.5 (7.0-15.8)	10.3 (6.9-22.4)
GFR ($ml/min/1.73m^2$)	31.2 (16.7-52.0)	35.0 (17.3-57.5)	25.9 (19.3-47.0)
	N=78	N=64	N=18
Hypertension§	69.2 (54)	59.4 (38)	33.3 (6)*
	N=79	N=64	N=18
CHD	65.8 (52)	71.9 (46)	61.1 (11)
LVH	62.0 (49)	67.2 (43)	55.6 (10)
CAS	49.4 (39)	56.3 (36)	55.6 (10)
CVA§	29.1 (23)	23.4 (15)	0.0 (0) **
	N=75	N=63	N=18
Diabetic nephropathy	85.3 (64)	76.2 (48)	72.2 (13)
	N=78	N=64	N=18
Death due to renal disease	9.0 (7)	3.1 (2)	0.0 (0)
Death due to Cardiovascular disease	52.6 (41)	43.8 (28)	44.4 (8)

Renal pathology	N=22	N=10	N=5
Glomerular lesion	86.4 (19)	80.0 (8)	80.0 (4)
GGS	63.6 (14)	80.0 (8)	80.0 (4)
NGS	18.2 (4)	40.0 (4)	0.0 (0)
DGS	4.5 (1)	20.0 (2)	0.0 (0)
Glomerular hypertrophy§	59.1 (13)	40.0 (4)	0.0 (0)†
Vascular lesions	100.0 (22)	90.0 (9)	100.0 (5)
Hyaline arteriolosclerosis	81.8 (18)	80.0 (8)	100.0 (5)
Arteriosclerosis	86.4 (19)	70.0 (7)	60.0 (3)
Tubulointerstitial lesions	50.0 (11)	60.0 (6)	80.0 (4)
Tubular lesion	27.3 (6)	50.0 (5)	60.0 (3)
Interstitial fibrosis	45.5 (10)	50.0 (5)	60.0 (3)
Vascular-tubulointerstitial lesions	100.0 (22)	100.0 (10)	100.0 (5)
Near normal structure	0.0 (0)	0.0 (0)	0.0 (0)
Pancreas lesions	N=37	N=23	N=9
Vascular lesions	81.1 (30)	87.0 (20)	77.8 (7)
Hyaline arteriolosclerosis	70.3 (26)	87.0 (20)	77.8 (7)
Arteriosclerosis	64.9 (24)	56.5 (13)	33.3 (3)
Interstitial lesions	89.2 (33)	100.0 (23)	88.9 (8)
Interstitial fibrosis	62.2 (23)	65.2 (15)	33.3 (3)
Interstitial fat infiltration	81.1 (30)	95.7 (22)	77.8 (7)
Islet amyloidosis	37.8 (14)	30.4 (7)	11.1 (1)
Vascular interstitial lesions	97.3 (36)	100.0 (23)	100.0 (9)
Near normal structure	2.7 (1)	0.0 (0)	0.0 (0)

*P=0.0046 versus AA genotype, compared with pearson's Chi-square test. **P=0.0055 and †P=0.0407 versus AA genotype, compared with Fisher's exact test. CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA:

cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as mean±SD, medians (interquartile range) or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Medians among three groups were compared using Kruskal-Wallis H test. Medians between two groups were compared using Mann–Whitney U test, a two-tailed *P*-value <0.025 was considered significant. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant. §P values for trend<0.0500, linear-by-linear test was used to compare categorical variables for trend.

Table 37 Associations of GLUT1 polymorphisms with hypertension

	Crude OR (95%CI)	P	Adjusted* OR (95%CI)	P
AA genotype	1	1	1	1
AT genotype	0.6 (0.3-1.3)	0.2223	0.6 (0.3-1.3)	0.1824
TT genotype	0.2 (0.1-0.7)	0.0069	0.2 (0.1-0.6)	0.0050
T carrier	0.5 (0.3-1.0)	0.0444	0.5 (0.3-0.9)	0.0329

* Adjusted by age, sex; OR: odds Ratio.

3.6.2.2. Correlation of GLUT1 polymorphism with clinicopathological characteristics in control cases

In the nondiabetic control cases, GLUT1 genotypes and polymorphisms were not associated with any clinicopathological features (Table 38, recessive model).

Table 38 Clinicopathological characteristics of GLUT1 rs710218 genotype in the control cases

	AA	AT	TT
	N=64	N=38	N=13
Age (year)	70.1±16.0	65.3±16.3	69.8±19.7
Female	53.1 (34)	44.7 (17)	46.2 (6)
Hypertension	14.1 (9)	15.8 (6)	15.4 (2)
CHD	15.6 (10)	2.6 (1)	7.7 (1)
LVH	4.7 (3)	5.3 (2)	0.0 (0)
CAS	15.6 (10)	2.6 (1)	7.7 (1)
Death due to renal disease	1.6 (1)	0.0 (0)	0.0 (0)
Death due to Cardiovascular disease	18.8 (12)	7.9 (3)	7.7 (1)
CVA	12.5 (8)	10.5 (4)	23.1 (3)
Renal pathology	N=64	N=38	N=13
Glomerular lesion	50.0 (32)	57.9 (22)	61.5 (8)
GGS	46.9 (30)	52.6 (20)	46.2 (6)
DGS	0 (0)	0 (0)	0 (0)
Glomerular hypertrophy	12.5 (8)	13.2 (5)	30.8 (4)
Vascular lesions	62.5 (40)	52.6 (20)	61.5 (8)
Hyaline arteriolosclerosis	12.5 (8)	7.9 (3)	15.4 (2)
Arteriosclerosis	60.9 (39)	52.6 (20)	61.5 (8)
Tubulointerstitial lesions	46.9 (30)	47.4 (18)	53.8 (7)
Tubular lesion	23.4 (15)	28.9 (11)	38.5 (5)
Interstitial fibrosis	42.2 (27)	42.1 (16)	46.2 (6)
Vascular-tubulointerstitial lesions	70.3 (45)	65.8 (25)	61.5 (8)
Near normal structure	25.0 (16)	26.3 (10)	30.8 (4)
Pancreas lesions	N=64	N=38	N=13
Vascular lesions	23.4 (15)	15.8 (6)	15.4 (2)

Hyaline arteriolosclerosis	7.8 (5)	5.3 (2)	0.0 (0)
Arteriosclerosis	20.3 13()	13.2 (5)	15.4 (2)
Interstitial lesions	59.4 (38)	65.8 (25)	61.5 (8)
Interstitial fibrosis	23.4 (15)	10.5 (4)	30.8 (4)
Interstitial fat infiltration	51.6 (33)	60.5 (23)	53.8 (7)
Islet amyloidosis	1.6 (1)	5.3 (2)	0.0 (0)
Vascular interstitial lesions	67.2 (43)	73.7 (28)	61.5 (8)
Near normal structure	28.1 (18)	15.8 (6)	30.8 (4)

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant. All P values for trend>0.0500, linear-by-linear test was used to compare categorical variables for trend.

3.6.2.3. Correlation of GLUT1 polymorphism with clinicopathological characteristics in the entire study population

There was no significant association of GLUT1 polymorphisms with clinicopathological characteristics in the entire study population (Table 39, recessive model).

Table 39 Clinicopathological characteristics of GLUT1 rs710218 genotype in the entire group

	AA	AT	TT
	N=143	N=102	N=31
Age (year)	70.5±13.3	69.0±13.6	71.2±15.2
Female	51.7 (74)	52.0 (53)	58.1 (18)
Hypertension	44.4 (63)	43.1 (44)	25.8 (8)
CHD	43.4 (62)	46.1 (47)	38.7 (12)
LVH	36.4 (52)	44.1 (45)	32.3 (10)
CAS	34.3 (49)	36.3 (37)	35.5 (11)
Diabetic nephropathy	85.3 (64)	76.2 (48)	72.2 (13)
Death due to renal disease	5.6 (8)	2.0 (2)	0.0 (0)
Death due to Cardiovascular disease	37.3 (53)	30.4 (31)	29.0 (9)
CVA	21.7 (31)	18.6 (19)	9.7 (3)
Renal pathology	N=86	N=48	N=18
Glomerular lesion	59.3 (51)	62.5 (30)	66.7 (12)
GGS	51.2 (44)	58.3 (28)	55.6 (10)
NGS	4.7 (4)	8.3 (4)	0.0 (0)
DGS	1.2 (1)	4.2 (2)	0.0 (0)
Glomerular hypertrophy	24.4 (21)	18.8 (9)	22.2 (4)
Vascular lesions	72.1 (62)	60.4 (29)	72.2 (13)
Hyaline arteriolosclerosis	30.2 (26)	22.9 (11)	38.9 (7)
Arteriosclerosis	67.4 (58)	56.3 (27)	61.1 (11)
Tubulointerstitial lesions	47.7 (41)	50.0 (24)	61.1 (11)
Tubular lesion	24.4 (21)	33.3 (16)	44.4 (8)
Interstitial fibrosis	43.0 (37)	43.8 (21)	50.0 (9)
Vascular-tubulointerstitial lesions	77.9 (67)	72.9 (35)	72.2 (13)

Near normal structure	18.6 (16)	20.8 (10)	22.2 (4)
Pancreas lesions	N=101	N=61	N=22
Vascular lesions	44.6 (45)	42.6 (26)	40.9 (9)
Hyaline arteriolosclerosis	30.7 (31)	36.1 (22)	31.8 (7)
Arteriosclerosis	36.6 (37)	29.5 (18)	22.7 (5)
Interstitial lesions	70.3 (71)	78.7 (48)	72.7 (16)
Interstitial fibrosis	37.6 (38)	31.1 (19)	31.8 (7)
Interstitial fat infiltration	62.4 (63)	73.8 (45)	63.6 (14)
Islet amyloidosis	14.9 (15)	14.8 (9)	4.5 (1)
Vascular interstitial lesions	78.2 (79)	83.6 (51)	77.3 (17)
Near normal structure	18.8 (19)	9.8 (6)	18.2 (4)

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant. All P values for trend>0.0500, linear-by-linear test was used to compare categorical variables for trend.

3.7. Gene interaction of ApoE and GLUT1 with renal glomerular hypertrophy

3.7.1. Gene interaction of ApoE and GLUT1 with renal glomerular hypertrophy in T2DM

Based on our study in GLUT1 and ApoE, ApoE ε2/ApoE ε4 genotypes and GLUT1 AA/AT genotypes were the risk-conferring genotypes for renal glomerulopathy. Hence, I scored 1 for each risk genotype and scored 0 for non-risk genotype carriers to study the additive effects of ApoE and GLUT1 risk variants. The cases that had ApoE and GLUT1 data were stratified into three groups based on their score from 0 to 2 (Table 40).

Table 40 showed a significantly increased and dose-dependent risk association with renal glomerular hypertrophy across these risk groups. Other clinicopathological characteristics were not significantly different among risk groups (Table 40).

Table 40 Additive effects of ApoE and GLUT1 risk conferring genotypes on renal and pancreatic pathological changes in T2DM

	Score=0	Score=1	Score=2
	N=11	N=97	N=40
Age (year)	71.4±12.1	71.9±10.6	70.5±9.8
Female	63.6 (7)	53.6 (52)	52.5 (21)
	N=4	N=24	N=11
BMI (kg/m^2)	24.6±5.7	22.0±2.9	23.6±3.2
	N=7	N=83	N=32
Fasting plasma glucose (mmol/L)	8.7±3.8	11.4±5.3	12.5±6.6
	N=7	N=84	N=33
Systolic blood pressure (mmHg)	126.9±29.7	151.1±38.5	148.2±42.6
Diastolic blood pressure (mmHg)	66.6±10.5	81.4±21.3	80.8±22.3
	N=6	N=78	N=30
Plasma creatinine (μmol/L)	186.0 (86.5-270.8)	156.5 (114.5-248.3)	112.0 (78.0-155.3)
Plasma urea (mmol/L)	13.7 (5.4-25.6)	12.1 (7.4-18.9)	9.2 (5.8-14.8)
GFR ($ml/min/1.73m^2$)	24.4 (15.4-59.1)	31.1 (15.8-46.5)	41.3 (28.1-67.9)
	N=11	N=96	N=40
Hypertension	36.4 (4)	66.7 (64)	62.5 (25)
	N=11	N=97	N=40
CHD	72.7 (8)	73.2 (71)	67.5 (27)
LVH	72.7 (8)	64.9 (63)	67.5 (27)
CAS	63.6 (7)	54.6 (53)	55.0 (22)
CVA	0.0 (0)	27.8 (27)	22.5 (9)
	N=11	N=94	N=38
Diabetic nephropathy	63.6 (7)	79.8 (75)	78.9 (30)
	N=11	N=96	N=40
Death due to renal disease	0.0 (0)	5.2 (5)	5.0 (2)
Death due to Cardiovascular disease	54.5 (6)	55.2 (53)	40.0 (16)
Renal pathology	N=5	N=18	N=14

Glomerular lesion	80.0 (4)	83.3 (15)	85.7 (12)
GGS	80.0 (4)	66.7 (12)	71.4 (10)
NGS	0.0 (0)	27.8 (5)	21.4 (3)
DGS	0.0 (0)	5.6 (1)	14.3 (2)
Glomerular hypertrophy§	0.0 (0)	38.9 (7)	71.4 (10)*
Vascular lesions	100.0 (5)	100.0 (18)	92.9 (13)
Hyaline arteriolosclerosis	100.0 (5)	77.8 (14)	85.7 (12)
Arteriosclerosis	60.0 (3)	83.3 (15)	78.6 (11)
Tubulointerstitial lesions	80.0 (4)	50.0 (9)	57.1 (8)
Tubular lesion	60.0 (3)	27.8 (5)	42.9 (6)
Interstitial fibrosis	60.0 (3)	38.9 (7)	57.1 (8)
Vascular-tubulointerstitial lesions	100.0 (5)	100.0 (18)	100.0 (14)
Near normal structure	0.0 (0)	0.0 (0)	0.0 (0)
Pancreas lesions	N=5	N=34	N=19
Vascular lesions	80.0 (4)	85.3 (29)	78.9 (15)
Hyaline arteriolosclerosis	80.0 (4)	76.5 (26)	73.7 (14)
Arteriosclerosis	20.0 (1)	55.9 (19)	68.4 (13)
Interstitial lesions	80.0 (4)	97.1 (33)	84.2 (16)
Interstitial fibrosis	20.0 (1)	70.6 (24)	68.4 (13)
Interstitial fat infiltration	80.0 (4)	91.2 (31)	78.9 (15)
Islet amyloidosis	0.0 (0)	32.4 (11)	36.8 (7)
Vascular interstitial lesions	100.0 (5)	97.1 (33)	100.0 (19)
Near normal structure	0.0 (0)	2.9 (1)	0.0 (0)

*P=0.0108 vs. score=0 group, calculated by Fisher's exact test. CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD, medians (interquartile range) or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Medians among three groups were compared using Kruskal-Wallis H test. Medians between two groups were compared using Mann–Whitney U test, a two-tailed *P*-value <0.025 was considered significant. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were

compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant. §P values for trend<0.0500, linear-by-linear test was used to compare categorical variables for trend.

3.7.2. Gene interaction of ApoE and GLUT1 with renal glomerular hypertrophy in control cases

In the control group, ApoE and GLUT1 risk-conferring genotypes had additive effects on pancreatic vascular interstitial changes. Other clinicopathological parameters were not significantly different among the risk groups (Table 41).

Table 41 Additive effects of ApoE and GLUT1 risk-conferring genotypes on renal and pancreatic pathological changes in control cases

	Score=0	Score=1	Score=2
	N=9	N=63	N=9
Age (year)	61.3±17.8	68.7±16.7	66.2±19.7
Female	33.3 (3)	49.2 (31)	55.6 (5)
Hypertension	22.2 (2)	15.9 (10)	11.1 (1)
CHD	0.0 (0)	7.9 (5)	0.0 (0)
LVH	0.0 (0)	6.3 (4)	0.0 (0)
CAS	0.0 (0)	7.9 (5)	11.1 (1)
Death due to renal disease	0.0 (0)	1.6 (1)	0.0 (0)
Death due to Cardiovascular disease	0.0 (0)	14.3 (9)	11.1 (1)
CVA	11.1 (1)	12.7 (8)	11.1 (1)
Renal pathology			
Glomerular lesion	55.6 (5)	55.6 (35)	33.3 (3)
GGS	33.3 (3)	50.8 (32)	22.2 (2)
DGS	0 (0)	0 (0)	0 (0)
Glomerular hypertrophy	33.3 (3)	12.7 (8)	22.2 (2)
Vascular lesions	44.4 (4)	61.9 (39)	55.6 (5)
Hyaline arteriolosclerosis	11.1 (1)	11.1 (7)	11.1 (1)
Arteriosclerosis	44.4 (4)	61.9 (39)	55.6 (5)
Tubulointerstitial lesions	33.3 (3)	47.6 (30)	44.4 (4)
Tubular lesion	22.2 (2)	28.6 (18)	44.4 (4)
Interstitial fibrosis	22.2 (2)	41.3 (26)	33.3 (3)
Vascular-tubulointerstitial lesions	44.4 (4)	68.3 (43)	66.7 (6)
Near normal structure	44.4 (4)	27.0 (17)	33.3 (3)
Pancreas lesions			

Vascular lesions	11.1 (1)	22.2 (14)	22.2 (2)
Hyaline arteriolosclerosis	0.0 (0)	7.9 (5)	11.1 (1)
Arteriosclerosis	11.1 (1)	20.6 (13)	11.1 (1)
Interstitial lesions	44.4 (4)	60.3 (38)	77.8 (7)
Interstitial fibrosis	22.2 (2)	17.5 (11)	11.1 (1)
Interstitial fat infiltration	44.4 (4)	52.4 (33)	66.7 (6)
Islet amyloidosis	0.0 (0)	3.2 (2)	0.0 (0)
Vascular interstitial lesions§	44.4 (4)	69.8 (44)	88.9 (8)
Near normal structure	44.4 (4)	23.8 (15)	11.1 (1)

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis.

Data are presented as or mean±SD or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant. §P values for trend<0.0500, linear-by-linear test was used to compare categorical variables for trend.

3.7.3. Gene interaction of ApoE and GLUT1 with renal glomerular hypertrophy in the entire study population

In the entire study population, the frequency of glomerular hypertrophy and hyaline arteriolosclerosis was higher in the group with score 2 than the group with score 0 with significant trend across the risk groups. The dose effects across risk groups were also found for pancreatic arteriosclerosis, islet amyloidosis and vacular interstitial changes (Table 42). After adjusting for confounding factors, pancreatic vascular interstitial changes were significantly higher in the score 2 group (P=0.0259, OR=14.8, 95%CI=1.4-158.7) than score 0 group (table 43).

Table 42 Additive effects of ApoE and GLUT1 risk-conferring genotypes on renal and pancreatic pathological changes in the entire study population

	Score=0	Score=1	Score=2
	N=20	N=160	N=49
Age (year)	66.9±15.4	70.7±13.4	69.7±12.0
Female	50.5 (10)	51.9 (83)	53.1 (26)
	N=20	N=159	N=49
Hypertension	30.0 (6)	46.5 (74)	53.1 (26)
	N=20	N=160	N=49
CHD	40.0 (8)	47.5 (76)	55.1 (27)
LVH	40.0 (8)	41.9 (67)	55.1 (27)
CAS	35.0 (7)	36.3 (58)	46.9 (23)
CVA	5.0 (1)	21.9 (35)	20.4 (10)
	N=11	N=94	N=38
Diabetic nephropathy	63.6 (7)	79.8 (75)	78.9 (30)
	N=20	N=159	N=49
Death due to renal disease	0.0 (0)	3.8 (6)	4.1 (2)
Death due to Cardiovascular disease	30.0 (6)	39.0 (62)	34.7 (17)
Renal pathology	N=14	N=81	N=23
Glomerular lesion	64.3 (9)	61.7 (50)	65.2 (15)
GGS	50.0 (7)	54.3 (44)	52.2 (12)
NGS	0.0 (0)	6.2 (5)	13.0 (3)
DGS	0.0 (0)	1.2 (1)	8.7 (2)
Glomerular hypertrophy§	21.4 (3)	18.5 (15)	52.2 (12)*
Vascular lesions	64.3 (9)	70.4 (57)	78.3 (18)
Hyaline arteriolosclerosis	42.9 (6)	25.9 (21)	56.5 (13)**
Arteriosclerosis	50.0 (7)	66.7 (54)	69.6 (16)
Tubulointerstitial lesions	50.0 (7)	48.1 (39)	52.2 (12)
Tubular lesion	35.7 (5)	28.4 (23)	43.5 (10)
Interstitial fibrosis	35.7 (5)	40.7 (33)	47.8 (11)
Vascular-tubulointerstitial lesions	64.3 (9)	75.3 (61)	87.0 (20)
Near normal structure	28.6 (4)	21.0 (17)	13.0 (3)
Pancreas lesions	N=14	N=97	N=28

Vascular lesions	35.7 (5)	44.3 (43)	60.7 (17)
Hyaline arteriolosclerosis	28.6 (4)	32.0 (31)	53.6 (15)
Arteriosclerosis§	14.3 (2)	33.0 (32)	50.0 (14)
Interstitial lesions	57.1 (8)	73.2 (71)	82.1 (23)
Interstitial fibrosis	21.4 (3)	36.1 (35)	50.0 (14)
Interstitial fat infiltration	57.1 (8)	66.0 (64)	75.0 (21)
Islet amyloidosis§	0.0 (0)	13.4 (13)	25.0 (7)
Vascular interstitial lesions§	64.3 (9)	79.4 (77)	96.4 (27)†
Near normal structure§	28.6 (4)	16.5 (16)	3.6 (1)

CHD: coronary heart disease; LVH: left ventricular hypertrophy; CAS: coronary arteriosclerosis; CVA: cerebrovascular accident; GGS: global glomerulosclerosis; NGS: nodular glomerulosclerosis; DGS: diffuse glomerulosclerosis. *P=0.0012, **P=0.0058 vs score=1 group, †P=0.0113 vs. score=0 group, compared using Fisher's exact test..

Data are presented as or mean±SD or % (n). Means were compared using the one-way ANOVA. Dunnett t adjustment was used in post-hoc test for multiple comparisons. Categorical variables among 3 groups were compared using the Fisher's exact test. Categorical variables between 2 groups were compared using the Chi-square test or Fisher's exact test, as appropriate, a two-tailed P-value <0.025 was considered significant. §P values for trend<0.0500, linear-by-linear test was used to compare categorical variables for trend.

Table 43 Associations of risk scores of ApoE and GLUT1 genotypes with renal and pancreatic changes in the entire study population

	Score=1				Score=2			
	Crude OR (95%CI)	P	Adjusted OR (95%CI)	P	Crude OR (95%CI)	P	Adjusted OR (95%CI)	P
Renal pathology								
Glomerulaar hypertrophy	0.8 (0.2-3.4)	0.7977	0.8 (0.2-3.7)*	0.8255	4.0 (0.9-18.2)	0.0731	3.9 (0.8-19.1)*	0.0928
Hyaline arteriolosclerosis	0.5 (0.1-1.5)	0.2014	0.3 (0.1-1.3)*	0.1121	1.7 (0.5-6.6)	0.4217	1.5 (0.4-6.3)*	0.5434
Pancreas								
Vascular interstitial lesions	2.1 (0.6-7.1)	0.2139	1.9 (0.5-7.4)**	0.3719	15.0 (1.5-146.0)	0.0197	14.8 (1.4-158.7)**	0.0259

* Adjusted by age, sex, hypertension and death due to renal disease; * *Adjusted by age, sex, and hypertension; OR: odds ratio. Referential group: score=0

CHAPTER 4 DISCUSSION

4. Discussion

4.1. Association of ApoE polymorphism with diabetic glomerulopathy

The kidney is an integrated organ comprising glomeruli, tubules, arteries and blood vessels. Based on pathological changes, Gambara et al suggested three distinct patterns of renal damages in T2DM. Class 1 is characterized by predominant glomerulopathy including glomerulosclerosis, glomerular hypertrophy, and arteriolar hyalinosis. The glomerulosclerosis in class 1 is more likely to be accompanied by arteriolar hyalinosis. Class 2 has mild glomerulosclerosis and arteriolar hyalinosis with ischemic glomerular lesions and arteriosclerosis as predominant feathers. Class 3 refers to glomerular disease superimposed on diabetic glomerulosclerosis. From a clinical perspective, there might not be marked differences in age, disease duration, renal function, urinary protein excretion and mean arterial pressure amongst these three forms of renal disease by pathological classification [38]. Indeed, other studies have shown that T2DM patients with microalbuminuria exhihited marked heterogeneity in terms of glomerular, vascular and tubulointerstitial damage [257].

In this autopsy study, using histopathological diagnostic criteria, we observed that over 80% of T2DM cases had glomerular changes compared to 40% in the control cases. Vascular changes were also higher in T2DM than control cases. Glomerulopathy such as nodular glomerulosclerosis and arteriolar hyalinosis mainly occurred in T2DM but not in control cases. ApoE ε2 carries were more likely to have glomerulopathy in T2DM whereas ApoE ε4 carriers had renal tubular changes in control cases.

4.1.1. Effect of ApoE isoforms on lipid metabolism

As a lipoprotein, Apo E is a lipid transporter and plays a key role in the synthesis, circulation and clearance of various lipid particles through binding to specific receptors [258]. The presence of the ApoE ε2 allele results in a change from Arg to Cys in residue 158 to form Apo E ε2 isoform. The binding affinity of ApoE ε2 to the LDL receptor is 50-100 times weaker than that of ε3 or ε4 [259]. It is also less competent than ε3 and ε4 in binding heparin which promotes remnant lipoprotein metabolism in the liver. Compared to ApoE ε4, ApoE ε3 and ε2 isoforms show greater affinity for binding to the smaller, more phospholipid-enriched HDL. Alteration in residue 112 of the ApoE ε4 isoform enhances its binding affinitiy to the larger, TG-enriched VLDL [174]. Due to these different binding affinities, Apo ε2 carriers

tend to have increased TG and remnant-like lipoprotein particles. In contrast, ApoE ε4 carriers have rapid clearance of TG and remnant particles with increased formation of LDL-C resulting in low TG but high LDL-C [232].

4.1.2. Effects of ApoE isoforms beyond lipid metabolism

These conformational changes due to alteration of amino acid also influence protein-protein interactions of these lipoproteins [260]. ApoE ε2 isoform has reduced ability to bind to heparin and is less capable than ε3 isoform in its ability to induce matrix heparin sulfate proteoglycan (HSPG), which can inhibit glomerular mesangial cell proliferation [261]. On the other hand, ApoE ε4 isoform can cause RAAS activation [187], endothelial dysfunction, macrophage infiltration [262], oxidative stress [263], and compromise nerve repair after damage [191]. ApoE also regulates immunological reaction [264] and promotes cell regeneration after tissue injuries [191]. In keeping with these pluripotent effects, ApoE ε4 is a known risk allele for atherosclerosis [265], cerebrovascular disease [266], and AD [267].

In the kidney, increased TG-rich lipoproteins levels in association with ApoE ε2 may enhance glycosylated or oxidized LDL accumulation in mesangial cells [232] and macrophages [235]. The latter can stimulate secretion of cytokines such as interleukin-6, platelet-derived growth factor (PDGF), intercellular adhesion molecule-1 (ICAM-1), transforming growth factor-β (TGF-β), monocyte colony-stimulating factor and matrix metalloproteinase-3 [120, 268-270]. These cytokines can lead to increased production of extracellular matrix proteins with mesangial expansion. Increased recruitment of macrophages may cause further oxidization of LDL particles to set up a vicious circle culminating in renal damage [120].

4.1.3. Clinical versus histopathological studies

Several studies have reported the predictive role of genetic variations of ApoE on progression of chronic kidney disease independent of diabetes, race, lipid and non-lipid risk factors [231, 232]. The ApoE ε2 genotype has been associated with early onset and progression of renal disease in T2DM [232, 271]. Other workers have reported higher frequency of ApoE ε2 allele in patients with diabetic nephropathy compared to those without nephropathy and in normal subjects [272]. ApoE ε2

carriers were also more likely to have macroalbuminuria [233], overt nephropathy [232], and renal failure [234]. In contrast, the frequency of ApoE ε4 allele is lower in the nephropathy group than control [273]. Other researchers have reported association of ApoE ε4 carrier status with normoalbuminuria [232], attenuated rate of progression of chronic kidney disease [237], and low morbidity of glomerulonephritis [274]. Although most studies reported increased risk of renal disease with ApoE ε2 variant and reduced risk with ε4 genotype [231, 237], accelerated rate of decline in renal disease has also been reported in ApoE ε4 carriers [229, 230].

The discrepancies in genetic association studies are often due to differences in patient selection, definitions and methodologies. In positive association study of ApoE ε2 with diabetic nephropathy, most of the cases may have predominant pathological changes of diabetic glomerulopathy (class 1). In contrast, studies showing positive association of ApoE ε4 and diabetic nephropathy may have recruited subjects with predominant vascular changes (class 2). In this autopsy study, we found increased severity of glomerulopathy in Apo E ε2 allele carriers. This association was accompanied by increased ApoE protein expression in the mesangial areas and nodular lesions. In addition, we showed association of ApoE ε4 allele with glomerular hypertrophy accompanied by ApoE expression predominantly in the vascular structure.

These genetic-pathological correlations confirmed the heterogeneity of renal changes in T2DM with vascular, glomerular, tubular and interstitial components. We further confirmed the association of ApoE ε2 with glomerulopathy. Glomerular hypertrophy was accompanied by ApoE protein expression in the glomerular compartment. Taken together, these findings strongly suggest that Apo E is an important target for risk prediction and possibly intervention in diabetic nephropathy.

4.2. Association of ApoE polymorphism with islet amyloidosis

There are close associations between T2DM, pancreatic amyloidosis and AD, the latter characterised by formation of Aβ fibrils [275]. Amylin and Aβ monomers share similar features and can form oligomers which aggregate to form insoluble fibrils in AD and in T2DM respectively [276, 277]. During the process of amyloidogenesis, non-fibril chaperone components such as Apo E and amyloid P proteins are involved in fibril transport, aggregation and clearance [278-280]. In this study, we postulated

that genetic variants of ApoE might be associated with pancreatic amyloidosis. We found association of ApoE ε4 genotypes with pancreatic amyloidosis accompanied by arteriosclerosis, arteriolar hyalinosis and fibrosis.

Compared to the major form of ApoE coded by ε3 allele, the change from Cys to Arg in the 112th residue of the ε4 isoform can lead to increased exposure of arginine at position 61 which interacts with glutamic acid at position 255 in its carboxyl-terminal [174]. These conformational changes enhance formation and reduce clearance of amyloid fibril [174]. In *in vitro* study, ApoE ε4 isoforms bind strongly to amylin peptide and promote aggregation in high concentration [281]. In AD, ApoE ε4 genotypes were associated with increased Aβ amyloid load, early onset and increased severity of disease [282]. In support of this notion, we found increased islet amyloidosis in T2DM cases with ApoE ε4 allele.

In AD, amyloid P initiates the aggregation and stabilization of Aβ fibrils and prevents them from proteolysis in brain [280]. In our autopsy cases, we found co-existing immunoreactivity of amyloid P and ApoE in both arteriosclerosis and islet amyloidosis [283]. Since amyloid P and ApoE can bind to all types of amyloid fibrils and extracellular matrix molecules [278-280], our findings suggest that these proteins may be common constituents of arteriosclerotic lesions and amyloid deposits in T2DM and AD with shared pathogenetic mechanism.

Besides, we observed structural damages of diabetic islets affected by amyloid deposits (Figures 9-11). In agreement with previous reports [27, 165], there were pronounced interstitial fibrosis and extensive arteriosclerotic changes of muscular arteries and small arterioles coexisting with islet amyloidosis. In this regard, ApoE ε4 is a known risk factor for arteriosclerosis [284], cerebrovascular disease and AD [186, 282]. In experimental studies, ApoE ε4 isoform can activate the RAAS and inhibit smooth muscle cell proliferation through its binding with heparan sulphate proteoglycans [187, 188]. In the nervous system, ApoE plays an important role in neuronal repair. While ApoE ε3 stimulates polymerization of β-tubulin and stabilizes the formation of microtubules *in vivo*, ApoE ε4 isoform destabilizes microtubules assembly which may enhance hyperphosphorylation of tau protein and impaired neurite outgrowth [174]. Against this background, isolated normal human islets with disrupted blood supply and innervation demonstrate diffuse amyloidosis during islet culture and islet transplantation [285]. Based on these observations, we postulate that

the ApoE ε4 polymorphism may influence the islet microenvironment by causing vasculopathy, reducing neuronal repair and altering interstitial extracellular matrix conditions.

In conclusion, we demonstrated for the first time that arteriosclerotic lesions in T2DM contained the same chaperone molecules of amyloid deposits. We also demonstrated for the first time the increased frequency of islet amyloid deposition in diabetic pancreas of ApoE ε4 carriers. These genetic-pathologic correlation studies strongly support the role of chaperone proteins in amyloidosis and arteriosclerosis and that misfolding of these proteins may serve as a common pathway for the frequent coexistence of arteriosclerosis, T2DM, and neurodegenerative disease.

4.3. Association of IDE polymorphism with diabetic vascular disorders

In a separate thesis, my colleague, Mr. Vincent Lam, used a tagged SNP approach to examine the risk associations of T2DM and genetic polymorphisms of candidate genes implicated in amyloid formation and clearance in multiple case-control cohorts. Based on a series of replication studies in Chinese, Koreans and Japanese (personal communication, Vincent Lam), he confirmed the risk association of T2DM with the C minor allele of rs6583813. In this part of my thesis, I collaborated with Mr. Vincent Lam and confirmed for the first time using these autopsy cases that the C allele carriers were more likely to have cardiovascular events as the cause of death than T allele cases.

IDE is a zinc metalloendopeptidase with high degree of evolutionary conservation [286, 287]. It is ubiquitously expressed with regulated activity in different conditions. The enzyme has a wide range of intracellular and extracellular substrates including insulin, amylin, Aβ, insulin-like growth factor I and II [178]. Although the amino acid sequences of these substrates are different, they often share a common three-dimensional comformation similar to amyloid fibril [184]. These characteristics of multi-substrate affinity, evolutionary conservation, and extensive expression suggested important functions of IDE with pluripotent biological significance.

To date, most of the knowledge about IDE came from studies in AD. IDE is expressed predominantly in brain and found in amyloid plaques and endothelial cells [288]. Under physiological conditions, IDE is secreted by microglial cell to degrade Aβ peptide [185]. Compared to control subjects, patients with AD had reduced IDE

protein expression in hippocampaus with attenuated degrading activity of IDE. In the same vein, the protein level of Aβ increased in IDE knock-out mice while in Amyloid precursor protein (APP) and IDE double transgenic model, Aβ level decreased and formation of plaque was prevented [178].

Apart from the brain, IDE was also expressed in vascular pericytes, endothelial cells and cerebrovascular smooth muscle cells in AD [288, 289]. Cases with severe cerebral amyloid angiopathy (CAA) had elevated IDE levels but reduced IDE degrading activity [289], suggesting posible association of IDE with vascular disease. Since AD and T2DM share similar features of amyloidosis with loss of parenchymal cell, I asked the question whether IDE genetic polymorphism might be implicated in pancreatic amyloidosis. Although I was not able to confirm this hypothesis which might be due to small sample size (Power=0.2159), amongst the T2DM cases, the C allele carrier state had two-fold higher risk of having CVD as a cause of death than T allele carrier. Interestingly, despite the small sample size of the control study population, while none of the control with TT/CT genotype had amyloid deposit, 1 in 10 of the CC genotype carriers had amyloid deposit with a p value of 0.02. However these observations might be chance findings which need independent replication in larger sample size.

The IDE gene is a strong positional and biological candidate for T2DM susceptibility. The gene coding for IDE is located in 10q with evident linkage to T2DM in several populations. In animal study, the rat homolog of IDE is directly implicated in T2DM susceptibility. In a T2DM model, the Goto-Kakizaki (GK) rat, IDE gene mutation can lead to decreased insulin degradation and diabetic phenotypes [290]. The biological function of IDE also supports its important role in glucose metabolism [291]. As a neutral thiol metalloprotease, the major function of IDE is degradation. Among its various substrates, the Km value of IDE for insulin is the lowest (only about 0.1μmol) compared to others, suggesting that IDE is specific to insulin [292]. Culture cells with overexpressed IDE showed increased rate of cellular insulin clearance [293]. Microinjection of anti-IDE antibody into hepatoma cells resulted in inhibition of insulin degradation [294]. The GK rats with diabetic phenotypes had two missense mutations in IDE gene, accompanied by reduced insulin degradation, postprandial hyperglycemia and hyperinsulinemia [295]. In this regard, insulin resistance and hyperinsulinemia have been shown to be risk factors for

coronary heart disease in both diabetic and non-diabetic subjects. In healthy individuals with normal glucose tolerance, hyperinsulinemia is often considered as the linking factor for CVD risk factors including high total and LDL-cholesterol levels, low HDL-cholesterol levels, and high blood pressure [296]. In the Atherosclerosis Risk In Communities (ARIC) Study, the risk ratio for ischemic stroke was increased by 1.19 for every 50pmol/L increase in basal insulin level [297]. Given the pluripotent effects of insulin on vascular growth, intermediary metabolism and blood pressure regulation, genetic variants of IDE may contribute to increased risk for vascular disease through reduced insulin clearance. In support of this notion and in keeping with my hypothesis, I found increased frequency of vascular complications in IDE C carriers in this autopsy study.

Like insulin, amylin is a substrate of IDE with high affinity. In cultured RIN-m5F insulinoma cells, treatment with IDE inhibitor, bacitracin, resulted in amylin degradation by 78%. In addition, bacitracin also increased amyloid formation and amylin-induced cytotoxicity [298]. Of note, other substrates such as insulin, glucagon and atrial natriuretic peptide, may competitively inhibit amylin degradation [180]. Since amylin and insulin are co-secreted from islet beta-cells and given the higher affinity of IDE for insulin, it is conceivable that there may be reduced degradation of amylin resulting in hyperamylinemia as reported in diabetic and prediabetic subjects.

Of note, high amylin levels have also been reported in hypertensive subjects [299]. In experimental studies, amylin activates renin at physiological doses [300] with modest pressor effect [32]. The correlation of amylin and blood pressure may be mediated by the high-affinity amylin binding site on the proximal tubules in renal cortex and RAAS activation [301]. On the other hand, amylin can have dilating effect on rat aortic rings by inhibiting the cholecystokinin octapeptide (CCK-8) induced contractile response on smooth muscle cells. These vasodilating effects are also found in the pulmonary circulation of rat [300]. These multiple vascular effects of amylin (and insulin) suggest that the IDE-induced hyperamylinemia, often accompanied by RAS activation within a context of hyperinsulinemia may increase the risk of hypertension, CVD and related deaths in T2DM. In this regard, C carriers in control cases also showed higher percentage of renal vascular damages. Similar findings were not found in diabetic cases which might be due to the confounding effects of the large number of risk factors in these cases,

The other high affinity substrate of IDE is IGF-1 which has been shown to be a

cardiac hormone. IGF-1 promotes cardiac growth and improves cardiac contractility, stroke volume and ejection fraction. After myocardial infarction, IGF-1 plays a key role in restoring cardiac function and tissue remodeling. Thus, alteration of IGF-1 levels or its activity may impair cardiac growth and function [302]. On the other hand, the other IDE substrate, glucagon, is a crucial hormone in regulation of glucose metabolism, has low affinity to IDE and may be less affected by these genetic variations [287].

In this study, immunostaining shows higher insulin expression and lower amylin expression in the pancreatic islets of C carriers than TT genotype carriers, albeit short of significance. In addition, ACE expression level in glomeruli and blood vessels was two-fold higher in C carriers than the TT carriers, although the difference in ACE expression was not statistically significant in T2DM (P=0.0744 in renal glomeruli, P=0.1329 in blood vessel). While these cross-sectional findings suggesting RAS activation in IDE C carriers may be epiphenomena, the differences in expression of amylin, insulin and vasoactive hormones which share the same degradation pathway of IDE between the different allele carriers support the importance of IDE as a candidate gene for T2DM and cardio-renal complications. Functional studies are needed to ascertain the significance of these observations.

4.4. Association of GLUT1 polymorphism with diabetic glomerulopathy

GLUT1 was the first glucose transporter found to have high expression in endothelial cells of glomerular filtration barrier and was considered as a candidate gene of diabetic nephropathy. Several studies have reported association of nephropathy with a polymorphism at position of +22999bp in GLUT1 intron 2 in both T1DM and T2DM [303, 304], although these results are not always replicable in other populations [305]. In T1DM, another polymorphism at the promoter region of GLUT1 (rs710218) was associated with diabetic nephropathy [306]. In this autopsy study, aside from glomerulopathy, we observed association of hypertension and diabetic glomerulopathy with this genetic variant in T2DM cases.

All glucose transporters facilitate glucose transport across plasma membrane into mammalian cells in a gradient-mediated manner [218]. Among the 12 glucose transporters, GLUT1 is the most predominant one and widely expressed in many tissues. Evolutionally, GLUT1 is highly conserved among species [307]. In T1DM, several genetic polymorphisms of GLUT1 including XbaI (rs841853), Enh2SNP1

(rs841847), and GLUT1 A-2841T (rs710218) were found to be associated with diabetic nephropathy [308].

In this study, the minor T allele of polymorphism of GLUT1 rs710218 was associated with decreased frequency of renal glomerular hypertrophy, suggesting a protective effect. Given its location in the promoter, it is plausible that T allele may be associated with reduced GLUT1 expression and reduced glucose uptake in insulin sensitive tissues. Other studies have shown that increased expression of GLUT1 may alter the structure and function of mesangial cells, which are crucial for maintenance of capillary structure and regulation of glomerular filtration. These include hypertrophy and proliferation of mesangial cells, expansion of mesangial matrix, and increased thickness of basement membrane [219]. In addition, enhanced expression of GLUT1 also damages podocytes that are indispensable in the filtration barrier and glomerular filtration function. The increased intracellular glucose induced by high expression of GLUT1 in podocytes may be responsible for the increased extracellular matrix and podocyte dysfunction and apoptosis [218]. Furthermore, it is plausible that endothelial cell may be impaired by high intracellular glucose through release of profibrogenic cytokines from glomerular endothelial cells [218]. Taken together, decreased GLUT1 expression in T carriers might reduce glucose uptake in glomerular cells and protects against glucotoxicity in T2DM.

In agreement with other reports [309], we observed a negative risk association of GLUT1 T allele with hypertension. The mechanisms underlying the association between GLUT1 and hypertension are largely unknown. Transgenic mice, overexpressing of GLUT1 in skeletal muscle cells showed 4-fold increase in basal glucose uptake whereas the insulin-induced glucose uptake was absent [310] or reduced markedly [311]. These findings imply that GLUT1 overexpression may lead to insulin resistance, the latter has been linked to hypertension in both epidemiological and experimental studies [143, 144, 312]. Taken together, these findings suggest that GLUT1 genetic polymorphisms may be implicated in insulin resistance and hypertension which can interact with other risk factors to influence risk of renal complications especially in diabetic subjects.

4.5. Additive effect of ApoE and GLUT1 on diabetic glomerulopathy

Clinically, T2DM is characterized by hyperglycemia and dyslipidemia, which may lead to long-term complications, such as diabetic nephropathy and CVD [105]. In this

study, univariate analysis demonstrated that both ApoE ε2 carriers and GLUT1 A carriers had increased risk of diabetic glomerulopathy. Interestingly, these two risk-conferring genotypes showed additive effects as indicated by the linear relationship between risk of diabetic glomeruolpathy and risk scores. These findings are in accord with the known effects of glucotoxicity and lipotoxicity on pathogenesis of diabetic kidney disease.

Hyperglycemia can causes glycation and subsequent development of toxic advanced glycation end products (AGE) including glycated apolipoproteins which are more likely to be oxidized. In support of this notion, oxidized LDL has been found in the lesions of glomerulonephritis. These paticles can cause damage to masangial cells [232], podocytes [313] and endothelial cells, suggesting a direct pathogenic role of oxidized LDL in glomerular injury [314]. In addition, oxidized LDL accumulated in macrophages can stimulate secretion of cytokines such as interleukin-6, PDGF, ICAM-1, and TGF-β [120, 235, 268-270]. Besides, glycation of lipoproteins can alter protein structure with reduced uptake through the LDL receptor. These multiple changes can worsen hyperglycemia [314] and contribute to the additive effects of glucotoxicity and lipotoxicity on glomerulopathy.

Consistent with the finding in diabetic glomerupathy, risk-conferring variants of ApoE and GLUT1 showed dose effect on glomerulopathy in the entire study population including nondiabetic control cases. Higher risk score was associated with higher percentage of vascular lesions in pancreas compared to the referent group (risk score=0). These novel results provide the first set of human data to support the additive effect of glucotoxicity and lipotoxicity on renal structure.

4.6. Value of autopsy study

Autopsy is an important process to ensure quality of medical care and clarify causes of death, especially in atypical situations [315]. In the field of cardiology, neurology and endocrinology, autopsy is almost the only means to obtain human samples for detailed investigations. In other medical disciplines where biopsy samples can be taken, autopsy samples are still useful. For example, kidney biopsies are only performed in patients with atypical clinical and biochemical phenotypes. In the case of diabetes, patients undergo renal biopsy for atypical clinical presentation, e.g. presence of haematuria and/or absence of retinopathy. Therefore, selection bias of renal biopsy in T2DM is unavoidable. Conversely, consecutive autopsy study

population may minimize this type of bias. In addition, biopsy only provides partial information while autopsy provides more comprehensive data on pathological changes in multiple organs. Unfortunately, due to the decline of autopsy rate in most institutions [316, 317], autopsy materials becomes an increasingly scarce resources for clinical care and research.

4.7. Limitation of this study

Autopsy is the only source to provide sufficient human pancreatic tissues for examination. While these specimens provide useful information on genetic-pathological correlations, the retrospective nature of the study has inherent limitations. Not all deaths were autopsied in our hospital and not all autopsy cases had well-preserved pancreas and renal tissues. We sought to minimize this bias by using consecutive autopsies. Due to a generally decreasing autopsy rate, we only recruited 328 T2DM cases and 209 non-diabetic controls in this study with limited power and possible type 2 error. Furthermore, not all clinical data were available in the selected cases which further reduced the robustness of the study to detect genotype-phenotype correlations. Despite these limitations, my study using autopsy specimens have provided new insights regarding the importance of 3 genes governing the lipid/glucose pathways and their potential impacts on pancreatic, vascular and renal structure and function. In support of the value of pathological studies, these positive findings despite the relatively small sample size, may be due to the more accurate classification based on pathological changes than clinical definitions, as in the case of diabetic nephropathy.

In this study, the diagnosis of T2DM was based on the clinical diagnosis of "T2DM" or "non-insulin dependent diabetes mellitus (NIDDM)" provided in the autopsy reports. Since many diabetic subjects are not clinically diagnosed, this selection criterion might lead to misclassification. For example, we found four control cases which had islet amyloidosis but not diagnosed to have T2DM (table 14). However, examination of clinical parameters as well as renal and pancreatic sections did not identify other biochemical or pathological changes diagnostic of diabetes. In some non-diabetic control subjects, there were diabetes-associated renal pathological changes, such as hyaline arteriolosclerosis and arteriosclerosis (table 13). Due to the average age of the control cases which was 69 years, it was difficult to find 'healthy' controls in this elderly population. For example, 13.4% control cases had

hypertension (table 12) which is known to be associated with hyaline arteriolosclerosis and arteriosclerosis. However, none of these control cases showed diabetes-specific pathological renal changes, such as nodular glomerulosclerosis or hyalinosis in afferent and efferent glomerular arterioles. Since some of these pathological changes were due to age and risk factors shared by diabetic and non-diabetic subjects, I have adopted a consistent approach to use the clinical diagnosis in the autopsy reports to classify cases and controls.

CHAPTER 5 CONCLUSIONS AND FUTURE PROSPECTS

5. Conclusions and future prospects

5.1. Conclusions

In this thesis, I have applied my training as a pathologist to discover new knowledge regarding certain genetic-pathological correlations in pancreatic and renal tissue from T2DM. Most of these findings are novel although independent replication is needed. These include a) the associations of ApoE genotype with pancreatic amyloidosis which is often accompanied by features of atherosclerosis, arterial hyalinosis and fibrosis; b) the assocations of ApoE ε2 with diabetic glomerulopathy; c) the immunolocalization of ApoE and amyloid P in both pancreatic amyloidosis and vascular hyalinosis suggesting the possibility of shared mechanisms; d) the associations of IDE rs6583813 C carriers (CT and CC genotype) with increased severity of vascular disorders and increased frequency of related death, possibly acting through differential expression of several hormones or cytokines including insulin, amylin, TNF α and IGF1; e) association of the GLUT1 TT genotype with reduced risk of glomerulopathy and hypertension and f) apparant gene-gene interactions for ApoE and IDE on cardiovascular events and that for ApoE and GLUT1 on vasculopathy and glomeurlopathy..

Apart from providing corroborative evidence for other clinical and experimental studies, these findings support the important roles of genetic factors in development of diabetes-specific pathological changes, notably in the pancreas and kidney. Due to the highly specific nature of these pathological findings and the multicausality of T2DM and associated complications, these findings have clarified some inconsistencies such as the association of different ApoE polymorphisms with diabetic nephropathy in that the ApoE ε2 allele was more closely associated with glomerulopathy and the ApoE ε4 allele, with vasculopathy.

5.2. Future prospects

While my study has generated novel findings, the functional significance of these genetic variants notably the ApoE, GLUT1 and IDE as well as the molecular mechanisms underlying islet amyloidosis and diabetic glomerulopathy need further study. In addition to ApoE, the pathogenetic roles of other common components of amyloid deposits such as serum amyloid P remain to be clarified. Amyloid P reportedly prevents proteolysis of amyloid fibrils in AD possibly by increasing the

stability of fibrillar structure [280]. In addition, heparin sulfate proteoglycans (HSPG) might contribute to amyloidogenesis by inducing conformational change which facilitates assembly of major proteins into fibrils [173]. Thus, serum amyloid P and HSPG are other candidate genes which may increase the susceptibility to islet amyloidosis in T2DM. Based on the pathological correlation between islet amyloidosis and arteriosclerosis, future studies should focus on the possible effects of blood vessel impairment in the pathogenesis of diabetic islet amyloidosis.

In future genetic studies of diabetic glomerulopathy, a tagged SNP approach may allow further haplotype analysis to ascertain risk associations with GLUT1 alleles. Finally, given the multiple substrates of IDE which include amylin-insulin pathway and vasoactive hormones which are implicated in arteriosclerosis and glomerulosclerosis, there may be cross talk between IDE and some of these candidate genes implicated in pancreatic amyloidosis and diabetic glomerulopathy.

APPENDICES

Appendix I Pathological assessment

1. Tissue precessing

Reagents:

1) Ethanol 70%-100%
2) Xylene
3) Paraffin wax

Procedure:

1) Anhydrate tissue in gradient ethanol (75% one hour, 85% one hour, 95% one hour × 2 times, 100% 30 minutes × 2 times).
2) Lucidify in xylene, 15 minutes × 2 times.
3) Immerse in wax one hour × 2 times.
4) Embed tissue into wax.

2. Hematoxylin and Eosin (H&E) staining

Regents:

1) Xyline
2) Ethanol
3) Acid ethanol: 1ml 5N HCl + 400 ml 70% ethanol
4) Bluing Agent: Sodium bicarbonate 1 gm + distilled water 1 litre
5) Hematoxylin (DAKO, S3309, CA, USA)
6) Eosin: Eosin Y (alcoholic C.I. 45380) 0.5 g + Ethanol 96% 100 ml + Glacial Acetic Acid - 2 drops
7) Histomount (VWR, Inc. Poole, BH15 1TD, England)

Procedure:

1) Place slides containing paraffin sections in a slide holder (metal)
2) Deparaffinize and rehydrate sections:

   Xylene (blot excess xylene before going into ethanol) 3 minutes × 3 times

   100% ethanol 3 minutes × 2 times

   95% ethanol 3 minutes × 1 time

   80% ethanol 3 minutes × 1 time

   deionized H2O 3 minutes × 3 times
3) While sections are in water, skim surface of hematoxalin with a Kimwipe to remove oxidized particles. Blot excess water from slide holder before going into

hematoxalin.

4) Hematoxalin staining:

Hematoxalin 3 minutes × 1 time

Rinse deionized water

Tap water (to allow stain to develop) 5 minutes × 1 time

Dip 8-12 times in (fast) Acid ethanol (to destain)

Rinse in Tap water 3 minutes × 1 times

Immerse in blue regent 2 minutes × 1 time

Rinse in Tap water 3 minutes × 1 times

Rinse in Deionized water 2 minutes × 1 time

5) Blot excess water from slide holder before going into eosin.

6) Eosin staining,

Eosin 30 seconds × 1 time

11) Dehydration and Lucidification:

75% ethanol 2 minutes × 1 time

85% ethanol 3 minutes × 1 times

95% ethanol 3 minutes × 2 times

100% ethanol 3 minutes × 2 times (blot excess ethanol before going into xylene)

Xylene 3 minutes × 3 times

12) Add a drop of histomount to the slide, cover the slide with a clean coverslip.

13) Dry overnight in the hood.

Results:

Blue: nuclei, Basophilic cytoplasm

Bed: red blood cells

Pink: Acidophilic cytoplasm, Muscle, Connective tissue

3. Thioflavin T stain

Regents:

1) 0.01M phosphate buffered saline (PBS) PH 7.2-7.4: NaCl 8g + KCl 0.2g + Na_2HPO_4 1.44g + KH_2PO_4 0.24g + ddH_2O 800 ml, adjust PH value to 7.4 with 5N HCl, metered volume to 1000 ml

2) 10 mmol Thioflavin T

3) 300nm DAPI (invitrogen, D21490, Oregon, USA,):

1:47000 (14.3mmol) store solution 1: 10 mg DAPI powder + 2 ml milli Q water,

1:100 store solution 2: 1:470 dilute store solution 1,

working solution: 1:100 dilute store solution 2

4) Buffered glycerin: PBS 10 ml + glycerin () 90 ml

Procedure:

1) Mount 4 μm tissue section on coated slides with polylysine
2) Bake for 30 minutes at 55 °C to ensure optimal adhesion of tissue section
3) Place slides containing paraffin sections in a slide holder (metal)
4) Deparaffinize and rehydrate sections:

   Xylene (blot excess xylene before going into ethanol) 3 minutes × 3 times

   100% ethanol 3 minutes × 2 times

   95% ethanol 3 minutes × 1 time

   80% ethanol 3 minutes × 1 time

   deionized H_2O 3 minutes × 3 times

3) Incubate in 10 mmol Thioflavin T 45 minutes.

   wash with PBS 2 minutes × 3 times

4) Counterstain with DAPI working solution 3-5 minutes.

   Wash with PBS 2 minutes × 3 times

5) Add a drop of buffered glycerin to the slide, cover the slide with a clean coverslip.
6) Conserve in 4°C.

Results:

Green: amyloid fibril

Blue: nuclei

4. Immunohistochemistry staining

Regents and solutions:

1) Xyline
2) Ethanol
3) Hematoxylin (DAKO, S3309, CA, USA)
4) Acid ethanol: 1ml 5N HCl + 400 ml 70% ethanol
5) Bluing Agent: Sodium bicarbonate 1 gm + distilled water 1 litre
6) 0.01M phosphate buffered saline (PBS) PH 7.2-7.4: NaCl 8g + KCl 0.2g + Na_2HPO_4 1.44g + KH_2PO_4 0.24g + ddH_2O 800 ml, adjust PH value to 7.4 with

5N HCl, metered volume to 1000 ml

7) 0.01M citrate buffer PH 6.0±0.1:

A: 0.1M Citric Acid: citric acid 21.01g + 1000ml dd H_2O

B: 0.1M Sodium Citrate 29.41g + 1000ml dd H_2O

Working solution: solution A 9ml + B solution 41 ml + 450 ml dd H_2O

8) 0.1% bovine serum albumin BSA (Sigma, 20K760, Steinheim, Germany):

100mg BSA + 100 ml ddH_2O

9) Histomount (VWR, Inc. Poole, BH15 1TD, England)

10) DAKO EnVision system (DAKO, K4006, CA, USA)

Procedure:

1) Mount 4 μm tissue section on coated slides with polylysine

2) Bake for 30 minutes at 55 °C to ensure optimal adhesion of tissue section

3) Place slides containing paraffin sections in a slide holder (metal)

4) Deparaffinize and rehydrate sections:

Xylene (blot excess xylene before going into ethanol) 3 minutes × 3 times

100% ethanol 3 minutes × 2 times

95% ethanol 3 minutes × 1 time

80% ethanol 3 minutes × 1 time

deionized H_2O 3 minutes × 3 times

5) Put slides into a beaker with 0.01M citrate buffer pH 6.0,heat with high power 3 minutes in microwave oven, followed by low power for 6.5 minutes, then cool at room temperature for 20 minutes.

6) Add peroxidase block reagent to diminish background staining for 10 minutes.

7) Prepare primary antibody (30ul/slide)

Antibody: mouse anti-ApoE monoclonal antibody (1:100 dilution, Abcam, Cambridge, MA, USA)

8) Incubate with primary antibody overnight at 4°C, negative were incubated with 0.1% BSA

Wash with PBS 2 minutes × 3 times

9) Incubate with peroxidase labeled polymer 30 minutes in room temperature.

Wash with PBS 2 minutes × 3 times

10) Apply enough prepared liquid DAB + substrate-chromogen solution to cover specimen, incubate for 5 minutes.

Collect substrate-chromogen waster in a hazardous materials container and wash slides with distilled water 5 minutes × 3 times

11) Hematoxalin counterstain:

Hematoxalin 3 minutes × 1 time

Rinse deionized water

Tap water (to allow stain to develop) 5 minutes × 1 time

Dip 8-12 times in (fast) Acid ethanol (to destain)

Rinse in Tap water 3 minutes × 1 times

Immerse in blue regent 2 minutes × 1 time

Rinse in Tap water 3 minutes × 1 times

Rinse in Deionized water 2 minutes × 1 time

12) Dehydration and Lucidification:

75% ethanol 2 minutes × 1 time

85% ethanol 3 minutes × 1 times

95% ethanol 3 minutes × 2 times

100% ethanol 3 minutes × 2 times (blot excess ethanol before going into xylene)

Xylene 3 minutes × 3 times

13) Add a drop of histomount to the slide, cover the slide with a clean coverslip.

14) Dry overnight in the hood.

Resuts:

Brown or yellow: specific protein expression

Blue: nuclei

5. Immunohistofluorescence staining

Regents and solutions:

1) Xyline
2) Ethanol
3) 0.01M phosphate buffered saline (PBS) PH 7.2-7.4: NaCl 8g + KCl 0.2g + Na_2HPO_4 1.44g + KH_2PO_4 0.24g + ddH_2O 800 ml, adjust PH value to 7.4 with 5N HCl, metered volume to 1000 ml
4) 0.01M citrate buffer PH 6.0±0.1:
   a) A: 0.1M Citric Acid: citric acid 21.01g + 1000ml dd H_2O
   b) B: 0.1M Sodium Citrate 29.41g + 1000ml dd H_2O
   c) Working solution: solution A 9ml + B solution 41 ml + 450 ml dd H_2O

5) 0.1% bovine serum albumin (BSA) (sigma, 20K7607, steinhein, germany): 100mg BSA + 100 ml ddH_2O

6) Primary antibodies

Table 44 Primary antibodies and dilution

Antibody	Source	Productor	Catalog No.	Dilution
Insulin	Mouse	Zymed	18-0066	1:100
Insulin	Guinea pig	DAKO	A0564	Ready to use
Amylin	Mouse	AbD Serotec	P10997	1:400
Amylin	Rabbilt	Phoenix Pharmaceuticals	H-017	1:400
Glucagon	Rabbit	Dako	A0565	1:400
Apo E	Mouse	Abcam	Ab1907-100	1:100
IDE	Rabbit	Abcam	Ab25970-50	1:400
Amyloid P	Rabbit	DAKO	A0302	1:100
IGF-1	Goat	Santa cruz	SC1422	1:200
ACE	Rabbit	Santa cruz	SC20791	1:300
Mitochrondria	Rabbit	Santa Cruz		1:400

7) Secondary antibodies (Invitrogen; A10036, A10040, A11056, A11055, A21202, and A21206; dilution: 1:400)

8) 300nm DAPI (invitrogen, D21490, Oregon, USA):

1:47000 (14.3mmol) store solution 1: 10 mg DAPI powder + 2 ml milli Q water,

1:100 store solution 2: 1:470 dilute store solution 1,

working solution: 1:100 dilute store solution 2

9) Anti-fade regent (invitrogen, P36935, Oregon, USA)

Procedure:

1) Mount 4 μm tissue section on coated slides with polylysine

2) Bake for 30 minutes at 55 °C to ensure optimal adhesion of tissue section

3) Place slides containing paraffin sections in a slide holder (metal)

4) Deparaffinize and rehydrate sections:

Xylene (blot excess xylene before going into ethanol) 3 minutes × 3 times

100% ethanol 3 minutes × 2 times

95% ethanol 3 minutes × 1 time

80% ethanol 3 minutes × 1 time

with same setting.

3) Open the metaMorph image analysis system.
4) Outline the target area (such as islet or glomeruli).
5) Highlight positive area and/or cells.
6) Count the pixel ratio to the area examined.
7) Repeat the above procedures 4)-6) for the all the images randomly obtained
8) Calculate the mean value of the images for each case.

Appendix II SNPs detection

1. DNA extraction

Regents:

1) Digestion buffer: 100 mM NaCl, 10mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS. Store at RT.
2) Proteinase K: Stored as 20 mg/ml aliquots at -20 C (can be refrozen a few times), working solution: 0.8 mg/ml in digestion buffer
3) PC: phenol/chloroform=1:1
4) CI: chloroform/isoamyl alcohol=24:1
5) sodium acetate (3M, PH 5.2): 24.6g sodium acetate in 100ml milli Q water

Procedure:

1) Cut 5 pieces 10 um section from formalin fixed paraffin blocks each sample into 1.5ml tubes.
2) Add 1 ml xylene, mix, incubate at RT for 5 mins, then spin down for 2 minutes in centrifuge, pipet off & discard supernatant.
3) Repeat Step 2) 2 times.
4) Add 100% ethanol, incubate at RT for 5 mins, Spin down and remove ethanol.
5) Repeat Step 4) 2 times, allow pellet to air dry.
6) Add up to 800μL of Proteinase K (PK) in digestion buffer to a final concentration of 0.8 mg/ml. Incubate overnight, shaking, at 65°C (If there are still some tissue in tubes, we can add fresh concentrated PK).
7) Add equal volume PC (phenol: chloroform = 1:1) to the PK digested aqueous solution, spin 2 mins, remove upper aqueous phase to new tube. Repeat 1 time.
8) Add equal volume CI (chloroform: isoamyl alcohol = 24:1) to the upper solution, spin 2 mins, remove upper aqueous phase to new tube.
9) Add 0.1 original volume sodium acetate (3M, PH 5.2) and 2.5X volume 100%

deionized H_2O 3 minutes × 3 times

5) Put slides into a beaker with 0.01M citrate buffer pH 6.0,heat with high power 3 minutes in microwave oven, followed by low power for 6.5 minutes, then cool at room temperature for 20 minutes.
6) Add 0.1% BSA to diminish background staining for 10 minutes.
7) Prepare primary antibody (30ul/slide)
8) Incubate with primary antibody overnight at 4°C, negative were incubated with 0.1% BSA

   Wash with PBS 2 minutes × 3 times
9) Incubate with secondary antibody (1:400) 30 minutes in room temperature.

   Wash with PBS 2 minutes × 3 times
10) Counterstain with DAPI working solution 3-5miutes.
11) Wash with PBS 2 minutes × 3 times
12) Add a drop of buffered glycerin to the slide, cover the slide with a clean coverslip.
13) Conserve in 4°C.

Resuts:

Red or Green: specific protein expression

Blue: nuclei

6. Image analysis

Device and software:

1) Zeiss Axioplan 2 imaging microscope (Carl Zeiss Inc., Gottingen, Germany)
2) Digital spot camera (version 3.1 forWindows 95/98/NT; Diagnostic Instruments Inc., Sterling Heights, MI, USA).
3) Computer: LAN ® computer, Pentium Pro ®, PII 350 MHz, 512.0 MB RAM; SONY monitor (18')
4) Operation system: Microsoft Windows 95 4.00.950B
5) Image analysis software: MetaMorph 4.0, 1992-1999 Universal Imaging Corporation

Procedure:

1) Turn on computer, Zeiss microscope and spot camera.
2) At least 10 images were automaticly obtained in a case at suitable magnification

ethanol, overnight at -20 C.

10) Spin for 20 minutes at 4°C, decant ethanol and air dry.
11) Carefully resuspend the pellets in small volumes of Milli Q water (20 ul) and let dissolve at RT.
12) Examine DNA quality on the Fluorometer. Ideal DNA OD value is from 1.7-1.9.

2. PCR amplification

Regents:

1) TBE (10×): Tris base 54g, boric acid 27.5g, 0.5M EDTA 20ml (PH 8.0) in 500ml distilled water
2) Primers: Stock solution is 100uM (primers in sterile, deionized water Store at -20°C.), working solution is 10uM.
3) dNTPs (10mM) (store at -20°C)
4) 10×PCR buffer (Store at -20°C)
5) MgCl2 (25mM) (Store at -20°C)
6) Milli Q water
7) Amplitaq DNA Polymerase (5U/ul) (Store at -20°C) (Applied Biosystems, N808-0245, CA, USA)
8) Loading Dye "Blue Juice": 0.25g bromophenol blue, 0.25g xylene cyanol, 1.0ml 1M Tris (pH 8.0), 45 ml deionized or distilled water, 50ml of glycerol, metered volume to 100 ml
9) Agarose (promega, V3125, WI, USA)

Procedure

1) Make up Master Mix solution in a 1.5ml tube.

Master Mix per reaction tube (25µl):

Mini Q water 16.9ul,

PCR buffer(10×) 2.5ul,

MgCl2 (25mM) 3.0ul,

Primer 1(10uM) 0.5ul,

Primer 2(10uM) 0.5ul,

dNTP (10mM) 0.5ul

Taq (5U/ul) 0.1ul

2) Using a filtered P-20 tip, pipet 24µl of master mix into each PCR tube.
3) Pipet 1µl of DNA (100 ng of genomic DNA) into the appropriate PCR tube using

filtered tips

4) Put tubes into Thermalcycler.
5) 94°C for 5 min, 40× (95°C for 0.5 min, annealing for 1 min, 72°C for 1 min), 72°C for 10 min.
6) Set up appropriate gel apparatus with enough wells to load samples and 50bp marker.

Weigh 2g of agarose out and add 100ml of 1XTBE into a 500ml Erlenmeyer flask and microwave on med (Use 2% agarose gel in 1X TBE). high 3-5min.

Once mixed, visually check if all the agarose is melted (clear solution, no opaque white agarose present or "swirly bubbles").

7) The samples run from negative to positive (black to red).
8) Run gel
9) Label a piece of parafilm with sample numbers and pipet 2μl of Blue juice under each number.
10) Pipet 5μl of sample onto appropriately labeled blue juice dot. Be sure and include a marker (3.5μl of 50bp/per comb).
11) Run gel between 83V and 121V until the front-running band (greenish-blue band) is about 3/4 of the way across
12) Staining with Ethidium Bromide and
13) The gel can be moved to a container of 400μg/ml Ethidium Bromide for 10 minutes.
14) The gel can be moved to the UV transilluminator and visualized to check that the bands are resolved enough to read.

3. LDR detection

Regents and equipments

DNA sequencer (ABI prism 377 DNA sequencer, CA, USA)

NEB Taq DNA ligase (Stock solution is 5U/ul, Store at -20°C.)

Probe mixture

Procedure

1) Make up Master Mix solution.

Master Mix per reaction tube (10ul):

Mini Q water 6.95ul

Buffer(10×) 1ul

Probe mix 1ul

Ligase (5U/ul) 0.05ul

2) Using a filtered tip, pipet 9μl of master mix into each PCR tube.
3) Pipet 1μl of PCR product (>100 ng/ul) into the appropriate PCR tube using filtered tips
4) Put tubes into Thermalcycler.
5) 94°C for 2 min, 15× (94°C for 0.5 min, 60°C for 2 min).
6) Sequencing in 377 sequencing electrophoresis apparatus.
7) Results analysis

BIBLIOGRAPHY

Journal Article

1. **Guan J**, Zhao HL, Baum L, Sui Y, He L, Wong H, Lai FM, Tong PC, Chan JC. Apolipoprotein E polymorphism and expression in type 2 diabetic patients with nephropathy: clinicopathological correlation. Nephrol Dial Transplant. 2009 Jun;24(6):1889-95.
2. Zhao HL, **Guan J**, Lee HM, Sui Y, He L, Siu J, Tse PP, Lai FM, Tong PC, Chan JC. Upregulated pancreatic tissue microRNA-375 associates with human type 2 diabetes through beta-cell deficit and islet amyloid deposition. Pancreas. (In press)
3. HL Zhao, **J Guan**, Y Sui, L He, Fernand MM Lai. Islet Amyloidosis and β-cell Neogenesis in Chronic Calcifying Pancreatitis with Non-Insulin-Dependent Diabetes Mellitus. Pancreas. 2008 Oct;37(3):e68-73.
4. Zhao HL, Sui Y, **Guan J**, He L, Lai FM, Zhong DR, Yang D, Baum L, Tong PC, Tomlinson B, Chan JC. Higher islet amyloid load in men than in women with type 2 diabetes mellitus. Pancreas. 2008 Oct;37(3):e68-73.
5. Zhao HL, Sui Y, **Guan J**, He L, Gu XM, Wong HK, Baum L, Lai FM, Tong PC, Chan JC. Amyloid oligomers in diabetic and nondiabetic human pancreas. Transl Res. 2009 Jan;153(1):24-32.
6. Zhao HL, Sui Y, **Guan J**, Lai FM, Gu XM, He L, Zhu X, Rowlands DK, Xu G, Tong PC, Chan JC. Topographical associations between islet endocrine cells and duct epithelial cells in the adult human pancreas.Clin Endocrinol (Oxf). 2008 Sep;69(3):400-6.

Submitted Article

1. **J Guan**, HL Zhao, Y Sui, Fernand MM Lai, P CY Tong, J CN Chan. Histopathological correlations of islet amyloidosis and hyaline arteriosclerosis with amylin gene mutations and apolipoprotein E polymorphisms in Chinese patients with type 2 diabetes.

Conference Abstract

1. **J Guan ***, Hai-Lu Zhao, Yi Sui, Lan He, Harriet Wong, Peter CY Tong, Juliana CN Chan. Apolipoprotein E polymorphism and expression in type 2 diabetic patients with nephropathy: Clinicopathological correlation. The 6th Congress of

the Asian Pacific Society of Atherosclerosis and Vascular Disease & 10th Hong Kong Diabetes and Cardiovascular Risk Factors – East Meets West Symposium, 25 – 28 September 2008, Hong Kong Convention and Exhibition Center, Hong Kong. Programme & Abstract Book, p48 (Ab62). * Young Investigator Award Winner

2. **J Guan**, Y Sui, HL Zhao, FM Lai, PC Tong, JC Chan. Extra-islet beta-cells in adult human pancreas. The American Diabetes Association 67th Scientific Sessions, 22-26 June 2007, Chicago, IL, USA. Diabetes 2007;56 (Suppl 1): A 422 (1666-P).
3. **J Guan**, Yi Sui, Hai-Lu Zhao, Fernand MM Lai, Peter CY Tong, Juliana CN Chan. Pancreatic fibrosis in diabetic patient with renal failure. The American Diabetes Association 68th Scientific Sessions, 6-10 June 2008, San Francisco, CA, USA. Diabetes 2008;57 (Suppl 1): A633 (2305-PO).
4. **J Guan**, HL Zhao, Y Sui, Fernand MM Lai, P CY Tong, J CN Chan. Histopathological correlations of islet amyloidosis and hyaline arteriosclerosis with amylin gene mutations and apolipoprotein E polymorphisms in Chinese patients with type 2 diabetes. The American Diabetes Association 69th Scientific Sessions, 5-9 June 2009, New Orleans, LA, USA. Diabetes 2009;58 (Suppl 1): A? (1418-P).
5. Hai-Lu Zhao, Yi Sui, **Jing Guan**, Fernand M Lai, Ding-Rong Zhong, Di Yang, Peter C Tong, Juliana C Chan. Gender differential in Type 2 diabetes-associated islet amyloid. The American Diabetes Association 67th Scientific Sessions, 22-26 June 2007, Chicago, IL, USA. Diabetes 2007;56 (Suppl 1): A669 (2642-PO).

REFERENCES

1. L M Tierney, S.J.M., M A Papadakis, *Current medical Diagnosis & Treatment. International edition.* 2002, New York: Lange Medical Books/McGraw-Hill. 1203-1215.
2. Stumvoll, M., B.J. Goldstein, and T.W. van Haeften, *Type 2 diabetes: principles of pathogenesis and therapy.* Lancet, 2005. **365**(9467): p. 1333-46.
3. Akerblom, H.K., et al., *Environmental factors in the etiology of type 1 diabetes.* Am J Med Genet, 2002. **115**(1): p. 18-29.
4. Gillespie, K.M., *Type 1 diabetes: pathogenesis and prevention.* Cmaj, 2006. **175**(2): p. 165-70.
5. Alberti, K.G. and P.Z. Zimmet, *Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation.* Diabet Med, 1998. **15**(7): p. 539-53.
6. *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2008. **31 Suppl 1**: p. S55-60.
7. Ossman, S., *Diabetic Nephropathy: Where We Have Been and Where We Are Going.* Diabetes Spectr., 2006. **19**(3): p. 153-156.
8. Dobson, M., *Nature of the urine in diabetes.* Medical Observations and Inquiries, 1776. **5**: p. 298-310.
9. Chast, F., *[Apollinaire Bouchardat, pharmacist, nutritionist].* Ann Pharm Fr, 2000. **58**(6 Suppl): p. 435-42.
10. Namazi, M.R., *Paul Langerhans: a tribute to an admirable life in science.* Arch Dermatol, 2008. **144**(9): p. 1109.
11. Patlak, M., *New weapons to combat an ancient disease: treating diabetes.* Faseb J, 2002. **16**(14): p. 1853.
12. Opie, E., *The relation of diabetes mellitus to lesions of the pancreas: hyaline degeneration of the islands of Langerhans.* J Exp Med, 1901. **5**: p. 527-540.
13. Ahronheim, J., *Nature of hyaline material in pancreatic islets in diabetes mellitus.* Am J Pathol, 1943. **19**: p. 873-882.
14. Ehrlich JC, R.I., *Amyloidosis of the islets of Langerhans. A restudy of islet hyaline in diabetic and non diabetic individuals.* Am J Pathol, 1961. **38**: p. 49-59.
15. Westermark, P., *Fine structure of islets of Langerhans in insular amyloidosis.* Virchows Arch A Pathol Pathol Anat, 1973. **359**(1): p. 1-18.
16. Westermark, P., et al., *Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells.* Proc Natl Acad Sci U S A, 1987. **84**(11): p. 3881-5.
17. Cooper, G.J., et al., *Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients.* Proc Natl Acad Sci U S A, 1987. **84**(23): p. 8628-32.
18. Koopmans, S.J., et al., *Biological action of pancreatic amylin: relationship with glucose metabolism, diabetes, obesity and calcium metabolism.* Neth J Med, 1992. **41**(1-2): p. 82-90.
19. Kimmelstiel P, W.C., *Intercapillary lesions in the glomeruli of the kidney.* American Journal of Pathology, 1936. **12**: p. 83-97.
20. Essue B, M.M., Leeder SR, Colagiuri R., *Epidemiology of Diabetes. The Serious and*

Continuing Illness Policy and Practice Study (SCIPPS). 2007, sydney: The Menzies Centre for Health Policy.

21. Huxley, R., F. Barzi, and M. Woodward, *Excess risk of fatal coronary heart disease associated with diabetes in men and women: meta-analysis of 37 prospective cohort studies.* Bmj, 2006. **332**(7533): p. 73-8.
22. Ottenbacher, K.J., et al., *Diabetes mellitus as a risk factor for stroke incidence and mortality in Mexican American older adults.* J Gerontol A Biol Sci Med Sci, 2004. **59**(6): p. M640-5.
23. Pugh, J.A., et al., *NIDDM is the major cause of diabetic end-stage renal disease. More evidence from a tri-ethnic community.* Diabetes, 1995. **44**(12): p. 1375-80.
24. Rosenfeld, L., *Insulin: discovery and controversy.* Clin Chem, 2002. **48**(12): p. 2270-88.
25. Hoppener, J.W., B. Ahren, and C.J. Lips, *Islet amyloid and type 2 diabetes mellitus.* N Engl J Med, 2000. **343**(6): p. 411-9.
26. Kahn, S.E., S. Andrikopoulos, and C.B. Verchere, *Islet amyloid: a long-recognized but underappreciated pathological feature of type 2 diabetes.* Diabetes, 1999. **48**(2): p. 241-53.
27. Zhao, H.L., et al., *Prevalence and clinicopathological characteristics of islet amyloid in chinese patients with type 2 diabetes.* Diabetes, 2003. **52**(11): p. 2759-66.
28. Zhao, H.L., et al., *Higher islet amyloid load in men than in women with type 2 diabetes mellitus.* Pancreas, 2008. **37**(3): p. e68-73.
29. Gross, J.L., et al., *Diabetic nephropathy: diagnosis, prevention, and treatment.* Diabetes Care, 2005. **28**(1): p. 164-76.
30. *US Renal Data System: USRDS 2003 Annual Data Report: Atlas of End-Stage Renal Disease in the United States.* 2003.
31. Chan, J.C. and C.S. Cockram, *Diabetes in the Chinese population and its implications for health care.* Diabetes Care, 1997. **20**(11): p. 1785-90.
32. Haynes, J.M., W.C. Hodgson, and M.E. Cooper, *Rat amylin mediates a pressor response in the anaesthetised rat: implications for the association between hypertension and diabetes mellitus.* Diabetologia, 1997. **40**(3): p. 256-61.
33. Mauer, S.M., et al., *Structural-functional relationships in diabetic nephropathy.* J Clin Invest, 1984. **74**(4): p. 1143-55.
34. Fioretto, P. and M. Mauer, *Histopathology of diabetic nephropathy.* Semin Nephrol, 2007. **27**(2): p. 195-207.
35. Hasslacher, C., *Diabetic nephropathy: structural-functional relationships.* Contrib Nephrol, 1989. **73**: p. 24-8; discussion 28-9.
36. Borch-Johnsen, K., *The costs of nephropathy in type II diabetes.* Pharmacoeconomics, 1995. **8 Suppl 1**: p. 40-5.
37. Parving, H.H., et al., *Prevalence and causes of albuminuria in non-insulin-dependent diabetic patients.* Kidney Int, 1992. **41**(4): p. 758-62.
38. Gambara, V., et al., *Heterogeneous nature of renal lesions in type II diabetes.* J Am Soc Nephrol, 1993. **3**(8): p. 1458-66.
39. Ruggenenti, P., et al., *The nephropathy of non-insulin-dependent diabetes: predictors of*

outcome relative to diverse patterns of renal injury. J Am Soc Nephrol, 1998. **9**(12): p. 2336-43.

40. Olsen, S. and C.E. Mogensen, *How often is NIDDM complicated with non-diabetic renal disease? An analysis of renal biopsies and the literature.* Diabetologia, 1996. **39**(12): p. 1638-45.
41. Waldherr, R., C. Ilkenhans, and E. Ritz, *How frequent is glomerulonephritis in diabetes mellitus type II?* Clin Nephrol, 1992. **37**(6): p. 271-3.
42. Fioretto, P., et al., *Patterns of renal injury in NIDDM patients with microalbuminuria.* Diabetologia, 1996. **39**(12): p. 1569-76.
43. Stout, L.C., S. Kumar, and E.B. Whorton, *Focal mesangiolysis and the pathogenesis of the Kimmelstiel-Wilson nodule.* Hum Pathol, 1993. **24**(1): p. 77-89.
44. Wirta, O., et al., *Renal findings and glomerular pathology in diabetic subjects.* Nephron, 2000. **84**(3): p. 236-42.
45. Schwartz, M.M., et al., *Renal pathology patterns in type II diabetes mellitus: relationship with retinopathy. The Collaborative Study Group.* Nephrol Dial Transplant, 1998. **13**(10): p. 2547-52.
46. Carpenter, A.M., et al., *Glomerulosclerosis in type 2 (non-insulin-dependent) diabetes mellitus: relationship to glycaemia in the University Group Diabetes Program (UGDP).* Diabetologia, 1993. **36**(10): p. 1057-63.
47. Bisi, H., et al., *Later complications on diabetic patients: nephropathies in necropsy material.* Rev Paul Med, 1993. **111**(6): p. 449-53.
48. Turcotte, L.P. and J.S. Fisher, *Skeletal muscle insulin resistance: roles of fatty acid metabolism and exercise.* Phys Ther, 2008. **88**(11): p. 1279-96.
49. Bluher, M., et al., *Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance.* Dev Cell, 2002. **3**(1): p. 25-38.
50. Choukem, S.P. and J.F. Gautier, *How to measure hepatic insulin resistance?* Diabetes Metab, 2008. **34**(6 Pt 2): p. 664-73.
51. Kasuga, M., *Insulin resistance and pancreatic beta cell failure.* J Clin Invest, 2006. **116**(7): p. 1756-60.
52. Newman, B., et al., *Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins.* Diabetologia, 1987. **30**(10): p. 763-8.
53. Kaprio, J., et al., *Concordance for type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland.* Diabetologia, 1992. **35**(11): p. 1060-7.
54. Goldfine, A.B., et al., *Insulin resistance is a poor predictor of type 2 diabetes in individuals with no family history of disease.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2724-9.
55. Groop, L.C. and T. Tuomi, *Non-insulin-dependent diabetes mellitus--a collision between thrifty genes and an affluent society.* Ann Med, 1997. **29**(1): p. 37-53.
56. Koberling J., T.H., *Empirical risk figures for first-degree relatives of noinsulin dependent diabetics.* Vol. The Genetics of Diabetes Mellitus. 1982, London.: Academic Press. 201-209.

57. Meigs, J.B., L.A. Cupples, and P.W. Wilson, *Parental transmission of type 2 diabetes: the Framingham Offspring Study.* Diabetes, 2000. **49**(12): p. 2201-7.

58. King, H., R.E. Aubert, and W.H. Herman, *Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections.* Diabetes Care, 1998. **21**(9): p. 1414-31.

59. Chan, J.C., et al., *Diabetes in Asia: epidemiology, risk factors, and pathophysiology.* Jama, 2009. **301**(20): p. 2129-40.

60. Barroso, I., *Genetics of Type 2 diabetes.* Diabet Med, 2005. **22**(5): p. 517-35.

61. McCarthy, M.I. and P. Froguel, *Genetic approaches to the molecular understanding of type 2 diabetes.* Am J Physiol Endocrinol Metab, 2002. **283**(2): p. E217-25.

62. Gat-Yablonski, G., S. Shalitin, and M. Phillip, *Maturity onset diabetes of the young--review.* Pediatr Endocrinol Rev, 2006. 3 **Suppl 3**: p. 514-20.

63. Tiret, L., *Gene-environment interaction: a central concept in multifactorial diseases.* Proc Nutr Soc, 2002. **61**(4): p. 457-63.

64. Neel, J.V., *Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"?* Am J Hum Genet, 1962. **14**: p. 353-62.

65. Deeb, S.S., et al., *A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity.* Nat Genet, 1998. **20**(3): p. 284-7.

66. Ridderstrale, M. and L. Groop, *Genetic dissection of type 2 diabetes.* Mol Cell Endocrinol, 2009. **297**(1-2): p. 10-7.

67. Luan, J., et al., *Evidence for gene-nutrient interaction at the PPARgamma locus.* Diabetes, 2001. **50**(3): p. 686-9.

68. Palmer, N.D., et al., *Association of TCF7L2 gene polymorphisms with reduced acute insulin response in Hispanic Americans.* J Clin Endocrinol Metab, 2008. **93**(1): p. 304-9.

69. Pearson, E.R., et al., *Variation in TCF7L2 influences therapeutic response to sulfonylureas: a GoDARTs study.* Diabetes, 2007. **56**(8): p. 2178-82.

70. Malecki, M.T., et al., *Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus.* Nat Genet, 1999. **23**(3): p. 323-8.

71. Froguel, P., et al., *Close linkage of glucokinase locus on chromosome 7p to early-onset non-insulin-dependent diabetes mellitus.* Nature, 1992. **356**(6365): p. 162-4.

72. Zeggini, E., et al., *Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes.* Nat Genet, 2008. **40**(5): p. 638-45.

73. Frayling, T.M., *Genome-wide association studies provide new insights into type 2 diabetes aetiology.* Nat Rev Genet, 2007. **8**(9): p. 657-62.

74. *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2004. **27 Suppl 1**: p. S5-S10.

75. Nissim, R., et al., *[Eating disturbances in adolescent girls with type 1 diabetes mellitus].* Harefuah, 2002. **141**(10): p. 902-7, 929.

76. Luna, B. and M.N. Feinglos, *Drug-induced hyperglycemia.* Jama, 2001. **286**(16): p. 1945-8.

77. Garcia Branco, R., et al., *Glycemic control and insulin therapy in sepsis and critical illness.* J Pediatr (Rio J), 2007. **83**(5 Suppl): p. S128-36.

78. Capes, S.E., et al., *Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: a systematic overview.* Stroke, 2001. **32**(10): p. 2426-32.

79. *Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group.* Lancet, 1998. **352**(9131): p. 837-53.

80. Kilpatrick, E.D. and R.P. Robertson, *Differentiation between glucose-induced desensitization of insulin secretion and beta-cell exhaustion in the HIT-T15 cell line.* Diabetes, 1998. **47**(4): p. 606-11.

81. Bjorklund, A., A. Lansner, and V.E. Grill, *Glucose-induced [Ca2+]i abnormalities in human pancreatic islets: important role of overstimulation.* Diabetes, 2000. **49**(11): p. 1840-8.

82. Rustenbeck, I., *Desensitization of insulin secretion.* Biochem Pharmacol, 2002. **63**(11): p. 1921-35.

83. Poitout, V. and R.P. Robertson, *Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity.* Endocrinology, 2002. **143**(2): p. 339-42.

84. Chang-Chen, K.J., R. Mullur, and E. Bernal-Mizrachi, *Beta-cell failure as a complication of diabetes.* Rev Endocr Metab Disord, 2008. **9**(4): p. 329-43.

85. Gleason, C.E., et al., *Determinants of glucose toxicity and its reversibility in the pancreatic islet beta-cell line, HIT-T15.* Am J Physiol Endocrinol Metab, 2000. **279**(5): p. E997-1002.

86. *The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group.* N Engl J Med, 1993. **329**(14): p. 977-86.

87. Eastman, R.C., et al., *Clinical review 51: Implications of the Diabetes Control and Complications Trial.* J Clin Endocrinol Metab, 1993. **77**(5): p. 1105-7.

88. *The absence of a glycemic threshold for the development of long-term complications: the perspective of the Diabetes Control and Complications Trial.* Diabetes, 1996. **45**(10): p. 1289-98.

89. Gugliucci, A., *Glycation as the glucose link to diabetic complications.* J Am Osteopath Assoc, 2000. **100**(10): p. 621-34.

90. Wong, T.Y., et al., *Relation between fasting glucose and retinopathy for diagnosis of diabetes: three population-based cross-sectional studies.* Lancet, 2008. **371**(9614): p. 736-43.

91. Gabbay, K.H., *The sorbitol pathway and the complications of diabetes.* N Engl J Med, 1973. **288**(16): p. 831-6.

92. Lachin, J.M., et al., *Effect of glycemic exposure on the risk of microvascular complications in the diabetes control and complications trial--revisited.* Diabetes, 2008. **57**(4): p. 995-1001.

93. Aronson, D., *Hyperglycemia and the pathobiology of diabetic complications.* Adv Cardiol, 2008. **45**: p. 1-16.

94. Raptis, A.E. and G. Viberti, *Pathogenesis of diabetic nephropathy.* Exp Clin Endocrinol Diabetes, 2001. **109 Suppl 2**: p. S424-37.

95. *Effect of intensive therapy on the development and progression of diabetic nephropathy in the Diabetes Control and Complications Trial. The Diabetes Control and Complications (DCCT)*

Research Group. Kidney Int, 1995. **47**(6): p. 1703-20.

96. Holman, R.R., et al., *10-year follow-up of intensive glucose control in type 2 diabetes.* N Engl J Med, 2008. **359**(15): p. 1577-89.

97. Nathan, D.M., et al., *Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes.* N Engl J Med, 2005. **353**(25): p. 2643-53.

98. Ceriello, A., *Postprandial hyperglycemia and diabetes complications: is it time to treat?* Diabetes, 2005. **54**(1): p. 1-7.

99. Piga, R., et al., *Short-term high glucose exposure induces monocyte-endothelial cells adhesion and transmigration by increasing VCAM-1 and MCP-1 expression in human aortic endothelial cells.* Atherosclerosis, 2007. **193**(2): p. 328-34.

100. Quagliaro, L., et al., *Intermittent high glucose enhances ICAM-1, VCAM-1 and E-selectin expression in human umbilical vein endothelial cells in culture: the distinct role of protein kinase C and mitochondrial superoxide production.* Atherosclerosis, 2005. **183**(2): p. 259-67.

101. Huang, C.N., et al., *Hibiscus sabdariffa inhibits vascular smooth muscle cell proliferation and migration induced by high glucose--a mechanism involves connective tissue growth factor signals.* J Agric Food Chem, 2009. **57**(8): p. 3073-9.

102. Srivastava, A.K., *High glucose-induced activation of protein kinase signaling pathways in vascular smooth muscle cells: a potential role in the pathogenesis of vascular dysfunction in diabetes (review).* Int J Mol Med, 2002. **9**(1): p. 85-9.

103. Dasu, M.R., S. Devaraj, and I. Jialal, *High glucose induces IL-1beta expression in human monocytes: mechanistic insights.* Am J Physiol Endocrinol Metab, 2007. **293**(1): p. E337-46.

104. Fredrickson, D.S. and R.S. Lees, *A System for Phenotyping Hyperlipoproteinemia.* Circulation, 1965. **31**: p. 321-7.

105. Krauss, R.M. and P.W. Siri, *Dyslipidemia in type 2 diabetes.* Med Clin North Am, 2004. **88**(4): p. 897-909, x.

106. Kannel, W.B., *Lipids, diabetes, and coronary heart disease: insights from the Framingham Study.* Am Heart J, 1985. **110**(5): p. 1100-7.

107. *U.K. Prospective Diabetes Study 27. Plasma lipids and lipoproteins at diagnosis of NIDDM by age and sex.* Diabetes Care, 1997. **20**(11): p. 1683-7.

108. Paolisso, G., et al., *A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM.* Diabetologia, 1995. **38**(10): p. 1213-7.

109. Branstrom, R., et al., *Long-chain CoA esters activate human pancreatic beta-cell KATP channels: potential role in Type 2 diabetes.* Diabetologia, 2004. **47**(2): p. 277-83.

110. Karaskov, E., et al., *Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis.* Endocrinology, 2006. **147**(7): p. 3398-407.

111. Kharroubi, I., et al., *Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress.* Endocrinology, 2004. **145**(11): p. 5087-96.

112. Gu, K., C.C. Cowie, and M.I. Harris, *Diabetes and decline in heart disease mortality in US*

adults. Jama, 1999. **281**(14): p. 1291-7.

113. Almdal, T., et al., *The independent effect of type 2 diabetes mellitus on ischemic heart disease, stroke, and death: a population-based study of 13,000 men and women with 20 years of follow-up.* Arch Intern Med, 2004. **164**(13): p. 1422-6.

114. Gaede, P., et al., *Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes.* N Engl J Med, 2003. **348**(5): p. 383-93.

115. Haffner, S.M., et al., *Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction.* N Engl J Med, 1998. **339**(4): p. 229-34.

116. Turner, R.C., et al., *Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23).* Bmj, 1998. **316**(7134): p. 823-8.

117. Sacks, F.M., et al., *The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators.* N Engl J Med, 1996. **335**(14): p. 1001-9.

118. Koskinen, P., et al., *Coronary heart disease incidence in NIDDM patients in the Helsinki Heart Study.* Diabetes Care, 1992. **15**(7): p. 820-5.

119. Haffner, S.M., *Dyslipidemia management in adults with diabetes.* Diabetes Care, 2004. **27 Suppl 1**: p. S68-71.

120. Bonnet, F. and M.E. Cooper, *Potential influence of lipids in diabetic nephropathy: insights from experimental data and clinical studies.* Diabetes Metab, 2000. **26**(4): p. 254-64.

121. Jenkins, A.J., et al., *Lipoproteins in the DCCT/EDIC cohort: associations with diabetic nephropathy.* Kidney Int, 2003. **64**(3): p. 817-28.

122. Jenkins, A.J., et al., *Lipoproteins and diabetic microvascular complications.* Curr Pharm Des, 2004. **10**(27): p. 3395-418.

123. Nofer, J.R., M.F. Brodde, and B.E. Kehrel, *High-density lipoproteins, platelets and the pathogenesis of atherosclerosis.* Clin Exp Pharmacol Physiol, 2010. **37**(7): p. 726-35.

124. Orchard, T.J., et al., *Nephropathy in type 1 diabetes: a manifestation of insulin resistance and multiple genetic susceptibilities? Further evidence from the Pittsburgh Epidemiology of Diabetes Complication Study.* Kidney Int, 2002. **62**(3): p. 963-70.

125. Coonrod, B.A., et al., *Predictors of microalbuminuria in individuals with IDDM. Pittsburgh Epidemiology of Diabetes Complications Study.* Diabetes Care, 1993. **16**(10): p. 1376-83.

126. Misra, A., et al., *The role of lipids in the development of diabetic microvascular complications: implications for therapy.* Am J Cardiovasc Drugs, 2003. **3**(5): p. 325-38.

127. Jenkins, A.J., et al., *'Lipoproteins, glycoxidation and diabetic angiopathy'.* Diabetes Metab Res Rev, 2004. **20**(5): p. 349-68.

128. Brosius, F.C., 3rd, *New insights into the mechanisms of fibrosis and sclerosis in diabetic nephropathy.* Rev Endocr Metab Disord, 2008. **9**(4): p. 245-54.

129. *National High Blood Pressure Education Program Working Group report on hypertension in diabetes.* Hypertension, 1994. **23**(2): p. 145-58; discussion 159-60.

130. Gress, T.W., et al., *Hypertension and antihypertensive therapy as risk factors for type 2 diabetes mellitus. Atherosclerosis Risk in Communities Study.* N Engl J Med, 2000. **342**(13): p. 905-12.

131. Lago, R.M., P.P. Singh, and R.W. Nesto, *Diabetes and hypertension.* Nat Clin Pract Endocrinol Metab, 2007. **3**(10): p. 667.

132. Williams, B., *The Hypertension in Diabetes Study (HDS): a catalyst for change.* Diabet Med, 2008. **25 Suppl 2**: p. 13-9.

133. Sowers, J.R., M. Epstein, and E.D. Frohlich, *Diabetes, hypertension, and cardiovascular disease: an update.* Hypertension, 2001. **37**(4): p. 1053-9.

134. Curb, J.D., et al., *Effect of diuretic-based antihypertensive treatment on cardiovascular disease risk in older diabetic patients with isolated systolic hypertension. Systolic Hypertension in the Elderly Program Cooperative Research Group.* Jama, 1996. **276**(23): p. 1886-92.

135. Bostom, A.G., et al., *Chronic renal transplantation: a model for the hyperhomocysteinemia of renal insufficiency.* Atherosclerosis, 2001. **156**(1): p. 227-30.

136. Hansson, L., et al., *Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. HOT Study Group.* Lancet, 1998. **351**(9118): p. 1755-62.

137. Tuomilehto, J., et al., *Effects of calcium-channel blockade in older patients with diabetes and systolic hypertension. Systolic Hypertension in Europe Trial Investigators.* N Engl J Med, 1999. **340**(9): p. 677-84.

138. Bakris, G.L., et al., *Preserving renal function in adults with hypertension and diabetes: a consensus approach. National Kidney Foundation Hypertension and Diabetes Executive Committees Working Group.* Am J Kidney Dis, 2000. **36**(3): p. 646-61.

139. Tesfaye, S., et al., *Vascular risk factors and diabetic neuropathy.* N Engl J Med, 2005. **352**(4): p. 341-50.

140. Mann, J.F., et al., *Renal insufficiency as a predictor of cardiovascular outcomes and the impact of ramipril: the HOPE randomized trial.* Ann Intern Med, 2001. **134**(8): p. 629-36.

141. Schiffrin, E.L., M.L. Lipman, and J.F. Mann, *Chronic kidney disease: effects on the cardiovascular system.* Circulation, 2007. **116**(1): p. 85-97.

142. Solomon, S.D., et al., *Renal function and effectiveness of angiotensin-converting enzyme inhibitor therapy in patients with chronic stable coronary disease in the Prevention of Events with ACE inhibition (PEACE) trial.* Circulation, 2006. **114**(1): p. 26-31.

143. Lind, L., C. Berne, and H. Lithell, *Prevalence of insulin resistance in essential hypertension.* J Hypertens, 1995. **13**(12 Pt 1): p. 1457-62.

144. Jonk, A.M., et al., *Microvascular dysfunction in obesity: a potential mechanism in the pathogenesis of obesity-associated insulin resistance and hypertension.* Physiology (Bethesda), 2007. **22**: p. 252-60.

145. de Jongh, R.T., et al., *Impaired microvascular function in obesity: implications for obesity-associated microangiopathy, hypertension, and insulin resistance.* Circulation, 2004.

109(21): p. 2529-35.

146. Sowers, J.R., *Insulin resistance and hypertension.* Am J Physiol Heart Circ Physiol, 2004. **286**(5): p. H1597-602.

147. Klimstra, D., *Histopathology for Pathologists*. 2nd ed. Vol. Pancreas. 1997, Philadelphia: Lippincott-Raven Publishers. 613-647.

148. Longnecker, D., *Anderson's Pathology*. Vol. Pancreas. 1996, St. Louis.: CV Mosby Co. 1891-1916.

149. Ernest, E., *Pathology of the pancreas, gallbladder, extrahepatic Biliary Tract, and Ampullary region.* 2003, Lack: Oxford. 19-40.

150. Bloodworth, J., *Bloodworth's Endocrine Pathology*. Vol. The endocrine pancreas. 1996, Baltimore: Lippincott Williams & Wilkins 519-557.

151. Kloppel, G., Int'Veld, PA, Komminoth, P, *Functional Endocrine Pathology*. Vol. The endocrine pancreas. 1998, Cambridg: Black-well Science. 415-487.

152. Bardeesy, N. and R.A. DePinho, *Pancreatic cancer biology and genetics.* Nat Rev Cancer, 2002. **2**(12): p. 897-909.

153. Rahier, J., R.M. Goebbels, and J.C. Henquin, *Cellular composition of the human diabetic pancreas.* Diabetologia, 1983. **24**(5): p. 366-71.

154. Maclean, N. and R.F. Ogilvie, *Quantitative estimation of the pancreatic islet tissue in diabetic subjects.* Diabetes, 1955. **4**(5): p. 367-76.

155. Clark, A., et al., *Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes.* Diabetes Res, 1988. **9**(4): p. 151-9.

156. Clark, A., et al., *Islet changes induced by hyperglycemia in rats. Effect of insulin or chlorpropamide therapy.* Diabetes, 1982. **31**(4 Pt 1): p. 319-25.

157. Westermark, P., *Mast cells in the islets of Langerhans in insular amyloidosis.* Virchows Arch A Pathol Pathol Anat, 1971. **354**(1): p. 17-23.

158. Haataja, L., et al., *Islet amyloid in type 2 diabetes, and the toxic oligomer hypothesis.* Endocr Rev, 2008. **29**(3): p. 303-16.

159. Gillmore, J.D., P.N. Hawkins, and M.B. Pepys, *Amyloidosis: a review of recent diagnostic and therapeutic developments.* Br J Haematol, 1997. **99**(2): p. 245-56.

160. Cherny, I. and E. Gazit, *Amyloids: not only pathological agents but also ordered nanomaterials.* Angew Chem Int Ed Engl, 2008. **47**(22): p. 4062-9.

161. Hayden, M.R. and S.C. Tyagi, *"A" is for amylin and amyloid in type 2 diabetes mellitus.* JOP, 2001. **2**(4): p. 124-39.

162. Westermark, P., et al., *Islet amyloid in type 2 human diabetes mellitus and adult diabetic cats contains a novel putative polypeptide hormone.* Am J Pathol, 1987. **127**(3): p. 414-7.

163. Hoppener, J.W. and C.J. Lips, *Role of islet amyloid in type 2 diabetes mellitus.* Int J Biochem Cell Biol, 2006. **38**(5-6): p. 726-36.

164. Cooper, G.J., et al., *Amylin and the amylin gene: structure, function and relationship to islet amyloid and to diabetes mellitus.* Biochim Biophys Acta, 1989. **1014**(3): p. 247-58.

165. Kauppila, L.I., P. Hekali, and A. Penttila, *Postmortem pancreatic angiography in 45 subjects*

with non-insulin-dependent diabetes mellitus and 51 controls. Pancreas, 1998. **16**(1): p. 60-5.

166. Lazarus, S.S. and B.W. Volk, *Pancreas in maturity-onset diabetes. Pathogenetic considerations.* Arch Pathol, 1961. **71**: p. 44-59.

167. Howard, C.F., Jr., *Longitudinal studies on the development of diabetes in individual Macaca nigra.* Diabetologia, 1986. **29**(5): p. 301-6.

168. Maloy, A.L., D.S. Longnecker, and E.R. Greenberg, *The relation of islet amyloid to the clinical type of diabetes.* Hum Pathol, 1981. **12**(10): p. 917-22.

169. Lupi, R. and S. Del Prato, *Beta-cell apoptosis in type 2 diabetes: quantitative and functional consequences.* Diabetes Metab, 2008. **34 Suppl 2**: p. S56-64.

170. Sakagashira, S., et al., *Missense mutation of amylin gene (S20G) in Japanese NIDDM patients.* Diabetes, 1996. **45**(9): p. 1279-81.

171. Sakagashira, S., et al., *S20G mutant amylin exhibits increased in vitro amyloidogenicity and increased intracellular cytotoxicity compared to wild-type amylin.* Am J Pathol, 2000. **157**(6): p. 2101-9.

172. Sempoux, C., et al., *Human type 2 diabetes: morphological evidence for abnormal beta-cell function.* Diabetes, 2001. **50 Suppl 1**: p. S172-7.

173. Ancsin, J.B., *Amyloidogenesis: historical and modern observations point to heparan sulfate proteoglycans as a major culprit.* Amyloid, 2003. **10**(2): p. 67-79.

174. Mahley, R.W. and S.C. Rall, Jr., *Apolipoprotein E: far more than a lipid transport protein.* Annu Rev Genomics Hum Genet, 2000. **1**: p. 507-37.

175. Wu, C.W., et al., *Brain region-dependent increases in beta-amyloid and apolipoprotein E levels in hypercholesterolemic rabbits.* J Neural Transm, 2003. **110**(6): p. 641-9.

176. Sjogren, M., et al., *Cholesterol and Alzheimer's disease--is there a relation?* Mech Ageing Dev, 2006. **127**(2): p. 138-47.

177. Peila, R., B.L. Rodriguez, and L.J. Launer, *Type 2 diabetes, APOE gene, and the risk for dementia and related pathologies: The Honolulu-Asia Aging Study.* Diabetes, 2002. **51**(4): p. 1256-62.

178. Qiu, W.Q. and M.F. Folstein, *Insulin, insulin-degrading enzyme and amyloid-beta peptide in Alzheimer's disease: review and hypothesis.* Neurobiol Aging, 2006. **27**(2): p. 190-8.

179. Duckworth, W.C. and A.E. Kitabchi, *Insulin and glucagon degradation by the same enzyme.* Diabetes, 1974. **23**(6): p. 536-43.

180. Bennett, R.G., W.C. Duckworth, and F.G. Hamel, *Degradation of amylin by insulin-degrading enzyme.* J Biol Chem, 2000. **275**(47): p. 36621-5.

181. Muller, D., et al., *Atrial natriuretic peptide (ANP) is a high-affinity substrate for rat insulin-degrading enzyme.* Eur J Biochem, 1991. **202**(2): p. 285-92.

182. Hamel, F.G., et al., *Identification of the cleavage sites of transforming growth factor alpha by insulin-degrading enzymes.* Biochim Biophys Acta, 1997. **1338**(2): p. 207-14.

183. Roth, R.A., et al., *Degradation of insulin-like growth factors I and II by a human insulin degrading enzyme.* Endocr Res, 1984. **10**(2): p. 101-12.

184. Miners, J.S., et al., *Abeta-degrading enzymes in Alzheimer's disease.* Brain Pathol, 2008. **18**(2):

p. 240-52.

185. Qiu, W.Q., et al., *Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation.* J Biol Chem, 1998. **273**(49): p. 32730-8.

186. McCarron, M.O., D. Delong, and M.J. Alberts, *APOE genotype as a risk factor for ischemic cerebrovascular disease: a meta-analysis.* Neurology, 1999. **53**(6): p. 1308-11.

187. Nickenig, G., et al., *Upregulation of vascular angiotensin II receptor gene expression by low-density lipoprotein in vascular smooth muscle cells.* Circulation, 1997. **95**(2): p. 473-8.

188. Zeleny, M., et al., *Distinct apolipoprotein E isoform preference for inhibition of smooth muscle cell migration and proliferation.* Biochemistry, 2002. **41**(39): p. 11820-3.

189. Slooter, A.J., et al., *Apolipoprotein E epsilon4 and the risk of dementia with stroke. A population-based investigation.* Jama, 1997. **277**(10): p. 818-21.

190. Nicoll, J.A., G.W. Roberts, and D.I. Graham, *Amyloid beta-protein, APOE genotype and head injury.* Ann N Y Acad Sci, 1996. **777**: p. 271-5.

191. Mahley, R.W. and Y. Huang, *Apolipoprotein E: from atherosclerosis to Alzheimer's disease and beyond.* Curr Opin Lipidol, 1999. **10**(3): p. 207-17.

192. Wilson, P.W., *Diabetes mellitus and coronary heart disease.* Am J Kidney Dis, 1998. **32**(5 Suppl 3): p. S89-100.

193. Baron, A.D., *Insulin resistance and vascular function.* J Diabetes Complications, 2002. **16**(1): p. 92-102.

194. Abbas, A., P.J. Grant, and M.T. Kearney, *Role of IGF-1 in glucose regulation and cardiovascular disease.* Expert Rev Cardiovasc Ther, 2008. **6**(8): p. 1135-49.

195. Tian, H., et al., *Correlative factors of insulin resistance in essential hypertension.* Hypertens Res, 2000. **23**(3): p. 265-70.

196. Gans, R.O. and A.J. Donker, *Insulin and blood pressure regulation.* J Intern Med Suppl, 1991. **735**: p. 49-64.

197. Chan, J.C., et al., *Atrial natriuretic peptide and urinary dopamine output in non-insulin-dependent diabetes mellitus.* Clin Sci (Lond), 1992. **83**(2): p. 247-53.

198. Sagnella, G.A., et al., *Raised circulating levels of atrial natriuretic peptides in essential hypertension.* Lancet, 1986. **1**(8474): p. 179-81.

199. Chan, J.C., et al., *Atrial natriuretic peptide and renin-angiotensin-aldosterone system in non-insulin-dependent diabetes mellitus.* J Hum Hypertens, 1994. **8**(6): p. 451-6.

200. Azizov, V.A. and S.R. Muradova, *Atrial natriuretic peptide and cardiovascular system.* Anadolu Kardiyol Derg, 2001. **1**(4): p. 297-300.

201. Cooper, M.E., et al., *Amylin stimulates plasma renin concentration in humans.* Hypertension, 1995. **26**(3): p. 460-4.

202. Ding, Y., et al., *Effects of simulated hyperglycemia, insulin, and glucagon on endothelial nitric oxide synthase expression.* Am J Physiol Endocrinol Metab, 2000. **279**(1): p. E11-7.

203. Merlet-Benichou, C., et al., *Nephron number: variability is the rule. Causes and consequences.* Lab Invest, 1999. **79**(5): p. 515-27.

204. Mills., S.E., *Histology for pathologists.* 3th ed. 2007, Philadephia: Lippincott Williams &

Wilkins. 862-907.

205. Madsen KM, T.C., *Brenner and Rector's The kidney.* 7th ed. Vol. Anatomy of the kidney. 2004, Philadelphia: WB Saunders. 3-72.

206. Osterby, R., *Structural changes in the diabetic kidney.* Clin Endocrinol Metab, 1986. **15**(4): p. 733-51.

207. J. Charles Jennette, J.L.O., Melvin M. Schwartz, Fred G. Silva., *Heptinstall's pathology of the kidney.* 6th ed ed. 2006, Philadelphia: Lippincott Williams & Wilkins 803-852.

208. Mac-Moune Lai, F., et al., *Isolate diffuse thickening of glomerular capillary basement membrane: a renal lesion in prediabetes?* Mod Pathol, 2004. **17**(12): p. 1506-12.

209. Mertz, D.P., *[Diabetic nephropathy (author's transl)].* MMW Munch Med Wochenschr, 1976. **118**(5): p. 123-8.

210. Falk, R.J., et al., *Polyantigenic expansion of basement membrane constituents in diabetic nephropathy.* Diabetes, 1983. **32 Suppl 2**: p. 34-9.

211. Bloodworth, J.M., Jr., *A re-evaluation of diabetic glomerulosclerosis 50 years after the discovery of insulin.* Hum Pathol, 1978. **9**(4): p. 439-53.

212. Paueksakon, P., et al., *Microangiopathic injury and augmented PAI-1 in human diabetic nephropathy.* Kidney Int, 2002. **61**(6): p. 2142-8.

213. Hall, G.F., *The significance of atheroma of the renal arteries in Kimmelstiel-Wilson's syndrome.* J Pathol Bacteriol, 1952. **64**(1): p. 103-20.

214. Barrie, H.J., C.L. Aszkanazy, and G.W. Smith, *More glomerular changes in diabetics.* Can Med Assoc J, 1952. **66**(5): p. 428-31.

215. Taft, J.L., et al., *Clinical and histological correlations of decline in renal function in diabetic patients with proteinuria.* Diabetes, 1994. **43**(8): p. 1046-51.

216. *Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy: the Epidemiology of Diabetes Interventions and Complications (EDIC) study.* JAMA, 2003. **290**(16): p. 2159-67.

217. Fioretto, P., et al., *Renal protection in diabetes: role of glycemic control.* J Am Soc Nephrol, 2006. **17**(4 Suppl 2): p. S86-9.

218. Heilig, C.W., F.C. Brosius, 3rd, and C. Cunningham, *Role for GLUT1 in diabetic glomerulosclerosis.* Expert Rev Mol Med, 2006. **8**(4): p. 1-18.

219. Heilig, C.W., et al., *D-glucose stimulates mesangial cell GLUT1 expression and basal and IGF-I-sensitive glucose uptake in rat mesangial cells: implications for diabetic nephropathy.* Diabetes, 1997. **46**(6): p. 1030-9.

220. Pfafflin, A., et al., *Increased glucose uptake and metabolism in mesangial cells overexpressing glucose transporter 1 increases interleukin-6 and vascular endothelial growth factor production: role of AP-1 and HIF-1alpha.* Cell Physiol Biochem, 2006. **18**(4-5): p. 199-210.

221. Rajah, T.T., A.L. Olson, and P. Grammas, *Differential glucose uptake in retina- and brain-derived endothelial cells.* Microvasc Res, 2001. **62**(3): p. 236-42.

222. Akis, N. and M.P. Madaio, *Isolation, culture, and characterization of endothelial cells from mouse glomeruli.* Kidney Int, 2004. **65**(6): p. 2223-7.

223. Bell, D.S., *Hypertension and diabetes: a toxic combination.* Endocr Pract, 2008. **14**(8): p. 1031-9.

224. Monhart, V., *[Diabetes mellitus, hypertension and kidney].* Vnitr Lek, 2008. **54**(5): p. 499-504, 507.

225. Jandeleit-Dahm, K. and M.E. Cooper, *Hypertension and diabetes: role of the renin-angiotensin system.* Endocrinol Metab Clin North Am, 2006. **35**(3): p. 469-90, vii.

226. Ravikumar, B., et al., *Raised intracellular glucose concentrations reduce aggregation and cell death caused by mutant huntingtin exon 1 by decreasing mTOR phosphorylation and inducing autophagy.* Hum Mol Genet, 2003. **12**(9): p. 985-94.

227. Totary-Jain, H., et al., *Calreticulin destabilizes glucose transporter-1 mRNA in vascular endothelial and smooth muscle cells under high-glucose conditions.* Circ Res, 2005. **97**(10): p. 1001-8.

228. Dronavalli, S., I. Duka, and G.L. Bakris, *The pathogenesis of diabetic nephropathy.* Nat Clin Pract Endocrinol Metab, 2008. **4**(8): p. 444-52.

229. Boizel, R., et al., *ApoE polymorphism and albuminuria in diabetes mellitus: a role for LDL in the development of nephropathy in NIDDM?* Nephrol Dial Transplant, 1998. **13**(1): p. 72-5.

230. Joss, N., et al., *Influence of apolipoprotein E genotype on progression of diabetic nephropathy.* Nephron Exp Nephrol, 2005. **101**(4): p. e127-33.

231. Liberopoulos, E., K. Siamopoulos, and M. Elisaf, *Apolipoprotein E and renal disease.* Am J Kidney Dis, 2004. **43**(2): p. 223-33.

232. Eto, M., et al., *Apolipoprotein E genetic polymorphism, remnant lipoproteins, and nephropathy in type 2 diabetic patients.* Am J Kidney Dis, 2002. **40**(2): p. 243-51.

233. Ha, S.K., et al., *Association between apolipoprotein E polymorphism and macroalbuminuria in patients with non-insulin dependent diabetes mellitus.* Nephrol Dial Transplant, 1999. **14**(9): p. 2144-9.

234. Eto, M., et al., *Increased frequency of apolipoprotein epsilon 2 allele in non-insulin dependent diabetic (NIDDM) patients with nephropathy.* Clin Genet, 1995. **48**(6): p. 288-92.

235. Saito, M., M. Eto, and I. Makino, *Triglyceride-rich lipoproteins from apolipoprotein E3/2 subjects with hypertriglyceridemia enhance cholesteryl ester synthesis in human macrophages.* Atherosclerosis, 1997. **129**(1): p. 73-7.

236. Luk, A.O., et al., *Metabolic syndrome predicts new onset of chronic kidney disease in 5,829 patients with type 2 diabetes: a 5-year prospective analysis of the Hong Kong Diabetes Registry.* Diabetes Care, 2008. **31**(12): p. 2357-61.

237. Hsu, C.C., et al., *Apolipoprotein E and progression of chronic kidney disease.* JAMA, 2005. **293**(23): p. 2892-9.

238. Lehtinen, S., et al., *Apolipoprotein E gene polymorphism, hypercholesterolemia and glomerular filtration rate in type 2 diabetic subjects: a 9-year follow-up study.* J Biomed Sci, 2003. **10**(2): p. 260-5.

239. Levey, A.S., et al., *A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group.*

Ann Intern Med, 1999. **130**(6): p. 461-70.

240. LeVine, H., 3rd, *Quantification of beta-sheet amyloid fibril structures with thioflavin T.* Methods Enzymol, 1999. **309**: p. 274-84.

241. LeVine, H., 3rd, *Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution.* Protein Sci, 1993. **2**(3): p. 404-10.

242. Lehmann, U. and H. Kreipe, *Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies.* Methods, 2001. **25**(4): p. 409-18.

243. Shi, S.R., et al., *DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: heating under the influence of pH.* J Histochem Cytochem, 2002. **50**(8): p. 1005-11.

244. Day, D.J., et al., *Detection of steroid 21-hydroxylase alleles using gene-specific PCR and a multiplexed ligation detection reaction.* Genomics, 1995. **29**(1): p. 152-62.

245. Eggerding, F.A., et al., *Fluorescence-based oligonucleotide ligation assay for analysis of cystic fibrosis transmembrane conductance regulator gene mutations.* Hum Mutat, 1995. **5**(2): p. 153-65.

246. Favis, R. and F. Barany, *Mutation detection in K-ras, BRCA1, BRCA2, and p53 using PCR/LDR and a universal DNA microarray.* Ann N Y Acad Sci, 2000. **906**: p. 39-43.

247. Khanna, M., et al., *Multiplex PCR/LDR for detection of K-ras mutations in primary colon tumors.* Oncogene, 1999. **18**(1): p. 27-38.

248. Zhao, H.L., et al., *Clinicopathologic characteristics of nodular glomerulosclerosis in Chinese patients with type 2 diabetes.* Am J Kidney Dis, 2004. **44**(6): p. 1039-49.

249. Ng, M.C., et al., *Association of lipoprotein lipase S447X, apolipoprotein E exon 4, and apoC3 -455T>C polymorphisms on the susceptibility to diabetic nephropathy.* Clin Genet, 2006. **70**(1): p. 20-8.

250. *Reference SNP (refSNP) cluster Report: rs710218.* 2009, The National Center for Biotechnology Information

251. *Reference SNP (refSNP) cluster Report: rs6583813.* 2009, The National Center for Biotechnology Information

252. Bennet, A.M., et al., *Association of apolipoprotein E genotypes with lipid levels and coronary risk.* Jama, 2007. **298**(11): p. 1300-11.

253. Ashavaid, T.F., S.P. Todur, and K.G. Nair, *Apolipoprotein E polymorphism and coronary heart disease.* J Assoc Physicians India, 2003. **51**: p. 784-8.

254. Richard, F. and P. Amouyel, *Genetic susceptibility factors for Alzheimer's disease.* Eur J Pharmacol, 2001. **412**(1): p. 1-12.

255. Cui, D., et al., *An immunohistochemical study of amyloid P component, apolipoprotein E and ubiquitin in human and murine amyloidoses.* Pathol Int, 1998. **48**(5): p. 362-7.

256. Kitabchi, A.E. and F.B. Stentz, *Degradation of insulin and proinsulin by various organ homogenates of rat.* Diabetes, 1972. **21**(11): p. 1091-101.

257. Brocco, E., et al., *Renal structure and function in non-insulin dependent diabetic patients with microalbuminuria.* Kidney Int Suppl, 1997. **63**: p. S40-4.

258. Hatters, D.M., C.A. Peters-Libeu, and K.H. Weisgraber, *Apolipoprotein E structure: insights into function.* Trends Biochem Sci, 2006. **31**(8): p. 445-54.

259. Weisgraber, K.H., T.L. Innerarity, and R.W. Mahley, *Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site.* J Biol Chem, 1982. **257**(5): p. 2518-21.

260. Dong, L.M. and K.H. Weisgraber, *Human apolipoprotein E4 domain interaction. Arginine 61 and glutamic acid 255 interact to direct the preference for very low density lipoproteins.* J Biol Chem, 1996. **271**(32): p. 19053-7.

261. Chen, G., et al., *A protective role for kidney apolipoprotein E. Regulation of mesangial cell proliferation and matrix expansion.* J Biol Chem, 2001. **276**(52): p. 49142-7.

262. Bruneval, P., et al., *Mesangial expansion associated with glomerular endothelial cell activation and macrophage recruitment is developing in hyperlipidaemic apoE null mice.* Nephrol Dial Transplant, 2002. **17**(12): p. 2099-107.

263. Tamaoka, A., et al., *Apolipoprotein E allele-dependent antioxidant activity in brains with Alzheimer's disease.* Neurology, 2000. **54**(12): p. 2319-21.

264. Tenger, C. and X. Zhou, *Apolipoprotein E modulates immune activation by acting on the antigen-presenting cell.* Immunology, 2003. **109**(3): p. 392-7.

265. Ilveskoski, E., et al., *Age-dependent association of apolipoprotein E genotype with coronary and aortic atherosclerosis in middle-aged men: an autopsy study.* Circulation, 1999. **100**(6): p. 608-13.

266. Baum, L., et al., *Associations of apolipoprotein E exon 4 and lipoprotein lipase S447X polymorphisms with acute ischemic stroke and myocardial infarction.* Clin Chem Lab Med, 2006. **44**(3): p. 274-81.

267. Mayeux, R., et al., *Utility of the apolipoprotein E genotype in the diagnosis of Alzheimer's disease. Alzheimer's Disease Centers Consortium on Apolipoprotein E and Alzheimer's Disease.* N Engl J Med, 1998. **338**(8): p. 506-11.

268. Nishida, Y., H. Oda, and N. Yorioka, *Effect of lipoproteins on mesangial cell proliferation.* Kidney Int Suppl, 1999. **71**: p. S51-3.

269. Saraswathi, V. and A.H. Hasty, *The role of lipolysis in mediating the proinflammatory effects of very low density lipoproteins in mouse peritoneal macrophages.* J Lipid Res, 2006. **47**(7): p. 1406-15.

270. Cooper, M.E., *Pathogenesis, prevention, and treatment of diabetic nephropathy.* Lancet, 1998. **352**(9123): p. 213-9.

271. Araki, S., et al., *APOE polymorphism and the progression of diabetic nephropathy in Japanese subjects with type 2 diabetes: results of a prospective observational follow-up study.* Diabetes Care, 2003. **26**(8): p. 2416-20.

272. Hsieh, M.C., et al., *Higher frequency of apolipoprotein E2 allele in type 2 diabetic patients with nephropathy in Taiwan.* J Nephrol, 2002. **15**(4): p. 368-73.

273. Leiva, E., et al., *Relationship between Apolipoprotein E polymorphism and nephropathy in type-2 diabetic patients.* Diabetes Res Clin Pract, 2007. **78**(2): p. 196-201.

274. Roussos, L., et al., *Apolipoprotein E polymorphism in 385 patients on renal replacement therapy in Sweden.* Scand J Urol Nephrol, 2004. **38**(6): p. 504-10.

275. Lorenzo, A. and B.A. Yankner, *Amyloid fibril toxicity in Alzheimer's disease and diabetes.* Ann N Y Acad Sci, 1996. 777: p. 89-95.

276. Lin, C.Y., et al., *Toxic human islet amyloid polypeptide (h-IAPP) oligomers are intracellular, and vaccination to induce anti-toxic oligomer antibodies does not prevent h-IAPP-induced beta-cell apoptosis in h-IAPP transgenic mice.* Diabetes, 2007. **56**(5): p. 1324-32.

277. Takahashi, R.H., et al., *Oligomerization of Alzheimer's beta-amyloid within processes and synapses of cultured neurons and brain.* J Neurosci, 2004. **24**(14): p. 3592-9.

278. Botto, M., et al., *Amyloid deposition is delayed in mice with targeted deletion of the serum amyloid P component gene.* Nat Med, 1997. **3**(8): p. 855-9.

279. Pepys, M.B., et al., *Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis.* Nature, 2002. **417**(6886): p. 254-9.

280. Tennent, G.A., L.B. Lovat, and M.B. Pepys, *Serum amyloid P component prevents proteolysis of the amyloid fibrils of Alzheimer disease and systemic amyloidosis.* Proc Natl Acad Sci U S A, 1995. **92**(10): p. 4299-303.

281. Lei, P., et al., *Prevention and promotion effects of apolipoprotein E4 on amylin aggregation.* Biochem Biophys Res Commun, 2008. **368**(2): p. 414-8.

282. Chen, L., et al., *Apolipoprotein E genotype and its pathological correlation in Chinese Alzheimer's disease with late onset.* Hum Pathol, 1999. **30**(10): p. 1172-7.

283. Dergunov, A.D., *Role of ApoE in conformation-prone diseases and atherosclerosis.* Biochemistry (Mosc), 2006. **71**(7): p. 707-12.

284. Yip, A.G., et al., *APOE, vascular pathology, and the AD brain.* Neurology, 2005. **65**(2): p. 259-65.

285. Westermark, G.T., et al., *Widespread amyloid deposition in transplanted human pancreatic islets.* N Engl J Med, 2008. **359**(9): p. 977-9.

286. Bennett, R.G., F.G. Hamel, and W.C. Duckworth, *Identification and isolation of a cytosolic proteolytic complex containing insulin degrading enzyme and the multicatalytic proteinase.* Biochem Biophys Res Commun, 1994. **202**(2): p. 1047-53.

287. Authier, F., B.I. Posner, and J.J. Bergeron, *Insulin-degrading enzyme.* Clin Invest Med, 1996. **19**(3): p. 149-60.

288. Morelli, L., et al., *Insulin-degrading enzyme in brain microvessels: proteolysis of amyloid {beta} vasculotropic variants and reduced activity in cerebral amyloid angiopathy.* J Biol Chem, 2004. **279**(53): p. 56004-13.

289. Gao, W., et al., *Insulin degrading enzyme is expressed in the human cerebrovascular endothelium and in cultured human cerebrovascular endothelial cells.* Neurosci Lett, 2004. **371**(1): p. 6-11.

290. Fakhrai-Rad, H., et al., *Insulin-degrading enzyme identified as a candidate diabetes susceptibility gene in GK rats.* Hum Mol Genet, 2000. **9**(14): p. 2149-58.

291. Groves, C.J., et al., *Association and haplotype analysis of the insulin-degrading enzyme (IDE)*

gene, a strong positional and biological candidate for type 2 diabetes susceptibility. Diabetes, 2003. **52**(5): p. 1300-5.

292. Shii, K., et al., *Purification and characterization of insulin-degrading enzyme from human erythrocytes.* Diabetes, 1986. **35**(6): p. 675-83.

293. Kuo, W.L., et al., *Inducible expression and cellular localization of insulin-degrading enzyme in a stably transfected cell line.* J Biol Chem, 1994. **269**(36): p. 22599-606.

294. Shii, K. and R.A. Roth, *Inhibition of insulin degradation by hepatoma cells after microinjection of monoclonal antibodies to a specific cytosolic protease.* Proc Natl Acad Sci U S A, 1986. **83**(12): p. 4147-51.

295. Farris, W., et al., *Partial loss-of-function mutations in insulin-degrading enzyme that induce diabetes also impair degradation of amyloid beta-protein.* Am J Pathol, 2004. **164**(4): p. 1425-34.

296. Marks, J.B. and P. Raskin, *Cardiovascular risk in diabetes: a brief review.* J Diabetes Complications, 2000. **14**(2): p. 108-15.

297. Air, E.L. and B.M. Kissela, *Diabetes, the metabolic syndrome, and ischemic stroke: epidemiology and possible mechanisms.* Diabetes Care, 2007. **30**(12): p. 3131-40.

298. Bennett, R.G., F.G. Hamel, and W.C. Duckworth, *An insulin-degrading enzyme inhibitor decreases amylin degradation, increases amylin-induced cytotoxicity, and increases amyloid formation in insulinoma cell cultures.* Diabetes, 2003. **52**(9): p. 2315-20.

299. Kailasam, M.T., et al., *Circulating amylin in human essential hypertension: heritability and early increase in individuals at genetic risk.* J Hypertens, 2000. **18**(11): p. 1611-20.

300. Wookey, P.J., et al., *Amylin in the periphery.* ScientificWorldJournal, 2003. **3**: p. 163-75.

301. Wookey, P.J., Z. Cao, and M.E. Cooper, *Interaction of the renal amylin and renin-angiotensin systems in animal models of diabetes and hypertension.* Miner Electrolyte Metab, 1998. **24**(6): p. 389-99.

302. Ren, J., W.K. Samson, and J.R. Sowers, *Insulin-like growth factor I as a cardiac hormone: physiological and pathophysiological implications in heart disease.* J Mol Cell Cardiol, 1999. **31**(11): p. 2049-61.

303. Grzeszczak, W., et al., *Role of GLUT1 gene in susceptibility to diabetic nephropathy in type 2 diabetes.* Kidney Int, 2001. **59**(2): p. 631-6.

304. Hodgkinson, A.D., B.A. Millward, and A.G. Demaine, *Polymorphisms of the glucose transporter (GLUT1) gene are associated with diabetic nephropathy.* Kidney Int, 2001. **59**(3): p. 985-9.

305. Tarnow, L., et al., *Diabetic microvascular complications are not associated with two polymorphisms in the GLUT-1 and PC-1 genes regulating glucose metabolism in Caucasian type 1 diabetic patients.* Nephrol Dial Transplant, 2001. **16**(8): p. 1653-6.

306. Hodgkinson, A.D., et al., *A novel polymorphism in the 5' flanking region of the glucose transporter (GLUT1) gene is strongly associated with diabetic nephropathy in patients with Type 1 diabetes mellitus.* J Diabetes Complications, 2005. **19**(2): p. 65-9.

307. Ebeling, P., H.A. Koistinen, and V.A. Koivisto, *Insulin-independent glucose transport*

regulates insulin sensitivity. FEBS Lett, 1998. **436**(3): p. 301-3.

308. Granier, C., et al., *Gene and protein markers of diabetic nephropathy.* Nephrol Dial Transplant, 2008. **23**(3): p. 792-9.

309. Gutierrez, C., et al., *GLUT1 gene polymorphism in non-insulin-dependent diabetes mellitus: genetic susceptibility relationship with cardiovascular risk factors and microangiopathic complications in a Mediterranean population.* Diabetes Res Clin Pract, 1998. **41**(2): p. 113-20.

310. Marshall, B.A., et al., *Germline manipulation of glucose homeostasis via alteration of glucose transporter levels in skeletal muscle.* J Biol Chem, 1993. **268**(25): p. 18442-5.

311. Marshall, B.A. and M.M. Mueckler, *Differential effects of GLUT-1 or GLUT-4 overexpression on insulin responsiveness in transgenic mice.* Am J Physiol, 1994. **267**(5 Pt 1): p. E738-44.

312. Sechi, L.A., A. Melis, and R. Tedde, *Insulin hypersecretion: a distinctive feature between essential and secondary hypertension.* Metabolism, 1992. **41**(11): p. 1261-6.

313. Bussolati, B., et al., *Statins prevent oxidized LDL-induced injury of glomerular podocytes by activating the phosphatidylinositol 3-kinase/AKT-signaling pathway.* J Am Soc Nephrol, 2005. **16**(7): p. 1936-47.

314. Veiraiah, A., *Hyperglycemia, lipoprotein glycation, and vascular disease.* Angiology, 2005. **56**(4): p. 431-8.

315. Landefeld, C.S., et al., *Diagnostic yield of the autopsy in a university hospital and a community hospital.* N Engl J Med, 1988. **318**(19): p. 1249-54.

316. Kleiner, D.E., M.R. Emmert-Buck, and L.A. Liotta, *Necropsy as a research method in the age of molecular pathology.* Lancet, 1995. **346**(8980): p. 945-8.

317. Roberts, W.C., *The autopsy: its decline and a suggestion for its revival.* N Engl J Med, 1978. **299**(7): p. 332-8.