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COMMON GENETIC VARIATION IN NEPHRIN (*NPHS1*) AND ITS ASSOCIATIONS WITH RENAL AND TYPE 2 DIABETES MELLITUS-RELATED TRAITS

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NATIONAL UNIVERSITY OF SINGAPORE

2012

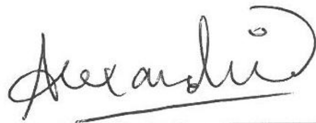
For *MUM* and *Aaron*

Love, Belle

DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

Nephrin (NPHS1) is a key structural component of the slit diaphragm (SD) and common genetic variation of *NPHS1* may influence SD function in diabetic nephropathy (DN). More recently, NPHS1 has also been reported in pancreatic β-cells and was involved in insulin secretion. Thus, common genetic variation of *NPHS1* may be associated with type 2 diabetes mellitus (T2DM) and its related traits. However, there are currently few studies investigating these potential roles of *NPHS1*. Therefore, this study investigated the association of *NPHS1*with both renal and T2DM-related traits. Six *NPHS1* SNPs were genotyped in both the Singapore Diabetes Cohort Study and 1998 Singapore National Health Survey subjects. There was significant evidence for interaction of *NPHS1* haplotypes with age on estimated glomerular filtration rate (eGFR) in T2DM patients. Specifically, with reference to the common haplotype, carriers of T/G/G/C/T/A and C/A/A/T/T/A had higher eGFR values among younger patients but had lower eGFR values among older patients. In contrast, carriers of T/G/A/T/T/G had lower eGFR values among younger patients with reference to the common haplotype. *NPHS1* was generally not associated with any of the T2DM-related traits investigated. However, there was borderline association of waist-to-hip ratio (WHR) with SNPs rs437168 and rs17777002 in the Chinese and Asian Indian populations respectively. In view of the studies implicating NPHS1 in β-cell function, this association with WHR is unexpected and its biological underpinning is less understood. In conclusion, our study has uncovered first evidence that *NPHS1* may be potentially involved in the modulation of eGFR over time in patients with T2DM. This may have significant implication in our understanding of DN and its treatment.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACTN4	Alpha-actinin 4
ACR	Albumin-to-creatinine ratio
BMI	Body mass index
CD2AP	CD2-associated protein
CG	Cockcroft-Gault
CKD-EPI	Chronic kidney disease epidemiology collaboration
CNF	Congenital nephrotic syndrome of the Finnish type
CysC	Cystatin C
DBP	Diastolic blood pressure
DM	Diabetes mellitus
DN	Diabetic nephropathy
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbant assay
ESRD	End-stage renal disease
FnIII	Fibronectin type III-like
FSGS	Focal segmental glomerulosclerosis
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
GSIR	Glucose-stimulated insulin release
HbA_{1C}	Glycated haemoglobin A_{1c}

HDL	High density lipoprotein
HOMA	Homeostatic model assessment
HWE	Hardy-Weinburg equilibrium
IDF	International Diabetes Federation
IFG	Impaired fasting glycemia
Ig	Immunoglobulin
IGT	Impaired glucose tolerance
IR	Insulin resistance
LD	Linkage disequilibrium
LDL	Low density lipoprotein
LnACR	Natural logarithmic of ACR
MCNS	Minimal change nephrotic syndrome
MDRD	Modification of diet in renal disease
MIN6	Mouse insulinoma 6
NHS98	Singapore National Health Survey 1998
NEPH1	Nephrin-related protein 1
NPHS1	Nephrin
NPHS2	Podocin
OGTT	Oral glucose tolerance test
SBP	Systolic blood pressure
SD	Slit diaphragm
SDCS	Singapore Diabetes Cohort Study

SNP	Single nucleotide polymorphism
SRNS	Steroid resistant nephrotic syndrome
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
UAE	Urinary albumin excretion
UTR	Untranslated region
WHO	World Health Organisation
WHR	Waist-to-hip ratio

CHAPTER ONE: INTRODUCTION

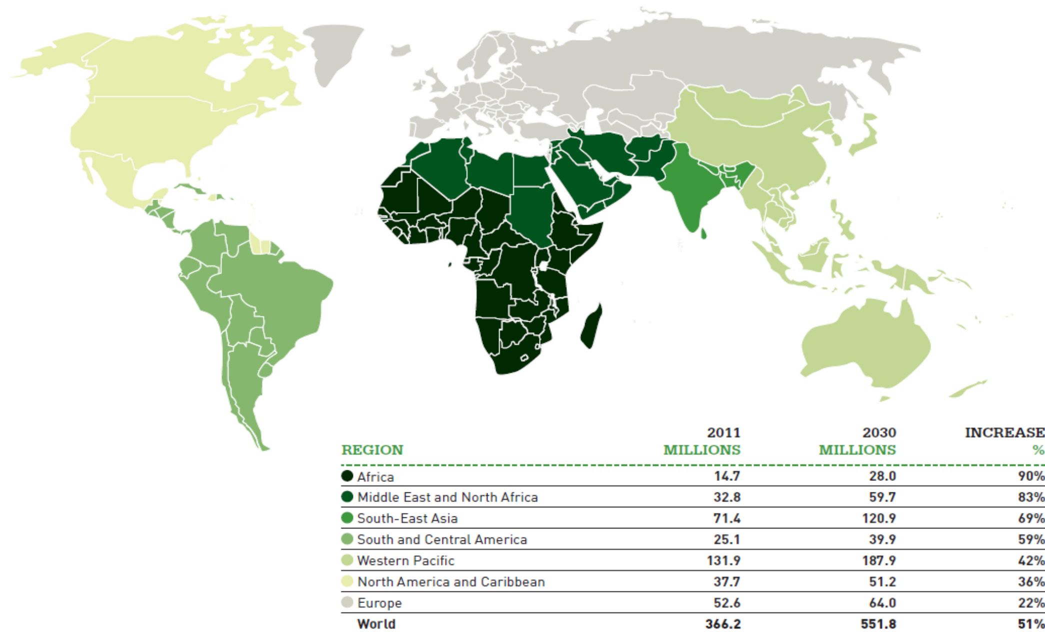
1.1 Type 2 diabetes mellitus (T2DM)

1.1.1 T2DM and its complications

Diabetes mellitus (DM) is a major public health problem worldwide and its prevalence will continue to increase over the next few decades [1]. T2DM presents itself in the long run with an onslaught of macrovascular complications like cardiovascular disease and microvascular complications including retinopathy, neuropathy and nephropathy. All these complications cause much morbidity and mortality to T2DM patients. At least 90% of diabetic cases worldwide are comprised of T2DM and hence there is a great urgency to reduce these numbers and curb the progression of its associated complications [2].

1.1.2 Prevalence of T2DM

The International Diabetes Federation (IDF) has estimated that there are more than 360 million people currently living with diabetes and this number is expected to rise to 552 million by 2030 (Figure 1). The Western Pacific region where China, Asia and Singapore are located has the greatest number of cases than any other region in the world [3]. Among adults aged 18 to 69 years in Singapore, 11.3% were diagnosed to be diabetic in 2010, a significant rise from 8.2% in 2004 [4].



REGION	2011 MILLIONS	2030 MILLIONS	INCREASE %
Africa	14.7	28.0	90%
Middle East and North Africa	32.8	59.7	83%
South-East Asia	71.4	120.9	69%
South and Central America	25.1	39.9	59%
Western Pacific	131.9	187.9	42%
North America and Caribbean	37.7	51.2	36%
Europe	52.6	64.0	22%
World	366.2	551.8	51%

Figure 1. International Diabetic Federation (IDF) regions and global projections of the number of people with diabetes (20-79 years), 2010-2030. Reprinted from Diabetes Atlas 5th edition [3], with kind permission from IDF.

1.1.3 T2DM-related traits and undiagnosed diabetes

Impaired glucose tolerance (IGT) and impaired fasting glycemia (IFG) are T2DM-related traits which place individuals at a higher risk of progressing to T2DM [2]. Singapore falls into the region with the highest prevalence of individuals with IGT at more than 14% (Figure 2). It is estimated that around half of those who have diabetes are not aware of their condition, a situation which proves worrying since many of these individuals would have started developing related complications upon diagnosis.

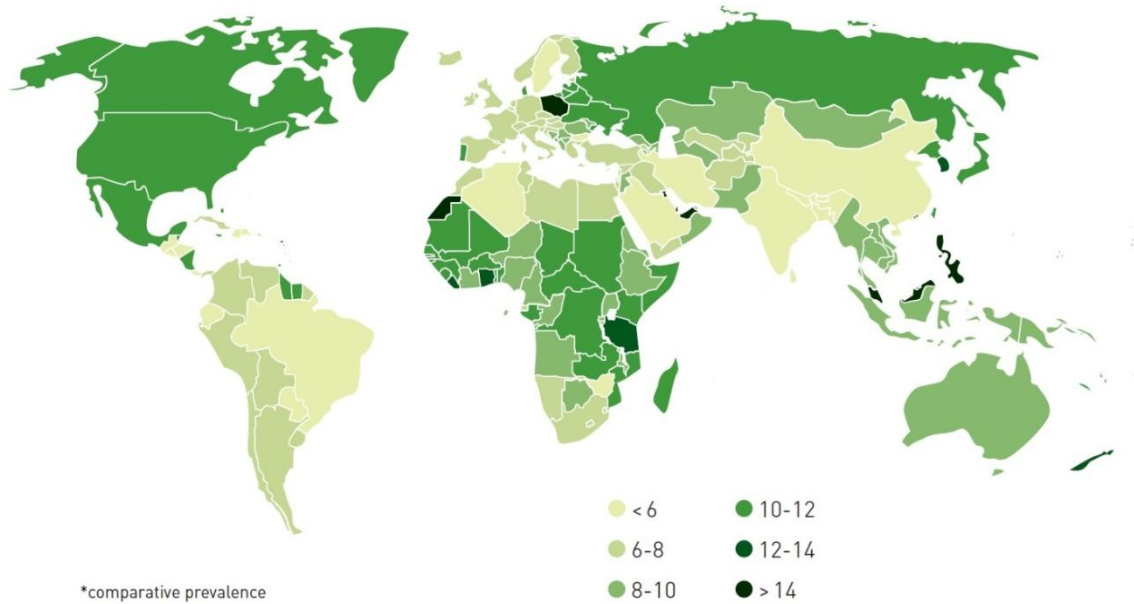


Figure 2. Comparative prevalence (%) of impaired glucose tolerance (20-79 years), 2011. Reprinted from Diabetes Atlas 5th edition [3], with kind permission from IDF.

1.2 Diabetic nephropathy (DN)

DN is a major complication of T2DM and it currently accounts for more cases of end-stage renal disease (ESRD) than any other cause of chronic kidney disease. It has been called a medical catastrophe of worldwide dimensions, likely due to an ever increasing prevalence of T2DM attributed to obesity, ageing and a sedentary lifestyle on one hand and improved survival resulting from better treatment for diabetic complications on the other [5, 6]. DN develops in about one third of diabetic patients [7]. Up to 63.5% of patients undergoing dialysis in Singapore are diabetic and this has been a disturbing upward trend over the years [8]. Patients with DN are at risk of progression to ESRD, by which time they would need to undergo dialysis or renal transplantation. Most of these patients do not receive the latter but depend on dialysis treatment for the rest of their lives which leads to a poor quality of life. Huge intervening efforts are needed to limit the

rising number of DN cases and this has fuelled an intense interest to search and discover markers for the early detection of DN.

1.2.1 Clinical pathology of DN

DN develops through several stages. In the earlier stages of DN, there is kidney hypertrophy where a thickened glomerular basement membrane (GBM), mild mesangial expansion and accumulation of hyaline in the arterioles are observed (Figure 3). Advanced nephropathy is characterised by the formation of Kimmelstiel-Wilson nodules, hyalinosis in both afferent and efferent arterioles and a markedly thickened GBM (Figure 4) [9]. These dramatic structural changes prevent the glomerulus from performing its filtration function.

Clinically, the onset of DN is characterised by a small to moderate increase in urinary albumin excretion (UAE) referred to as microalbuminuria and/or transient rise in glomerular filtration rate (GFR) called hyperfiltration. Without intervention, UAE rises dramatically to result in macroalbuminuria. GFR begins to decrease with this onset of overt DN [10].

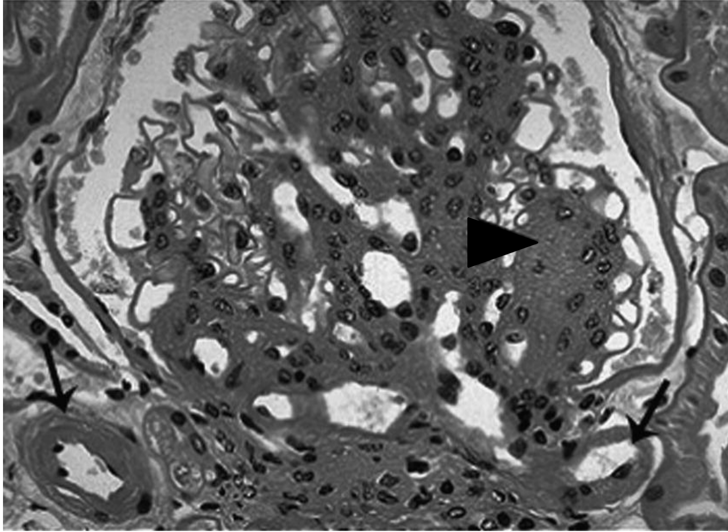


Figure 3. A glomerulus with diabetic nephropathy characterised by nodular mesangial expansion (arrowhead) and hyalinosis of afferent and efferent arterioles (arrows). Reprinted from Najafian and Mauer [11], with kind permission from Elsevier.

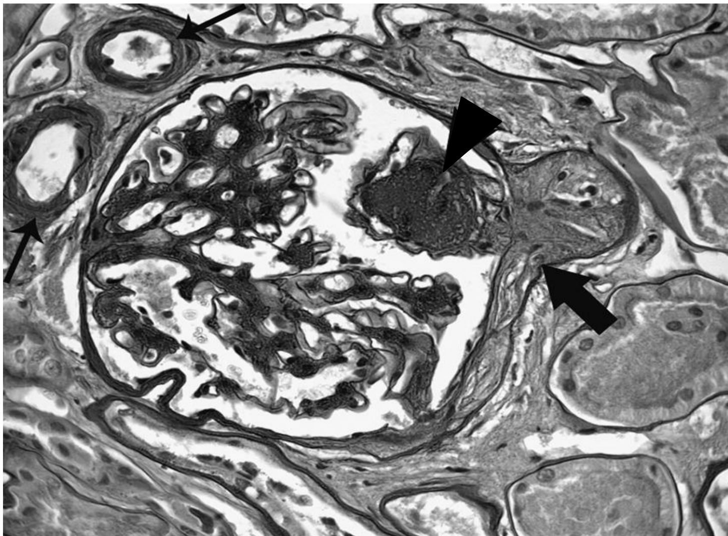


Figure 4. A glomerulus with a Kimmelstiel-Wilson nodule (arrowhead) which has completely occluded the glomerulotubular junction (thick arrow). Bowman's capsule is thickened and reduplicated. There is also hyalinosis of the arterioles (thin arrows). Reprinted from Najafian and Mauer [11], with kind permission from Elsevier.

1.2.2 Renal trait - GFR

GFR provides a measurement of the filtering capacity of the kidneys. It estimates the amount of plasma filtered by all nephrons in both kidneys per minute. However, GFR cannot be measured directly.

A substance that is inert, freely filtered at the glomerulus, but not secreted, reabsorbed, synthesised or metabolised by the kidney is a suitable candidate substance for GFR estimation as the amount of that substance filtered is equal to the amount excreted in the urine [12]. Inulin is one such substance and its clearance is currently the gold standard for measurement of GFR. Iohexol, technetium-labelled diethylene-triamine-penta-acetic acid and ethylene diamine-tetra-acetic acid are also able to achieve similar accuracy. However, measuring clearance using these methods is time consuming and costly especially for measurements done in a large number of individuals.

Therefore, alternative methods have emerged. The surrogate for GFR measurements based on plasma creatinine is currently widely employed. The Cockcroft-Gault (CG) and Modification of Diet in Renal Disease (MDRD) formulae are two conventional equations that make use of creatinine clearance to estimate GFR and takes into account variables like age, sex, race and body size. The MDRD formula may perform better than the CG equation in adults but the data are limited [12]. However, the MDRD underestimates GFR if it is greater than 60ml/min/1.73m^2 [13].

Cystatin C (CysC), unlike serum creatinine, is not secreted by proximal tubular cells and is thus less affected by extrarenal modulators. Equations have been derived to estimate GFR from CysC but proved to have no advantage over the MDRD in diabetic patients

[10, 14]. At the moment, the MDRD formula is still widely used for the estimation of GFR in adult patients although newer equations are being derived including the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [15, 16].

In early diabetes, patients experience glomerular hyperfiltration (>140ml/min/1.73m^2) and this is associated with a poor prognosis in the development of DN [17]. An increased plasma flow and intraglomerular pressure are established causes for hyperfiltration in early diabetes [18]. As duration of T2DM increases, estimated glomerular filtration rate (eGFR) declines gradually over the next 15 years before ESRD ensues by which time dialysis and renal transplantation are needed [19].

1.2.3 Urinary marker of DN - albumin

In normal individuals, very little albumin emerges in the urine. However, when there is renal damage, initially small amounts (microalbuminuria) and subsequently larger amounts (macroalbuminuria) of albumin excretion occurs. Microalbuminuria is a well-established biomarker of DN. UAE over a 24 h period is the current gold standard for determining the presence of microalbuminuria. However, this method is inconvenient and a more practical alternative like the random spot urine sample is sought. Although the latter method is susceptible to variation in urine concentration due to hydration, physical activity and other factors, nomalisation by dividing with creatinine concentration minimises some of these issues [20]. While a first morning void sample is a better alternative to 24 h collections than spot urine sample in the assessment of albuminuria, the spot urine sample for the measurement of albumin-to-creatinine ratio (ACR) is still widely used especially in out-patient diabetic clinics [15, 21, 22].

The earliest clinical evidence of DN is most often microalbuminuria (UAE, 20-200 μg/min in an overnight urine sample; ACR, 30-300 mg/g in a spot urine sample). Microalbuminuria affects 20-40% of patients 10-15 years after the onset of diabetes. Progression to macroalbuminuria (UAE, >200 μg/min; ACR,>300 mg/g) happens in 20-40% of patients over 15-20 years after diabetes onset. Hypertension and macroalbuminuria are likely to hasten the decline in GFR and eventual ESRD [23].

DN enters a vicious cycle once a certain degree of injury exists. Microalbuminuria triggers the progression of DN as continuous protein leakage overloads the tubular cells in the reabsorption pathway and this has been postulated to cause tubulointerstitial damage [24].

Encouragingly, remission to normoalbuminuria is possible with multifactorial intervention comprising intensive glycemic and blood pressure control in T2DM patients with microalbuminuria [25, 26]. However, with up to 50% undiagnosed diabetic patients and the asymptomatic nature of microalbuminuria, kidney damage may have started for a period of time before diagnosis and the opportunity for remission to normoalbuminuria could be less optimistic [26]. The transition from normal to micro- and on to macroalbuminuria is much more rapid than expected and possesses much heterogeneity among individuals [27]. When T2DM patients become macroalbuminuric, their renal function declines much more rapidly than in normoalbuminuric patients [28]. Hence, much focus is placed on the discovery of biomarkers which are able to provide early detection of DN.

1.2.4 Glomerular filtration barrier

The glomerular filtration barrier is made up of three layers namely a fenestrated endothelium, GBM and the slit diaphragm (SD) (Figure 5). The SD is located between the interdigitating secondary foot processes of podocytes that cover the endothelial surface (Figure 6).

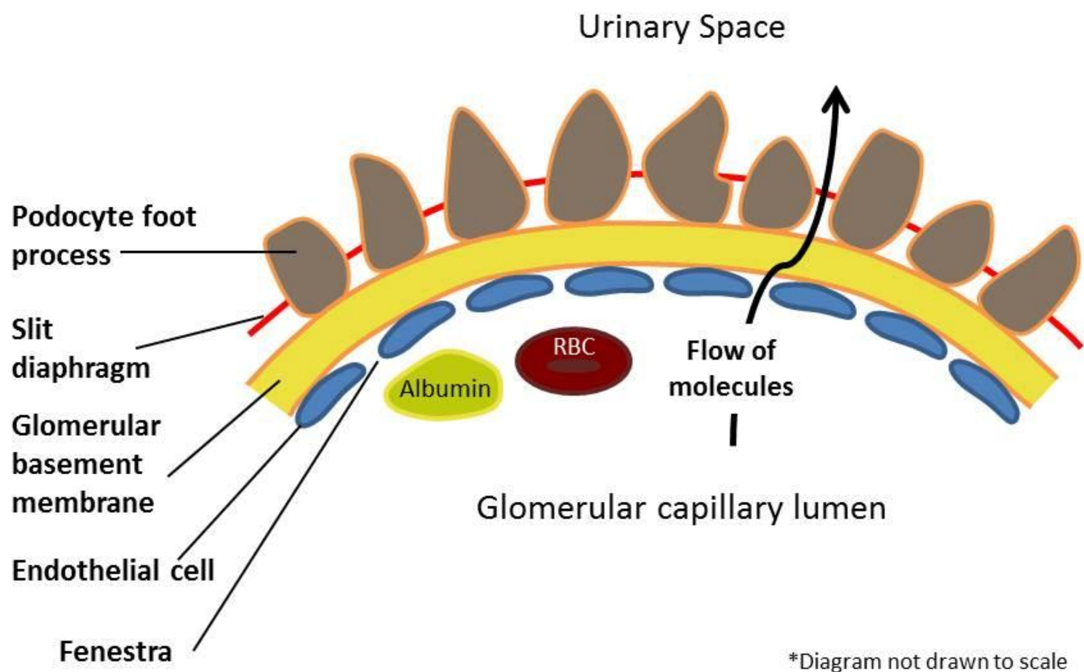


Figure 5. Cross sectional illustration of the glomerular filtration barrier.

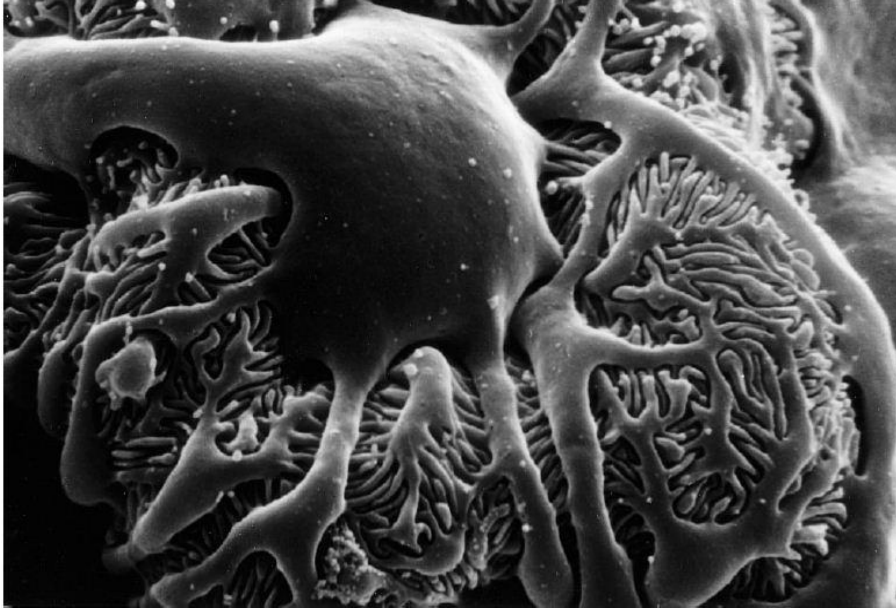


Figure 6. Scanning electron micrograph of a podocyte viewed from the urinary space and the spaces between the foot processes are the slit diaphragms (SDs). Reprinted from Smoyer and Mundel [29], with kind permission from Springer Science and Business Media.

The fenestrae of the endothelium is permeable to water and small solutes but is impermeable to red blood cells. Furthermore, due to the expression of negatively charged glycoproteins, it repels very large anionic proteins like albumin. Similarly, the GBM functions as a charge-selective filter.

The SD is made up of a zipper-like structure containing tiny pores [30].The extracellular domains of nephrin (NPHS1) interact with each other to form the structural scaffold of the SD (Figure 7) [31]. Other proteins like podocin (NPHS2), NPHS1 related protein 1 (NEPH1), alpha-actinin 4 (ACTN4) and CD2-associated protein (CD2AP) associate with NPHS1 to form the SD complex [32-36].

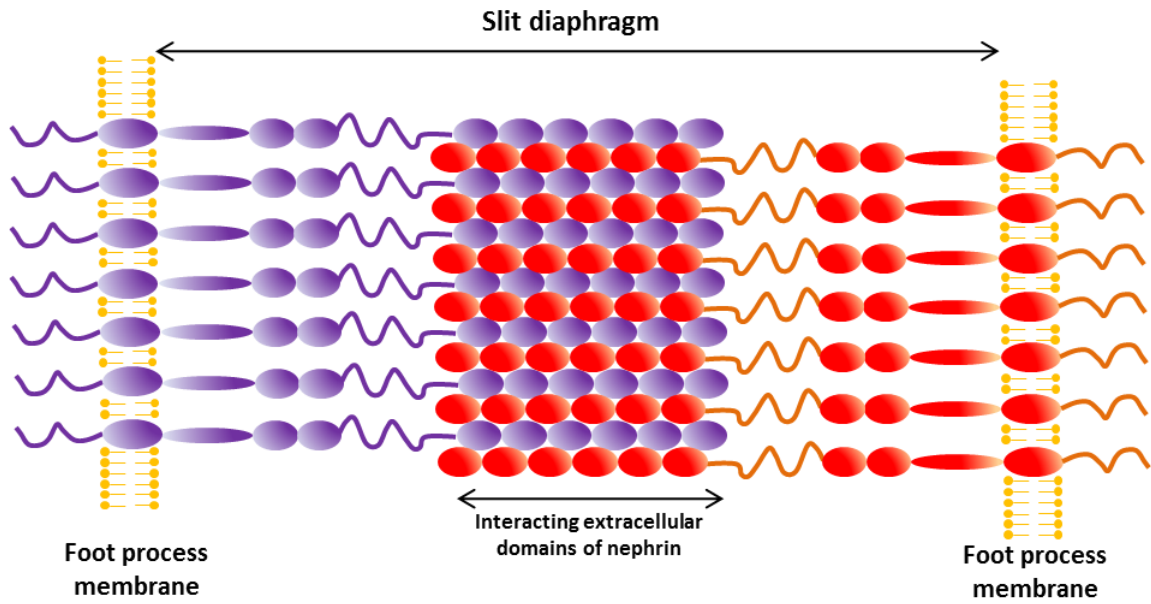


Figure 7. Simplified illustration of nephrin (NPHS1) assembly showing homophilic interaction of NPHS1 molecules from opposite foot processes at the centre of the SD. Interaction of NPHS1 with other proteins is not shown.

1.2.5 Genetics of DN

Genetic susceptibility is a critical factor for the development and progression of DN. While the search for DN genes is ongoing, it is widely accepted that angiotensin-converting enzyme (ACE) is the proto-typical DN gene [37].

The most common genetic variations in the human genome are single nucleotide polymorphisms (SNPs). These are sites in the DNA sequence where individuals differ at a single DNA base at a frequency of more than 1% in the population. Testing numerous individual SNPs along a candidate gene is a tedious and expensive process. On the other hand, haplotype analysis which takes into account multiple SNPs simultaneously could serve the same purpose with little loss of statistical power [38]. These SNPs may be selected according to the observation that some appear to be located in “blocks” which are regions with little historical recombination but demarcated by areas with an inferred high frequency of recombination events. A reduced set of tagging SNPs can then be identified and genotyped [39].

1.3 *NPHS1* gene

1.3.1 Congenital nephrotic syndrome of the Finnish type

Congenital nephrotic syndrome of the Finnish type (CNF) is an autosomal recessive disorder that is caused by mutations in *NPHS1*. The common mutations are Fin-major and Fin-minor. CNF is characterised by massive proteinuria even *in utero*, indicating early damage of the glomerular filtration barrier that leads on to fatality in the first couple years of life unless renal transplantation is performed [40]. Compound heterozygotes for Fin-major/Fin-minor human fetal kidneys had an absence of NPHS1 expression and missing SDs while expression of other SD proteins like ZO-1 and P-cadherin were similar to normal kidneys [41]. In another study, homozygous Fin-major and compound heterozygous Fin-major/Fin-minor kidneys had no NPHS1 expression, podocytes exhibited fusion and SDs of irregular sizes were seen along with missing ones while ZO-1 was detected in normal amounts [41].

A variety of *NPHS1* mutations have also been reported in patients with a spectrum of nephrotic disorders like focal segmental glomerulosclerosis (FSGS), steroid resistant nephrotic syndrome (SRNS) and minimal change nephrotic syndrome (MCNS) [42-44].

1.3.2 Experimental models

NPHS1 gene inactivation by homologous recombination in embryonic stem cells of mice resulted in neonatal death within 24 h. Affected murine kidneys had enlarged Bowman's spaces, dilated tubules, effacement of foot processes and missing SDs akin to that observed in CNF patients [45]. Absence of SDs and podocyte effacement was evident in *NPHS1* knockout mice while expression of GBM (type IV collagen, laminin) and SD proteins (NPHS2, CD2AP, ACTN4) were not altered [46]. Similarly, mutant *NPHS1*

mice generated by gene-trapping were proteinuric and died soon after birth but CD2AP and ZO-1 were unaltered [47]. Compared to wild type mice, NPHS1 deficient mice had fusion of foot processes and lacked SDs [47]. All these indicate that NPHS1 is a principle component of the SD structure and is crucial for the normal architecture and function of the glomerular filtration apparatus [46, 48].

1.3.3 Discovery of the NPHS1 gene

A gene search was initiated in 1989 among Finnish families due to the high frequency of CNF in Finland. The candidate gene approach was unfeasible at that time because there were no known basement membrane genes that were localised to the critical region 19q13.1. Positional cloning was the alternative approach and after years of combing the chromosomal region 19q13.1, the group finally pinned down the *NPHS1* gene in 1998 [32, 49].

1.3.4 Structure of the NPHS1 gene

NPHS1 has a size of 26,466 bp and contains 29 exons with sizes ranging from 25 to 216 bp. The gene product, NPHS1, is a transmembrane protein that contains eight immunoglobulin (Ig) C2-like motifs. The first exon codes for the signal peptide, and exons 2-21 codes for the region containing the Ig domains. A fibronectin type III-like (FnIII) domain is encoded by exons 22-23 while exon 24 codes for the helical transmembrane domain. Exons 25-29 encode the intracellular domain and 3'untranslated region (UTR) [49, 50].

1.3.5 NPHS1 structure

NPHS1 is a putative member of the large Ig-like superfamily which comprises cell surface and soluble proteins that are involved in the recognition, binding or adhesion processes of cells [51]. It is a type 1 single pass membrane protein of 1241 amino acid residues with a theoretical molecular weight of about 137 kDa but an apparent molecular weight of 180 kDa on Western blot [52]. NPHS1 is composed of an extracellular domain with eight distal Ig-like domains and one proximal FnIII domain, a short helical transmembrane domain and an intracellular domain (Figure 8).

A major splice variant of NPHS1 called NPHS1-α has an identical sequence with NPHS1 apart from a missing exon 24 which encodes the helical transmembrane portion of NPHS1 (Figure 9) [53]. NPHS1-α is potentially a secreted form of NPHS1 but its functional significance has not been elucidated.

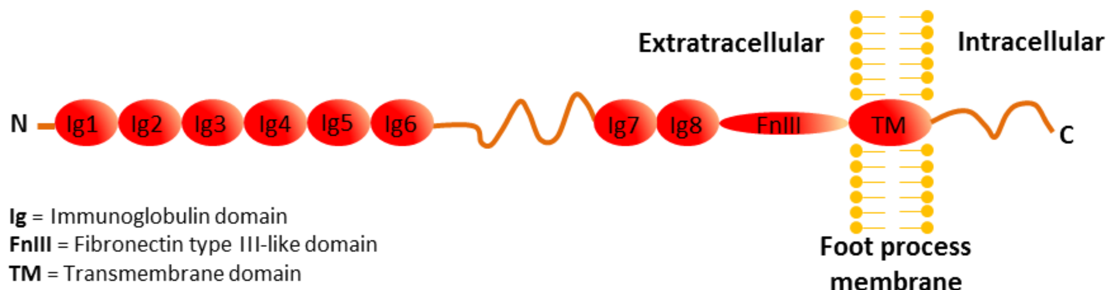


Figure 8. NPHS1 protein domains.

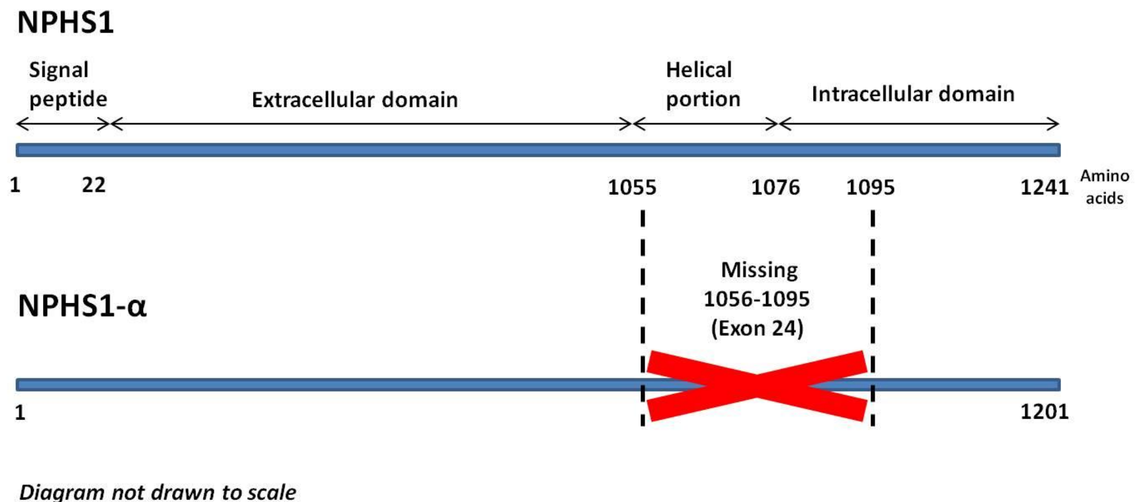


Figure 9. Protein structure of NPHS1 and NPHS1-α.

1.3.6 Impact of mutations on NPHS1 function

More than 170 *NPHS1* mutations have been reported to date [54]. In the Finnish population, 90% of CNF cases are of Fin-major (2 bp deletion in exon 2) and Fin-minor (nonsense mutation in exon 26) mutations [49, 50, 55]. These two mutations result in truncated forms of the protein. Other mutations that have been reported are missense mutations resulting in single amino acid substitutions which typically results in defective NPHS1 transport to the cell surface due to misfolding of the protein and are found accumulated in the endoplasmic reticulum [56]. An interesting missense mutation V882M results in the NPHS1 molecule being able to reach the cell surface but was restricted in lateral movement and trafficking at the plasma membrane [57].

1.3.7 Primary and extrarenal NPHS1 expression

The primary expression of NPHS1 is in the renal glomerulus, specifically in the podocytes and SDs (Figure 10). In rodents, the *NPHS1* gene promoter was also found to be activated in the brain, spinal cord and β-cells of the pancreas [45]. In addition, expression of NPHS1 was detected in the testis, spleen and thymus [58]. Cardiac expression in human fetal and mouse hearts was also reported recently, and purportedly played a role in mouse heart development [59]. However, the only extrarenal expression of NPHS1 confirmed in adult humans is that found in the pancreatic β- and microvascular endothelial cells [60, 61]. Clinically, children with the two major mutations in *NPHS1* do not have major defects in other organs except for the kidney. Minor neurological problems and mild cardiac hypertrophy are seen in these children but are most likely due to protein deficiency as a result of massive proteinuria [41]. However, subtle changes which do not give rise to obvious clinical defects should not be easily ruled out.

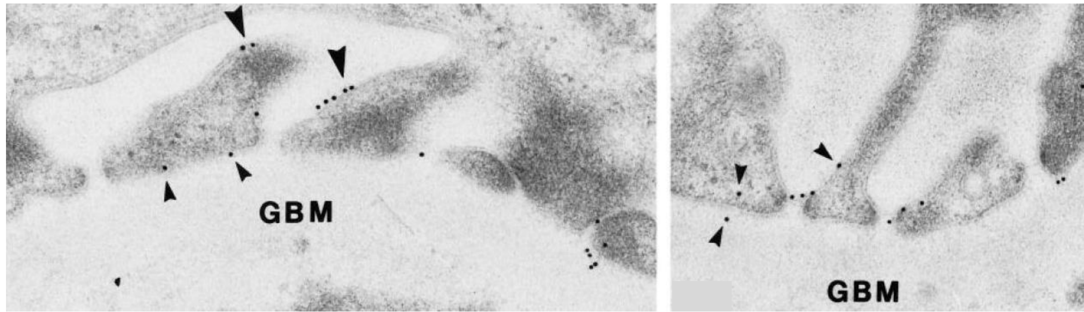


Figure 10. Localisation of NPHS1 (arrowheads) on podocytes and SDs in a normal kidney. Reprinted from Holthöfer *et al.* [53], with kind permission from Elsevier.

1.4 DN and NPHS1

1.4.1 In vitro studies

NPHS1 expression was attenuated in most podocyte cell cultures exposed to a hyperglycemic environment. Exposure of immortalised human podocytes to glycated albumin resulted in NPHS1 downregulation at the cell surface and NPHS1 mRNA expression was decreased by 42.6% in cells treated for 24 h. This phenomenon was absent in control cells that were treated with native albumin [62].

1.4.2 Experimental models

Several animal studies have generally shown that a reduction in NPHS1 expression in the glomerulus is predictive of albuminuria [63, 64]. Treatment with ACE inhibitors or anti-inflammatory drugs rescued low NPHS1 expression and also reduced or prevented albuminuria [64, 65]. NPHS1 mRNA and protein expression was markedly low while UAE was significantly higher in streptozotocin (STZ)-induced diabetic rats compared to non-diabetic control rats [63, 64]. Interestingly, in another study, STZ-induced diabetic rats and non-obese diabetic mice both had increased levels of NPHS1 mRNA in the early stages and albuminuria was detected [66]. At present, it is unclear how the conflicting results should be reconciled but there are some who propose that an upregulation of NPHS1 could also disrupt the structure of the SD due to an upset of the stoichiometry of the proteins working together to form it [67].

1.4.3 NPHS1 in kidneys of diabetic patients

There are few studies investigating NPHS1 expression in human subjects due to the invasive nature of kidney biopsies. These results have to be interpreted with care as sample sizes are small and there may be bias in the selection of patients for biopsy. There

was a significant reduction in NPHS1 expression in the glomeruli of T2DM patients compared with control human renal cortex sections obtained from patients who underwent surgery for renal malignancy (62% reduction, *P*=0.0003) [68]. A markedly low NPHS1 expression was observed in the renal biopsies of type 1 diabetes mellitus (T1DM) and T2DM patients with DN compared to normal controls (up to 67% reduction, *P*<0.001). Similarly, T1 and T2DM patients with microalbuminuria also showed a reduction in NPHS1 staining compared to controls (up to 69% reduction, *P*<0.001) [62]. The proportion of cells with NPHS1 mRNA was substantially lower in 13 T2DM patients with DN compared to five non-diabetic MCNS patients as well as five normal controls [69]. Gene expression profiles of normal and DN kidneys revealed that a total of 96 genes were upregulated in DN while 519 genes, including *NPHS1*, were downregulated [70]. NPHS1 protein and mRNA expression were reported to be greatly reduced in diabetic patients in contrast with non-diabetic MCNS and normal control patients. However, protein and mRNA expression of CD2AP and NPHS2 were similar in all three groups of patients. There were also significantly fewer SDs in diabetic patients [71]. There was an apparent inverse relationship between the degree of NPHS1 expression and level of proteinuria in these studies.

1.4.4 Nephrinuria

The presence of NPHS1 was successfully detected in the urine of STZ-induced diabetic rats as early as week 4 after induction, implicating this molecule in early kidney damage. It can thus be inferred that NPHS1 is modulated in the early stages of kidney damage. In that study, nephrinuria corresponded to an increase of NPHS1 mRNA levels [66]. An increase in NPHS1 mRNA might have allowed for more protein to be available for

shedding into the urine. Alternatively, the sudden increase in NPHS1 expression could have disrupted the stoichiometry of SD proteins and resulted in nephrinuria [67]. Regardless, this finding raised the possibility of using nephrinuria as a biomarker of early kidney damage.

Immunoreactive NPHS1 fragments were first detected in the urine of Finnish T1DM patients. It was observed that 28% of macroalbuminuric, 17% of microalbuminuric and 30% of normoalbuminuric patients presented with nephrinuria while none of the non-diabetic healthy controls were nephrinuric. Up to a third of normoalbuminuric diabetic patients have symptoms of filtration barrier damage as indicated by the manifestation of nephrinuria [72]. A subsequent study by Ng *et al.* (2011) found corroborating evidence for this earlier finding in Singaporean Chinese patients with T2DM. Furthermore, nephrinuria was found to be associated with a decline in eGFR even in normoalbuminuric patients [73]. A recent study also reported that 100% of micro- and macroalbuminuric Caucasian T2DM patients presented with nephrinuria while 54% of normoalbuminuric patients were nephrinuric [74]. These three studies highlighted the potential of nephrinuria as a predictor of early glomerular filtration barrier damage before its clinical manifestation as microalbuminuria.

1.4.5 NPHS1 variants and DN

There have been few studies looking at the association of *NPHS1* SNPs with DN. Three exonic SNPs namely, rs3814995, rs33950747 and rs4806213 were investigated in 996 Finnish T1DM patients for their association with DN. Results were largely negative [75]. The *NPHS1* promoter region was sequenced in 100 Caucasian T2DM patients in search of genetic variants. However, none were found in all groups of patients (T2DM

normoalbuminuric, T2DM macroalbuminuric, non-diabetic controls with proteinuric nephropathies and healthy controls) [76]. An intronic SNP rs466452 was genotyped in 231 Caucasian T1 and T2DM patients and there was an absence of association between the SNP and DN in both groups of patients [77].

1.5 T2DM and NPHS1

1.5.1 In vitro studies

NPHS1 was expressed specifically both on the plasma membrane and the surface of insulin vesicles in the β-cells and promotes insulin secretion in response to glucose [78]. A higher expression of NPHS1 in mouse insulinoma 6 (MIN6) cells was associated with greater insulin content and improved glucose-stimulated insulin release (GSIR) compared to control cells. Downregulation of NPHS1 using small interfering RNAs resulted in insulin content and GSIR decrement in MIN6 cells *in vitro*. Additionally, NPHS1-transfected cells had a markedly higher amount of secretory granules and secretory vesicles than control cells [78].

1.5.2 Experimental models

In vivo, transplantation of NPHS1-positive MIN6 pseudoislets into diabetic mice caused a significantly lower glycemia and an earlier reversal to normoglycemia compared to mice which had MIN6 pseudoislets transfected with an empty vector. Diabetic mice became hyperglycemic again after removal of the kidney bearing the NPHS1-positive pseudoislets [78].

1.5.3 NPHS1 in pancreatic islets of diabetic patients

NPHS1 expression in the pancreatic islets of T2DM cadaveric donors was reduced compared to age-, sex- and cold ischemia time-matched non-diabetic controls [78]. NPHS1 was inversely correlated with BMI (r^2=0.75, P<0.001) but this was perhaps not unexpected since BMI was significantly higher in diabetic cases than non-diabetic controls.

1.5.4 NPHS1 and insulin resistance in humans

Although NPHS1 from the pancreas would unlikely be excreted in the urine, a 100 kDa urinary protein immunoreactive to an anti-NPHS1 antibody was found to be associated with insulin resistance in 128 offspring of T2DM patients compared to nine controls. Offspring with strongly positive 100 kDa bands were more insulin resistant in terms of reduced rates of non-oxidative glucose disposal than offspring who were weakly positive or negative for this 100 kDa band [79]. The identity of this band cannot be confirmed as its size does not fit the typical size profile of those immunoreactive NPHS1 fragments detected previously in T1DM patients [72]. However, the group explained that this protein could be a degradation product of full-sized NPHS1 due to incomplete protease inhibition. The usefulness of nephrinuria in the prediction of T2DM remains to be seen.

1.5.5 NPHS1 variants with T2DM and T2DM-related traits

The Funagata Study investigated the association of two exonic SNPs rs2285450, rs437168 and one intronic SNP rs2267588 with T2DM in Japanese patients [80]. The group found that all three SNPs were associated with T2DM. The frequencies of at-risk genotypes in the IGT and T2DM group were higher than the normal glucose tolerance (NGT) group [80]. This suggested that *NPHS1* was not only associated with T2DM, but

potentially with T2DM-related traits like IGT as well. A single intronic SNP rs466452 was studied in the Caucasians and an absence of association with both T1 and T2DM was reported [77].

Experimental studies on the function of *NPHS1* SNPs in the pancreatic islets are lacking. It is probable that certain *NPHS1* variants cause NPHS1 in the β-cells to be downregulated which could result in IGT and eventual T2DM. The genetic involvement of *NPHS1* in T2DM remains to be fully understood.

1.6 Summary and rationale for present work

1.6.1 NPHS1 and DN

NPHS1 is essential for a healthy glomerular filtration system. It is a key structural component of the SD which is the last barrier to glomerular permeability. The absence of NPHS1 as shown clinically in CNF patients and experimental models gave rise to severe proteinuria and proved to be fatal in the neonate. There are many common genetic variations of*NPHS1*in the healthy population and these potentially play an important role in chronic kidney diseases, like DN. There are a few studies which investigated the association of *NPHS1* SNPs with DN. However, none of the studies have thoroughly reported the associations of SNPs in the full length gene. All of them investigated a limited number of SNPs either in the exonic or intronic region and had a fairly small sample size. Moreover, these studies were done on Caucasian patients and their relevance to the Asian population is unclear.

Hence there was a need to conduct a large study on Singaporean patients covering SNPs in the full length gene as well as substantial regions up- and downstream of the coding

region. In addition, genetic analysis involving the haplotypes would strengthen the findings of such a study, extending beyond that focused on single SNPs [38].

1.6.2 NPHS1 and T2DM

Extrarenal NPHS1 expression has been reported in the pancreatic β-cells of humans. NPHS1 was found to be localised to the plasma membrane and surface of insulin vesicles of the β-cells. A role of NPHS1 in the pathway of insulin exocytosis has been demonstrated using *in vitro* and *in vivo* methods. A greater NPHS1 expression translated to higher insulin content in cells and significantly lowered glycemia in mice. All these findings were consistent with the observation that NPHS1 expression was lower in islets of T2DM patients compared to non-diabetic controls in the same study [78]. Although this is the only study which has demonstrated the role of NPHS1 in insulin secretion, the evidence presented from human, animal and cell culture investigations seem to strongly implicate NPHS1in insulin secretion and thus β-cell function. Furthermore, an earlier study in Japanese had detected the association of three *NPHS1* variants with T2DM [80]. While these results were encouraging and potentially suggested that *NPHS1* may have a vital role to play in the pathophysiology of the development of T2DM, reports of genetic replication have not been forthcoming. Therefore this present study seeks to examine the association of *NPHS1* SNPs in the full length gene with T2DM-related traits as well as T2DM in Singaporeans.

1.6.3 Aims of study

Aim 1: To investigate the association of *NPHS1* SNPs with renal traits including eGFR and albuminuria in T2DM Chinese patients in Singapore. These patients were from the Singapore Diabetes Cohort Study (SDCS) described in Methods 2.1.1.

Aim 2: To investigate the association of *NPHS1* SNPs with T2DM-related traits in non-diabetic Chinese, Malay and Indian subjects in Singapore. These subjects were from the 1998 Singapore National Health Survey (NHS98) described in Methods 2.1.2.

Aim3: To investigate the association of *NPHS1* SNPs with T2DM in Chinese subjects in Singapore. Cases with T2DM were from both the SDCS and NHS98 (Methods 2.1.1 and 2.1.2) and non-diabetic controls were from the NHS98 (Methods 2.1.2).

CHAPTER 2: MATERIALS AND METHODS

2.1 Patient populations

2.1.1 Singapore Diabetes Cohort Study (SDCS)

T2DM patients included in this study were from patients recruited into the SDCS. Both the National University of Singapore Institutional Review Board (NUS-012) and the National Healthcare Group Domain-Specific Review Board (C/05/118) approved the research protocol and patients participating in this study gave informed consent. Briefly, since 2004 all patients previously diagnosed with T2DM and who were being treated at primary care facilities of the National Healthcare Group Polyclinics (NHGP) in Singapore were invited to join the SDCS. Of the patients approached, 91% agreed to participate in the study and formed a part of our SDCS patient group. Consenting patients completed a questionnaire to elicit information on demographics, lifestyle factors and family medical history. They also had their physical measurements taken. Medical records were reviewed to obtain information on their metabolic control and the presence of comorbidities and complications. Genotype data were only available for Chinese patients in this part of the study (n=1363).

2.1.2 1998 Singapore National Health Survey (NHS98)

NHS98 is a population based, cross-sectional study comprising Chinese, Malays, and Asian Indians aged between 18 and 69 years. Previously described survey methods were based on the World Health Organization (WHO) recommended model for field surveys of diabetes and other non-communicable diseases, and the WHO Multinational Monitoring of Trends and Determinants in Cardiovascular Disease protocol for

population surveys [80]. At the time of this study, DNA samples from 2953 Chinese, 792 Malay, and 603 Asian Indian subjects were available for analysis (n=4348).

2.1.3 Laboratory methods

2.1.3.1 Albumin and creatinine measurements

Spot urine samples were collected at the centre of recruitment. Sample aliquots were stored in the -80°C freezer until needed. When required, aliquots were thawed in an ice bath before centrifugation at 13000 rpm for 5 min to spin down all the sediments. Urine from the supernatant was used to measure albumin concentration by employing a competitive enzyme-linked immunosorbant assay (ELISA) (Albuwell II Albumin ELISA kit; Exocell Inc., Philadelphia, PA, USA). Likewise, creatinine concentration was determined by using a chemical colorimetric assay (The Creatinine Companion; Exocell Inc., Philadelphia, PA, USA) following the manufacturer's protocol. The albumin value (mg) was divided by the creatinine value (g) to obtain the albumin-to-creatinine ratio (ACR) (mg/g).

2.1.3.2 DNA extraction

Venous blood samples were collected and centrifuged at 3500 rpm for 15 min to separate blood into three layers namely plasma, buffy coat and red blood cells. All three layers were stored as aliquots in a -80°C freezer until required. DNA were isolated from blood samples using DNA blood Midi kits (QIAGEN, Hilden, Germany) following the manufacturer's protocol.

2.1.3.3 Estimated GFR

Renal function was estimated by the simplified MDRD equation [82]:

$$\text{eGFR} = 186.3\times(\text{plasma creatinine in } \mu\text{mol/L} \times 0.011)^{-1.154}\times(\text{age in years})^{-0.203}\times 0.742 \text{ for women} \times 1.21 \text{ if the individual is black}$$

*2.1.3.4 Plasma glucose and insulin measurements**

Fasting blood samples were drawn for measurement of plasma glucose (Boehringer Manheim, Mannheim, Germany) and insulin (immunoassay using an Abbott AxSYM; Abbott Laboratories, Chicago, Illinois) in NHS98 subjects after fasting overnight for at least 10 h.

*2.1.3.5 Oral glucose tolerance test**

NHS98 participants, except diabetic subjects on oral hypoglycemic agents or insulin, had an oral glucose tolerance test (OGTT). Subjects were considered to have T2DM if they gave a history of T2DM or if their fasting glucose was ≥7.0 mmol/L or if their OGTT was ≥11.1 mmol/L. IFG was defined as fasting glucose between 6.0 to 7.0 mmol/L and OGTT <7.8 mmol/L, and IGT was defined as fasting glucose >7.0 mmol/L and OGTT between 7.8 to 11.1 mmol/L.

*2.1.3.6 Physical measurements**

Blood pressure, height, weight, waist and hip circumference were measured for all subjects. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m^2).

** Measurements and tests were performed by staff of the NHGP.*

2.1.3.7 Homeostatic model assessment

Homeostatic model assessment (HOMA) for insulin resistance and β-cell function were calculated respectively based on the following equations [83]:

$$\text{HOMA-insulin resistance} = (\text{FPI} \times \text{FPG}) \div 22.5$$

$$\text{HOMA-}\beta\text{-cell function} = (20 \times \text{FPI}) \div (\text{FPG} - 3.5)\ \%$$

where FPI is fasting plasma insulin concentration (mU/L) and FPG is fasting plasma glucose (mmol/L) .

2.1.4 *Selection of NPHS1 SNPs*

NPHS1 SNPs rs2267588 and rs33950747 were studied in the Japanese and Caucasian respectively in relation to diabetes but lacked genotype data in the Han Beijing Chinese samples (CHB) from HapMap [75, 80]. They were assessed for their degree of polymorphism before the decision to include or exclude them from the genotyping exercise was made. The traditional restriction fragment length polymorphism method was employed for this purpose and it was found that these two variants were not polymorphic in the CHB samples. Therefore, they were excluded from further analysis.

For haplotype analysis, genotypic data of 30 SNPs in the chromosomal region Chr19: 41003706..41039579 (encompassing *NPHS1* gene as well as 5 kb up- and downstream of it) belonging to the CHB population were obtained from the HapMap Genome Browser release #27 (www.hapmap.org). 14 of the SNPs were non-polymorphic (minor allele frequency <0.05) and were excluded. Pairwise linkage disequilibrium (LD) was therefore measured in the remaining 16 SNPs between each SNP and its nearest neighbour as proposed by Gabriel *et al.* (2002) [38] (Table 1). 15 SNPs with Lewontin's |D'|>0.8 were located in haplotype blocks while rs10409299 (M7) which was not in strong LD with its neighbouring SNPs was treated as a singleton (Tables 2 and 3). Five tagging SNPs were needed to capture the haplotype diversity within the blocks. Together with the singleton, rs10409299, a total of six SNPs were genotyped in the SDCS and NHS98 samples.

Table 1. 16 biallelic SNPs selected for LD and haplotype block analyses.

Marker	SNP ID	Location	Marker	SNP ID	Location
M1	rs17777002	Downstream	M9	rs2073901	Exon 17
M2	rs11084831	Intron 28	M10	rs7248157	Intron 16
M3	rs460560	Intron 28	M11	rs392702	Exon 11
M4	rs731934	Intron 27	M12	rs3814995	Exon 3
M5	rs2071327	Exon 26	M13	rs2285450	Exon 3
M6	rs4806213	Exon 24	M14	rs401824	Promoter
M7	rs10409299	Intron 23	M15	rs443186	*KIRREL2* 5'UTR
M8	rs437168	Exon 17	M16	rs435605	*KIRREL2* 5'UTR

Table 2. Haplotype block 1.

No.	M1	M2	M3	M4	M5	M6	Freq
1	A	G	C	G	C	T	0.103
2	A	G	T	G	C	T	0.161
3	A	T	T	A	T	T	0.619
4	G	T	T	A	T	T	0.111
						Total	0.994

*Markers M1 (rs17777002), M3 (rs460560) and M5 (rs2071327) were selected from Block 1 to be genotyped.

Table 3. Haplotype block 2.

No.	M8	M9	M10	M11	M12	M13	M14	M15	M16	Freq
1	A	G	A	G	C	A	G	C	T	0.054
2	G	G	G	G	C	G	A	A	T	0.177
3	G	G	G	G	T	G	A	A	T	0.667
									Total	0.899

*Markers M8 (rs437168) and M12 (rs3814995) were selected from Block 2 to be genotyped.

2.1.5 Genotyping

The six SNPs were genotyped using short amplicon-based high resolution DNA melting assays. Each primer pair was designed by Primer 3 software (http://frodo.wi.mit.edu/primer3/) (Table 4). The PCR conditions were: initial denaturation performed at 95 °C for 2 min, followed by 50-65 cycles of denaturation at 98 °C for 20 s, annealing at respective temperature for 15 s, extension at 68 °C for 30 s, and final extension at 68 °C for 5 min. To maximise the incorporation of double stranded DNA-binding dye, LC-green Plus (Idaho Technology Inc., Salt Lake City, UT, USA), all samples were subjected to an additional denaturation at 95 °C for 30 s and annealing at 25 °C for 30 s after the final extension step. Fluorescence monitoring during thermal denaturation from 45 °C to 98 °C was done immediately using LightScanner (Idaho Technology Inc.). Genotyping success rate was more than 95% for all six SNPs.

Table 4. Genotyping conditions for *NPHS1* SNPs using high resolution DNA melting.

SNP	Forward Primer (5'-3')	Reverse Primer (5'-3')	TA (°C)	Amplicon size (bp)
rs3814995 (T>C)	GATGACGCGGAGTATGAGTG	CATACCCAGGATGGAGAGGA	56	90
rs437168 (G>A)	AGGACCCCACTGAGGTGAAC	GCTGGATCCTCACCAGTCTC	56	111
rs10409299 (A>G)	GTTGGGTCAAATGCCTGTGT	TTCAGGCAGGACTCCACAGT	68	102
rs2071327 (T>C)	GGCAGCACTCAACTTCC	TGAGTGTCCCGCTCTCCT	58	104
rs460560 (T>C)	GATCGCTTGAGCCCATAAG	TACAGGTGCACACCTCCAT	62	103
rs17777002 (A>G)	CGAAGTGCTGGGATTACGA	TGAATTTTCAGGAAGTGACCAG	62	100

TA= Annealing temperature

2.1.6 Statistical analysis

Data were presented as means and standard deviation for normally distributed variables and as median (25th and 75th percentile) for skewed data. Differences in characteristics among groups were compared using the χ^2 test for categorical variables. For continuous variables that were normally distributed, analysis of variance was used to compare differences in mean among groups. Otherwise, the non-parametric Kruskal-Wallis test was implemented and the medians were compared.

Hardy-Weinberg equilibrium (HWE) tests and estimation of LD strength for the SNPs were both performed using Haploview version 4.2 (http://www.broadinstitute.org/) [84].

Distribution of ACR was skewed hence natural logarithmic transformation was performed for the purpose of analysis. SNP association analyses for DN according to ACR were calculated using logistic regression reporting odds ratio. An additive genetic model was assumed where individuals were assigned as 0/1/2 according to the number of minor alleles. SNP association analyses for lnACR and eGFR traits were performed using linear regression assuming an additive model of inheritance.

The haplotype analyses were performed using *haplo.glm* function in R package *haplo.stats* (http://cran.stat.nus.edu.sg/). Haplotype frequency cut-off was set at 5%. The posterior probabilities of pairs of haplotypes for each subject were estimated using the maximum likelihood method and the estimated probabilities were subsequently used as weights in the regression model with lnACR or eGFR as dependent variables, and haplotypes and environmental variables as independent variables. Interaction between haplotypes and the environmental variable (e.g. age and DM duration) was investigated and significant

interactions were retained in the regression model. Haplotype association analysis with DN according to albuminuric status was performed using *haplo.score* function in R. Effects of statistically significant confounders were adjusted for by including the confounders in the regression model.

The T2DM-related traits that were considered were BMI, waist-to-hip ratio (WHR), fasting glucose, fasting insulin, HOMA-insulin resistance and HOMA-β-cell function. The distributions of fasting glucose, fasting insulin, HOMA-insulin resistance and HOMA-β-cell function were not normally distributed and were presented as geometric mean (range) as recommended [83]. Skewed values were normalised by natural logarithmic transformation for regression analysis and subsequently back transformed for presentation. SNP association analyses for T2DM-related traits were performed using linear regression assuming an additive genetic model. SNP association analyses for T2DM according to glucose tolerance status were performed using logistic regression. Analyses were stratified according to ethnic group and adjusted for age and gender. BMI and HOMA-insulin resistance were additionally adjusted for when testing SNP association with HOMA-β-cell function [83].

Haplotype association analysis with T2DM according to glucose tolerance status was performed using *haplo.score* function in R. Effects of potential confounders which were significantly different among the case-control groupings were adjusted for by including them in the statistical models.

For all statistical analyses, type I error was set to 5%. In order to mimimise false positive findings, Bonferroni correction for multiple comparisons was made according to the number of SNPs or haplotypes tested. All statistical analyses were performed using Stata V.11 (http://www.stata.com) unless otherwise stated.

CHAPTER 3: RESULTS

3.1 Aim 1

To investigate the association of *NPHS1* SNPs with renal traits including eGFR and albuminuria in T2DM Chinese patients in Singapore. These patients were from the SDCS described in Methods 2.1.1.

Only genotype data of Chinese subjects from the SDCS were available. This formed a case-control study where T2DM subjects were grouped according to their albuminuric status (stages of DN): controls were normoalbuminuric (ACR<30mg/g) and cases were microalbuminuric (30≤ACR<300mg/g) and macroalbuminuric (ACR≥300mg/g). SNP and haplotype association analyses with stages of DN were performed. Treating the renal traits, lnACR and eGFR as continuous variables, their association with *NPHS1* SNPs and haplotypes were also investigated.

3.1.1 Clinical characteristics of SDCS subjects

Subjects were grouped according to albuminuric status. There were 870 normoalbuminuric controls, 339 microalbuminuric and 95 macroalbuminuric cases (Table 5). There were 59 patients with missing ACR values who were excluded from analysis but subsequently included for eGFR analysis. The three groups were similar in terms of gender composition, body mass index (BMI), diastolic blood pressure (DBP), high density lipoprotein (HDL) levels and the proportion of patients taking lipid lowering medication. The cases were slightly older, had marginally higher waist-to-hip ratio (WHR), systolic blood pressure (SBP), triglycerides, total cholesterol and low density lipoprotein (LDL) levels ($P<0.05$). Cases also had a significantly longer median duration

of diabetes, higher glycatedhaemoglobin (HbA_{1c}) levels, lower eGFR and a greater proportion of individuals on antihypertensive and diabetes medication (P<0.0001).

Table 5.Clinical characteristics of SDCS patients stratified by albuminuric status.

	CTRLS	MICRO	MACRO	*P*
Number (n)	870	339	95	
Male gender, n (%)	432 (49.7)	183 (54.0)	45 (47.4)	0.323
At enrolment				
Age (years)	63.3 (9.1)	64.9 (10.2)	66.1 (11.3)	0.007
Body mass index (kg/m^2)	25.2 (3.7)	25.8 (4.1)	25.5 (4.1)	0.158
Waist-to-hip ratio	0.90 (0.07)	0.91 (0.07)	0.91 (0.07)	0.012
Diabetes duration (years)	5.5 (2-12)	8 (3-17)	10 (4-18)	<0.0001
Haemoglobin A_{1c}(%)	7.26 (0.94)	7.51 (1.05)	7.79 (1.47)	<0.0001
Systolic blood pressure (mmHg)	133.66 (12.25)	134.52 (12.12)	139.25 (14.33)	0.001
Diastolic blood pressure (mmHg)	77.32 (7.56)	77.40 (7.46)	79.08 (8.92)	0.302
Triglyceride (mmol/L)	1.42 (0.78)	1.56 (0.74)	1.63 (0.76)	0.004
Total cholesterol (mmol/L)	4.69 (0.75)	4.76 (0.76)	4.87 (1.18)	0.005
High density lipoprotein (mmol/L)	1.28 (0.33)	1.23 (0.36)	1.22 (0.35)	0.145
Low density lipoprotein (mmol/L)	2.78 (0.64)	2.84 (0.63)	2.88 (1.01)	0.005
eGFR (ml/min/1.73m^2)	80.54 (21.61)	75.24 (23.18)	68.88 (25.77)	<0.0001
Antihypertensive medication (Y/N)	702/154	295/41	85/9	<0.0001
Lipid lowering medication (Y/N)	741/115	281/55	81/13	0.862
Diabetes medication (Y/N)	639/219	283/53	81/14	<0.0001
Albumin creatinine ratio (mg/g)	8.1 (4.1-14.6)	66.6 (42.4-128.3)	694.0 (413.5-970.4)	NA

CTRL, normoalbuminuriccontrols; MICRO, cases with microalbuminuria; MACRO, cases with macroalbuminuria

3.1.2 HWE and LD of NPHS1 SNPs in SDCS subjects

Genotype data was available for 1363 Chinese patients from SDCS at the time of this study. All six SNPs did not deviate from that expected under HWE among SDCS subjects as well as among subjects stratified by albuminuric status (Supplementary Tables 1 and 2). As expected, the six SNPs were in low LD with each other (Supplementary Table 3).

3.1.3 NPHS1 and albuminuria

3.1.3.1 Association of NPHS1 SNPs with stages of DN

All six common variants of *NPHS1* were not associated with DN in the additive genetic model with adjustment for significant clinical covariates (Table 6). The dominant and recessive models of inheritance also did not reveal any significant associations (Supplementary Tables 4 and 5).

Table 6. Association of *NPHS1* SNPs with stages of DN (additive model).

SNP		CTRL (1)	MICRO (2)	MACRO (3)	(2) vs (1) OR (95% CI)	*P**	(3) vs (1) OR (95% CI)	*P**
rs3814995	*TT*	311 (36.6)	126 (37.9)	34 (36.6)				
	CT	399 (46.9)	159 (47.9)	47 (50.5)	0.95 (0.78-1.17)	0.628	0.97 (0.67-1.41)	0.889
	CC	140 (16.5)	47 (14.2)	12 (12.9)				
rs437168	*GG*	613 (71.0)	244 (72.4)	72 (76.6)				
	AG	225 (26.1)	86 (25.5)	19 (20.2)	0.81 (0.61-1.08)	0.155	0.70 (0.41-1.20)	0.193
	AA	25 (2.9)	7 (2.1)	3 (3.2)				
rs10409299	*AA*	469 (55.4)	189 (57.8)	50 (53.2)				
	AG	321 (37.9)	117 (35.8)	37 (39.4)	0.96 (0.76-1.21)	0.725	1.17 (0.78-1.76)	0.437
	GG	57 (6.7)	21 (6.4)	7 (7.4)				
rs2071327	*TT*	340 (40.4)	144 (43.1)	39 (41.5)				
	CT	387 (46.0)	157 (47.0)	44 (46.8)	0.87 (0.70-1.08)	0.207	0.96 (0.66-1.40)	0.828
	CC	115 (13.7)	33 (9.9)	11 (11.7)				
rs460560	*TT*	746 (87.2)	299 (89.3)	85 (91.4)				
	CT	108 (12.6)	36 (10.7)	8 (8.6)	0.83 (0.54-1.28)	0.380	0.59 (0.25-1.44)	0.251
	CC	2 (0.2)	0 (0.0)	0 (0.0)				
rs17777002	*AA*	671 (79.2)	267 (80.2)	76 (81.7)				
	AG	167 (19.7)	61 (18.3)	17 (18.3)	0.95 (0.69-1.31)	0.763	0.79 (0.73-1.45)	0.440
	GG	9 (1.1)	5 (1.5)	0 (0.0)				

*Adjusted for age, WHR, DM duration, HbA_{1c}%, SBP, triglycerides, cholesterol, LDL, eGFR, antihypertensive medication and diabetes medication.

3.1.3.2 Association of NPHS1 SNPs with lnACR

None of the SNPs were associated with lnACR across all patients with or without adjustment for significant clinical covariates (Table 7).

Table 7. Association of *NPHS1*SNPs with lnACR among all patients.

SNP	Genotype	N	Mean (SD)	95% CI	*P*	*P**
rs3814995	*TT*	471	2.93 (1.67)	2.78 - 3.08	0.492	0.602
	CT	605	2.96 (1.67)	2.82 - 3.10		
	CC	199	2.80 (1.58)	2.58 - 3.02		
rs437168	*GG*	929	2.95 (1.68)	2.85 - 3.06	0.246	0.052
	AG	330	2.88 (1.59)	2.71 - 3.06		
	AA	35	2.61 (1.74)	2.01 - 3.21		
rs10409299	*AA*	708	2.90 (1.65)	2.78 - 3.02	0.455	0.211
	AG	475	2.96 (1.66)	2.81 - 3.11		
	GG	85	3.00 (1.79)	2.61 - 3.39		
rs2071327	*TT*	523	2.96 (1.64)	2.82 - 3.10	0.540	0.800
	CT	588	2.94 (1.68)	2.80 - 3.08		
	CC	159	2.86 (1.62)	2.61 - 3.12		
rs460560	*TT*	1130	2.96 (1.67)	2.86 - 3.05	0.112	0.127
	CT	152	2.75 (1.59)	2.49 - 3.00		
	CC	2	2.12 (0.16)	0.67 - 3.57		
rs17777002	*AA*	1014	2.96 (1.67)	2.86 - 3.06	0.262	0.216
	AG	245	2.81 (1.59)	2.62 - 3.01		
	GG	14	2.93 (1.83)	1.87 - 3.99		

*Adjusted for DM duration, HbA_{1c}%, SBP, cholesterol, HDL and eGFR.

3.1.3.3 Association of NPHS1 haplotypes with stages of DN

With reference to the common haplotype T/G/A/T/T/A, none of the haplotypes showed significant association with stages of DN (Table 8).

Table 8. Association of *NPHS1* haplotypes with stages of DN.

							(2) vs (1)		(3) vs (1)	
	CTRL (1)		MICRO (2)		MACRO (3)		*P**		*P**	
Haplotype	Freq (%)	95%CI	Freq (%)	95%CI	Freq (%)	95%CI	Specific	Global	Specific	Global
T/G/A/T/T/A	33.0	29.9-36.2	34.8	29.7-40.1	32.4	23.4-43.0	0.705	0.746	0.636	0.884
T/G/A/T/T/G	5.2	3.9-7.0	5.6	3.4-8.6	7.6	3.0-14.6	0.633		0.808	
T/G/G/C/T/A	15.1	12.7-17.6	15.6	11.9-19.9	17.5	10.8-27.1	0.858		0.336	
C/G/A/T/T/A	14.4	12.1-16.9	16.8	13.0-21.2	18.2	10.8-27.1	0.126		0.143	
C/A/A/T/T/A	7.0	5.4-8.9	6.4	4.1-9.7	5.6	1.7-11.9	0.470		0.314	
Total	74.7		79.2		81.3					

*Adjusted for age, WHR, DM duration, HbA_{1c}%, SBP, triglycerides, cholesterol, LDL, eGFR, antihypertensive medication and DM medication.

3.1.3.4 Association of NPHS1 haplotypes with lnACR

The cut-off frequency for haplotype analysis was set at 5% and five haplotypes were obtained (Table 9). These haplotypes account for 76% of all haplotypes. Haplotypes of *NPHS1* were generally not associated with lnACR. One haplotype C/G/A/T/T/A showed borderline association but this did not remain significant after taking into account of multiple comparisons.

Table 9. Association of *NPHS1*haplotypes with lnACR among all patients.

Haplotype	Freq (%)	Mean (se) of lnACR	95%CI	*P**	
				Specific	Global
T/G/A/T/T/A	33.4	2.80 (0.7)	1.45 - 4.16	0.916	0.336
T/G/G/C/T/A	15.3	2.97 (0.7)	1.60 - 4.35	0.177	
C/G/A/T/T/A	15.2	3.03 (0.7)	1.66 - 4.41	0.049	
C/A/A/T/T/A	6.7	2.68 (0.7)	1.29 - 4.08	0.122	
T/G/A/T/T/G	5.4	2.92 (0.7)	1.52 - 4.32	0.706	

* Adjusted for SBP, HbA_{1c}%, DM duration, HDL, cholesterol and eGFR.

3.1.3.5 Interaction of NPHS1 haplotypes with age on lnACR

The potential for haplotype interactions with patient characteristics on lnACR were investigated. Particularly, we focused on age and DM duration. Using the most common haplotype as the reference, none of the remaining haplotypes showed significant interaction with age (Table 10). Rare haplotypes grouped as a single category exhibited significant interaction but this could not be interpreted meaningfully (P=0.004, $P_{corrected}$=0.024).

Table 10. Interaction of *NPHS1* haplotypes with age on lnACR.

Haplotype	Freq (%)	Slope/unit age (yrs)	95% CI	*P**
T/G/A/T/T/A	33.3	-0.013	-0.040;0.013	(reference)
T/G/G/C/T/A	15.3	0.003	-0.037;0.043	0.279
C/G/A/T/T/A	15.3	0.003	-0.036;0.042	0.249
C/A/A/T/T/A	6.7	-0.034	-0.083;0.014	0.312
T/G/A/T/T/G	5.4	-0.014	-0.058;0.029	0.946
Rare	N/A	0.014	-0.018;0.047	0.004

* Adjusted for SBP, HbA_{1c}%, DM duration, eGFR, cholesterol and HDL.

3.1.3.6 Interaction of NPHS1 haplotypes with DM duration on lnACR

Relative to the common haplotype, none of the remaining haplotypes showed any significant interaction with DM duration on lnACR (Table 11).

Table 11. Interaction of *NPHS1* haplotypes with DM duration on lnACR.

Haplotype	Freq (%)	Slope/unit DM duration (yrs)	95% CI	*P**
T/G/A/T/T/A	33.3	0.012	-0.016;0.039	(reference)
T/G/G/C/T/A	15.3	0.020	-0.018;0.057	0.544
C/G/A/T/T/A	15.2	0.035	-0.006;0.076	0.127
C/A/A/T/T/A	6.7	0.028	-0.020;0.076	0.408
T/G/A/T/T/G	5.4	0.018	-0.034;0.069	0.780
Rare	N/A	0.012	-0.022;0.047	0.934

*Adjusted for SBP, HbA_{1c}%, eGFR, cholesterol and HDL.

3.1.4 NPHS1 and eGFR

3.1.4.1 Association of NPHS1 SNPs with eGFR

None of the SNPs were associated with eGFR across all patients with or without adjustment for significant clinical covariates (Table 12).

Table 12. Association of *NPHS1*SNPs with eGFR among all patients.

SNP	Genotype	N	Mean (SD)	95% CI	*P*	*P**
rs3814995	*TT*	492	77.77 (22.40)	75.79 - 79.76	0.702	0.724
	CT	627	78.70 (23.61)	76.85 - 80.55		
	CC	208	78.15 (20.44)	75.36 - 80.94		
rs437168	*GG*	965	78.48 (22.42)	77.07 - 79.90	0.559	0.366
	AG	343	77.63 (23.68)	75.11 - 80.14		
	AA	40	77.68 (21.45)	70.82 - 84.54		
rs10409299	*AA*	732	78.05 (22.78)	76.40 - 79.70	0.613	0.366
	AG	502	78.42 (22.34)	76.46 - 80.38		
	GG	88	79.32 (22.85)	74.48 - 84.17		
rs2071327	*TT*	539	77.69 (22.28)	75.80 - 79.57	0.426	0.473
	CT	620	78.17 (23.32)	76.33 - 80.01		
	CC	164	79.39 (21.65)	76.05 - 82.72		
rs460560	*TT*	1176	78.13 (22.62)	76.84 - 79.43	0.979	0.390
	CT	159	77.69 (23.14)	74.06 - 81.31		
	CC	2	96.68 (34.70)	-215.06 - 408.42		
rs17777002	*AA*	1056	78.03 (22.63)	76.67 - 79.40	0.945	0.473
	AG	254	78.15 (22.76)	75.33 - 80.96		
	GG	16	78.11 (25.29)	64.64 - 91.59		

*Adjusted for gender, age, DBP, lnACR, triglycerides, HDL and antihypertensive medication.

3.1.4.2 Association of NPHS1 haplotypes with eGFR

There were no significant associations between *NPHS1* haplotypes with eGFR among all patients (Table 13).

Table 13. Association of *NPHS1* haplotypes with eGFR among all patients.

Haplotype	Freq (%)	Mean (se) of eGFR	95%CI	*P**	
				Specific	Global
T/G/A/T/T/A	33.5	77.13 (0.2)	76.77 - 77.49	0.344	1.000
T/G/G/C/T/A	15.4	78.58 (1.4)	75.79 - 81.36	0.391	
C/G/A/T/T/A	15.3	78.27 (1.5)	75.41 - 81.14	0.602	
C/A/A/T/T/A	6.7	77.25 (2.0)	73.32 - 81.17	0.766	
T/G/A/T/T/G	5.4	75.84 (2.2)	71.52 - 80.16	0.320	

*Adjusted for age, gender, DBP, HDL, triglycerides, lnACR and antihypertensive medication.

3.1.4.3 Interaction of NPHS1 haplotypes with age on eGFR

Negative slopes of eGFR over age were observed for all haplotypes (Table 14 and Figure 11). There was significant evidence for interaction of *NPHS1* haplotypes with age on eGFR. Specifically, with reference to the common T/G/A/T/T/A haplotype, carriers of T/G/G/C/T/A had higher eGFR values among younger patients but had lower eGFR values among older patients (P= 2.9 x 10^{-8}, $P_{corrected}$= 1.7 x 10^{-7}). C/A/A/T/T/A carriers also reported high eGFR values in younger patients compared to the common haplotype (P<0.0001). In contrast, carriers of T/G/A/T/T/G had lower eGFR values among younger patients with reference to the common haplotype (P= 1.9 x 10^{-5}, $P_{corrected}$= 1.1 x 10^{-4}). These interactions were still significant after adjusting for possible confounding clinical covariates and correcting for multiple comparisons.

Table 14. Interaction of *NPHS1* haplotypes with age on eGFR.

Haplotype	Freq (%)	Slope/unit Age (yrs)	95% CI	*P**
T/G/A/T/T/A	33.5	-0.86	-0.97;-0.75	(reference)
T/G/G/C/T/A	15.4	-1.01	-1.13;-0.89	2.9×10^{-8}
C/G/A/T/T/A	15.3	-0.9	-1.02;-0.78	1.8×10^{-1}
C/A/A/T/T/A	6.8	-1.16	-1.29;-1.04	<0.0001
T/G/A/T/T/G	5.5	-0.72	-0.84;-0.59	1.9×10^{-5}
Rare	N/A	-0.79	-0.97;-0.61	3.0×10^{-1}

*Adjusted for gender, DBP, HDL, triglycerides, lnACR and antihypertensive medication.

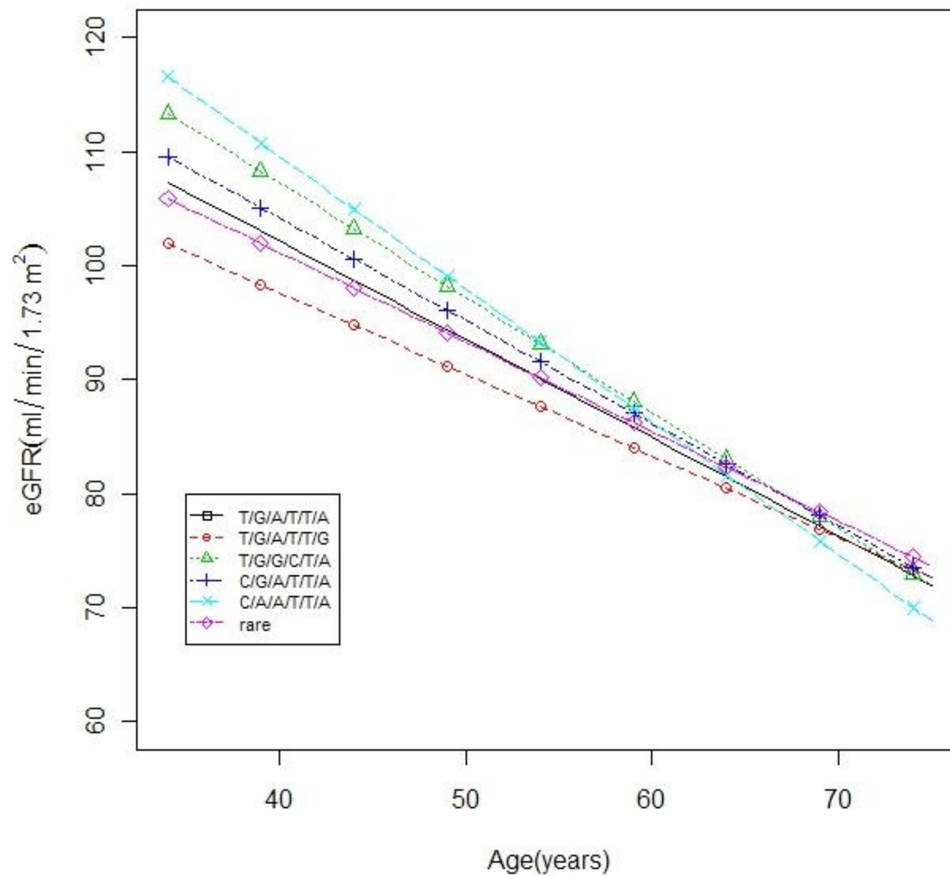


Figure 11. Slopes of eGFR over age according to *NPHS1* haplotypes.

3.1.4.4 Interaction of NPHS1 haplotypes with DM duration on eGFR

There was no significant haplotype interaction with DM duration on slope of eGFR for all haplotypes (Table 15).

Table 15. Interaction of *NPHS1* haplotypes with DM duration on eGFR.

Haplotype	Freq (%)	Slope/unit DM duration (yrs)	95% CI	*P**
T/G/A/T/T/A	33.6	0.05	-0.26;0.37	(ref category)
T/G/G/C/T/A	15.4	-0.26	-0.71;0.19	0.056
C/G/A/T/T/A	15.3	-0.04	-0.50;0.42	0.580
C/A/A/T/T/A	6.7	-0.07	-0.62;0.48	0.584
T/G/A/T/T/G	5.4	-0.30	-0.92;0.32	0.188
Rare	N/A	0.11	-0.29;0.52	0.646

*Adjusted for gender, DBP, HDL, triglycerides, lnACR and antihypertensive medication.

3.2 AIM 2

To investigate the association of *NPHS1* SNPs with T2DM-related traits in non-diabetic Chinese, Malay and Indian subjects in Singapore. These subjects were from the NHS98 described in Methods 2.1.2.

All subjects from the NHS98 were included. This was a cross-sectional study comprising of Chinese, Malay and Asian Indian subjects. The subjects were analysed for the association of *NPHS1* SNPs with T2DM-related traits like BMI, WHR, fasting glucose, fasting insulin, HOMA-insulin resistance and HOMA-β-cell function according to ethnicity.

3.2.1 Clinical Characteristics of NHS98 subjects

There were a total of 2952 Chinese, 792 Malay and 603 Asian Indian subjects (Table 16). Among these subjects, Malay and Asian Indian subjects were slightly older and had a higher BMI and WHR than the Chinese population. There were slightly fewer males in the Chinese subjects while gender composition was similar for the Malay and Indian subjects. There was a higher prevalence of T2DM in the Malay and Indian populations. The Malay population had higher blood pressure values, total cholesterol, LDL and HbA_{1c}% compared to the rest. The Asian Indians had the highest fasting glucose, fasting insulin, HOMA-insulin resistance, HOMA-β-cell function followed by the Malay and Chinese populations.

Table 16. Clinical characteristics of NHS98 subjects stratified by ethnicity.

	Chinese	Malay	Asian Indian
Number (n)	2953	792	603
Age (years)	37.9 (12.2)	38.9 (12.6)	40.5 (12.0)
Male gender, n (%)	1342 (45.4)	380 (48.0)	289 (47.9)
Weight (kg)	60.5 (12.1)	65.7 (13.5)	66.2 (13.0)
Height (m)	162.8 (8.4)	160.3 (8.6)	162.5 (9.3)
Body mass index (kg/m^2)	22.7 (3.7)	25.6 (5.0)	25.1 (4.7)
Waist-to-hip ratio	0.82 (0.07)	0.83 (0.08)	0.85 (0.08)
Systolic blood pressure (mmHg)	120.7 (16.4)	124.7 (19.3)	121.2 (17.1)
Diastolic blood pressure (mmHg)	73.8 (11.3)	76.1 (12.1)	73.6 (12.0)
Total Cholesterol (mmol/L)	5.42 (1.0)	5.81 (1.2)	5.50 (1.1)
Triglycerides (mmol/L)	1.40 (1.2)	1.68 (1.3)	1.68 (1.4)
High density lipoprotein (mmol/L)	1.42 (0.4)	1.30 (0.3)	1.15 (0.3)
Low density lipoprotein (mmol/L)	3.39 (1.0)	3.86 (1.1)	3.70 (1.0)
Haemoglobin A_{1c} (%)	8.5 (2.1)	8.9 (2.1)	8.0 (2.0)
Haemoglobin (mmol/L)	13.9 (1.6)	14.0 (1.7)	13.8 (1.9)
Fasting Glucose (mmol/L)*^	5.5 (0 - 17.6)	5.7 (3.5 - 23)	5.8 (4.1 - 19.9)
Fasting Insulin (mU/L)*^	6.1 (0.2 - 53.3)	7.2 (0.7 - 83.4)	8.6 (1 - 119)
HOMA Insulin resistance*^	1.48 (0 - 11.9)	1.84 (0.1 - 22.2)	2.19 (0.2 - 31.2)
HOMA β-cell function (%)*^	64.2 (2.1 - 761.4)	69.3 (2.8 - 667.2)	80.7 (6.1 - 991.7)
Diabetes medication, n	59	35	42
Glucose tolerance, n (%)			
NGT	2205 (74.7)	475 (60.0)	367 (60.9)
IFG	162 (5.5)	60 (7.6)	36 (6.0)
IGT	360 (12.2)	143 (18.0)	84 (13.9)
T2DM	225 (7.6)	114 (14.4)	116 (19.2)

*Geometric mean (range) shown due to skewed nature of data; ^Subjects taking diabetes medication were excluded (59 Chinese, 35 Malays and 42 Asian Indians); NGT, normal glucose tolerance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; T2DM, type 2 diabetes mellitus.

3.2.2 HWE and LD of NPHS1 SNPs in NHS98 subjects

Genotype data from 4348 subjects from NHS98 were available at the time of this study. Genotype distributions of all six SNPs were in HWE across all ethnic groups (Supplementary Table 6). LD was low for all SNPs in all populations and displayed similar trends for all races (Supplementary Table 7).

3.2.3 NPHS1 and T2DM-related traits

3.2.3.1 Association of NPHS1 SNPs with T2DM-related traits in all subjects

The risk allele for SNP rs437168 was associated with a lower WHR among Chinese and was able to withstand Bonferroni correction (P=0.004, $P_{corrected}$=0.024) (Table 17). This association became stronger after analysis was restricted to include only non-diabetic subjects (P=0.002, $P_{corrected}$=0.012) (Supplementary Table 8). On the other hand, the Malays and Asian Indians reported a higher WHR as number of risk alleles increased but the association failed to reach statistical significance.

There was an association of risk allele for SNP rs17777002 with higher WHR in the Asian Indian population (P=0.005, $P_{corrected}$=0.030) (Table 17). This association remained significant with the exclusion of diabetic subjects (P=0.003, $P_{corrected}$=0.018) (Supplementary Table 8).

SNPs rs3814995, rs10409299, rs2071327 and rs460560 did not appear to have any associations with any of the T2DM-related traits in all races that could withstand correction for multiple comparisons.

Table 17. Association of *NPHS1* SNPs with T2DM-related traits in NHS98 subjects.

	Chinese			*P**	Malay			*P**	Asian Indian			*P**
Number of risk alleles	**0**	**1**	**2**		**0**	**1**	**2**		**0**	**1**	**2**	
rs3814995 (T>C)												
BMI (kg/m^2)	22.9	22.7	22.4	0.011	25.7	25.6	25.5	0.626	24.2	24.8	25.4	0.030
WHR	0.826	0.824	0.821	0.092	0.832	0.833	0.834	0.723	0.843	0.852	0.860	0.022
Fasting glucose (mmol/l)†^	5.4	5.5	5.5	0.299	5.7	5.7	5.7	0.634	5.7	5.7	5.8	0.414
Fasting insulin (mmol/l)†^	6.1	6.1	6.1	0.878	7.3	7.3	7.2	0.748	8.8	8.7	8.6	0.633
HOMA-insulin resistance†^	1.5	1.5	1.5	0.983	1.9	1.8	1.8	0.906	2.2	2.2	2.2	0.811
HOMA-β-cell function (%)†^#	65.0	64.3	63.6	0.315	70.7	69.9	69.1	0.632	84.9	82.6	80.4	0.367
rs437168 (G>A)												
BMI (kg/m^2)	22.8	22.6	22.5	0.265	25.5	26.1	26.7	0.096	25.1	25.3	25.5	0.669
WHR	0.826	0.821	0.815	**0.004**	0.831	0.838	0.845	0.089	0.856	0.858	0.860	0.738
Fasting glucose (mmol/l)†^	5.5	5.5	5.5	0.709	5.7	5.7	5.7	0.844	5.8	5.7	5.5	0.278
Fasting insulin (mmol/l)†^	6.1	6.0	5.9	0.215	7.3	7.0	6.8	0.405	8.8	8.0	7.3	0.119
HOMA-insulin resistance†^	1.5	1.5	1.4	0.253	1.9	1.8	1.7	0.468	2.2	2.0	1.8	0.068
HOMA-β-cell function (%)†^#	64.5	64	63.5	0.579	70.2	69.3	68.4	0.741	81.8	80.7	79.6	0.792
rs10409299 (A>G)												
BMI (kg/m^2)	22.7	22.7	22.7	0.747	25.7	25.5	25.3	0.414	25.4	25.2	25.3	0.781
WHR	0.824	0.824	0.825	0.626	0.832	0.833	0.834	0.781	0.856	0.854	0.851	0.489
Fasting glucose (mmol/l)†^	5.4	5.5	5.5	0.196	5.7	5.7	5.6	0.491	5.8	5.7	5.6	0.316
Fasting insulin (mmol/l)†^	6.0	6.1	6.3	0.188	7.1	7.4	7.7	0.167	8.7	8.6	8.5	0.763
HOMA-insulin resistance†^	1.5	1.5	1.5	0.131	1.8	1.9	1.9	0.329	2.2	2.2	2.1	0.588
HOMA-β-cell function (%)†^#	64.5	64.0	63.6	0.537	68.7	71.2	73.8	0.200	81.7	82.7	83.7	0.721
rs2071327 (T>C)												
BMI (kg/m^2)	22.7	22.7	22.7	0.973	25.8	25.5	25.2	0.266	25.3	25.1	24.9	0.504
WHR	0.824	0.824	0.825	0.706	0.834	0.831	0.828	0.314	0.855	0.855	0.856	0.826
Fasting glucose (mmol/l)†^	5.5	5.5	5.5	0.609	5.8	5.7	5.6	0.129	5.7	5.8	5.8	0.542
Fasting insulin (mmol/l)†^	6.1	6.1	6.2	0.354	7.2	7.3	7.3	0.765	8.8	8.6	8.5	0.476
HOMA-insulin resistance†^	1.5	1.5	1.5	0.342	1.8	1.8	1.8	0.898	2.2	2.2	2.2	0.689
HOMA-β-cell function (%)†^#	64.5	64.5	64.4	0.929	68.1	70.7	73.5	0.138	84.2	81.7	79.2	0.276
rs460560 (T>C)												
BMI (kg/m^2)	22.7	22.7	22.7	0.988	25.5	26.2	26.9	0.172	25.2	24.9	24.6	0.365
WHR	0.824	0.823	0.822	0.590	0.833	0.835	0.836	0.754	0.857	0.853	0.848	0.334
Fasting glucose (mmol/l)†^	5.5	5.4	5.4	0.488	5.7	5.6	5.4	0.101	5.8	5.8	5.8	0.934
Fasting insulin (mmol/l)†^	6.1	6.0	5.9	0.386	7.3	7.0	6.8	0.587	8.8	8.4	8.1	0.257
HOMA-insulin resistance†^	1.5	1.4	1.4	0.329	1.8	1.7	1.6	0.344	2.2	2.1	2.0	0.280
HOMA-β-cell function (%)†^#	64.3	64.7	65.1	0.746	69.3	74.4	80.0	0.152	82.6	81.4	80.3	0.689
rs17777002 (A>G)												
BMI (kg/m^2)	22.7	22.9	23.2	0.072	25.6	25.7	25.9	0.751	25.0	25.3	25.6	0.360
WHR	0.824	0.824	0.824	0.922	0.832	0.835	0.838	0.549	0.850	0.863	0.875	**0.005**
Fasting glucose (mmol/l)†^	5.5	5.5	5.5	0.797	5.7	5.7	5.7	0.888	5.7	5.8	6.0	0.174
Fasting insulin (mmol/l)†^	6.1	6.1	6.1	0.791	7.3	7.1	7.0	0.721	8.7	8.5	8.4	0.701
HOMA-insulin resistance†^	1.5	1.5	1.5	0.740	1.8	1.8	1.8	0.790	2.2	2.2	2.2	0.813
HOMA-β-cell function (%)†^#	64.4	64.7	64.9	0.805	70.2	70.5	70.9	0.917	83	78.9	75.1	0.177

*All analyses were gender and age adjusted; †values were natural log transformed to improve normality in regression analysis, and adjusted means were subsequently back transformed; ^additionally adjusted for BMI and excluding subjects taking diabetic medication (59 Chinese, 35 Malays and 42 Asian Indians); #additionally adjusted for insulin resistance; bold-faced *P*-values are <0.05 after Bonferroni correction.

3.3 AIM 3

To investigate the association of *NPHS1* SNPs with T2DM in Chinese subjects in Singapore. Cases with T2DM were from both SDCS and NHS98 (Methods 2.1.1 and 2.1.2) and non-diabetic controls were from NHS98 (Methods 2.1.2).

The number of T2DM cases in NHS98 was relatively limited. Hence by taking in Chinese cases from SDCS, a larger case-control study for T2DM could be conducted for the Chinese population. This formed a case-control study where cases were T2DM patients from both SDCS and NHS98, and controls were NGT and IFG/IGT subjects from NHS98. The association of single *NPHS1* SNPs and haplotypes with T2DM was examined and the odds ratios were reported.

3.3.1 Clinical characteristics of Chinese SDCS and NHS98 subjects

Patients were grouped according to their glucose tolerance status. Subjects with NGT were controls while individuals with IFG/IGT and T2DM were cases. Controls had slightly fewer males compared to cases (Table 18). Cases were much older than controls and had higher BMI, WHR, SBP and DBP values. In addition, the proportion of subjects on antihypertensive medication was significantly larger in cases than controls.

Table 18. Clinical characteristics of Chinese patients from NHS98 and SDCS stratified by glucose tolerance status.

	NGT	IFG/IGT	T2DM	*P*
Number (n)	2205	522	1588	
Age (years)	35.3 (11.2)	43.2 (11.4)	62.1 (11.0)	<0.0001
Male gender, n (%)	969 (43.9)	263 (50.4)	786 (49.5)	0.002
Body mass index (kg/m^2)	22.1 (3.4)	24.3 (3.6)	25.4 (3.9)	<0.0001
Waist-to-hip ratio	0.81 (0.07)	0.85 (0.07)	0.90 (0.07)	<0.0001
Systolic blood pressure (mmHg)	117.5 (13.8)	127.4 (17.3)	134.6 (14.2)	<0.0001
Diastolic blood pressure (mmHg)	71.8 (10.5)	78.5 (10.9)	78.0 (8.6)	<0.0001
Total cholesterol (mmol/L)	5.3 (1.0)	5.8 (1.1)	4.9 (1.0)	<0.0001
Triglyceride (mmol/L)	1.2 (0.8)	1.8 (1.4)	1.6 (1.2)	<0.0001
High density lipoprotein (mmol/L)	1.5 (0.4)	1.3 (0.4)	1.3 (0.3)	<0.0001
Low density lipoprotein (mmol/L)	3.2 (0.9)	3.7 (1.0)	3.0 (0.8)	<0.0001
Haemoglobin A_{1C} (%)	NA	NA	7.4 (1.1)	NA
Diabetes duration (years)	0	0	6 (2-14)	<0.0001
Diabetes medication, n	0	0	1110	<0.0001
Antihypertensive medication (Y/N)	79/2126	56/466	1198/371	<0.0001

3.3.2 HWE of NPHS1 SNPs in Chinese SDCS and NHS98 subjects

All SNPs did not deviate from HWE among subjects grouped according to glucose tolerance status except for SNP rs3814995 of the NGT group (P=0.0002) (Supplementary Table 9). It had a slight excess of heterozygotes than expected but due to the large sample size of the NGT group, it became significant. However, SNP rs3814995 was retained in the analysis for completeness.

3.3.3 NPHS1 and glucose tolerance status in Chinese

3.3.3.1 Association of NPHS1 SNPs with IGT/IFG and T2DM in Chinese

In the additive model, there was no significant association of any *NPHS1* variants with IGT/IFG and T2DM (Table 19). Similarly, the dominant and recessive models did not report any significant results (Supplementary Tables 10 and 11).

Table 19. Association of *NPHS1* SNPs with T2DM among Chinese (additive model).

SNP		NGT (1)	IGT/IFG (2)	T2DM (3)	(2) vs (1) OR (95% CI)	*P**	(3) vs (1) OR (95% CI)	*P**
rs3814995	*TT*	810 (37.7)	210 (41.8)	595 (38.4)				
(T>C)	*CT*	1084 (50.4)	233 (46.3)	711 (45.9)	0.94 (0.80-1.11)	0.460	1.05 (0.86-1.28)	0.632
	CC	256 (11.9)	60 (11.9)	243 (15.7)				
rs437168	*GG*	1548 (71.9)	380 (75.7)	1132 (71.9)				
(G>A)	*AG*	556 (25.8)	107 (21.3)	396 (25.1)	0.94 (0.76-1.16)	0.538	1.17 (0.90-1.53)	0.240
	AA	49 (2.3)	15 (3.0)	47 (3.0)				
rs10409299	*AA*	1204 (55.7)	290 (57.5)	847 (54.9)				
(A>G)	*AG*	822 (38.0)	184 (36.5)	594 (38.5)	0.93 (0.78-1.10)	0.390	1.07 (0.86-1.33)	0.567
	GG	135 (6.3)	30 (6.0)	103 (6.7)				
rs2071327	*TT*	921 (42.8)	210 (42.2)	631 (40.7)				
(T>C)	*CT*	948 (44.1)	216 (43.4)	724 (46.7)	1.03 (0.86-1.21)	0.680	0.99 (0.81-1.21)	0.949
	CC	283 (13.1)	72 (14.4)	195 (12.6)				
rs460560	*TT*	1895 (86.8)	436 (85.2)	1377 (88.1)				
(T>C)	*CT*	279 (12.8)	74 (14.4)	185 (11.8)	1.17(0.88-1.56)	0.283	0.80 (0.54-1.19)	0.268
	CC	9 (0.4)	2 (0.4)	2 (0.1)				
rs17777002	*AA*	1667 (76.7)	368 (72.3)	1228 (79.1)				
(A>G)	*AG*	469 (21.6)	135 (26.5)	305 (19.7)	1.16 (0.94-1.45)	0.168	0.95 (0.71-1.26)	0.700
	GG	36 (1.7)	6 (1.2)	19 (1.2)				

*Adjusted for age, gender, BMI, WHR, SBP, DBP, total cholesterol, triglyceride, HDL, LDL and antihypertensive medication.

3.3.3.2 Association of NPHS1 haplotypes with IGT/IFG and T2DM in Chinese

With reference to the most common haplotype, none of the remaining haplotypes were significantly associated with IGT/IFG or T2DM after correction (Table 20).

Table 20. Association of *NPHS1* haplotypes with T2DM among Chinese.

	NGT (1)		IGT/IFG (2)		T2DM (3)		(2) vs (1) *P**		(3) vs (1) *P**	
Haplotype	Freq (%)	95%CI	Freq (%)	95%CI	Freq (%)	95%CI	Specific	Global	Specific	Global
T/G/A/T/C/G	32.9	31-34.9	31.6	27.6-35.8	33.1	30.8-35.5	0.483	0.699	0.959	0.412
T/G/G/C/T/A	14.8	13.3-16.3	13.6	10.8-16.8	15.6	13.9-17.5	0.334		0.774	
C/G/A/T/T/A	13.0	11.7-14.5	11.3	8.7-14.3	14.5	12.8-16.3	0.438		0.715	
T/G/A/T/T/G	5.9	4.9-7	8.5	6.2-11.2	7.0	5.8-8.4	0.034		0.588	
C/A/A/T/T/A	6.3	5.4-7.4	6.0	4.1-8.3	5.4	4.3-6.6	0.752		0.021	
Total	72.9		71.0		75.6					

*Adjusted for age, gender, BMI, WHR, SBP, DBP cholesterol, triglycerides, LDL, HDL and antihypertensive medication.

CHAPTER 4: DISCUSSION

4.1 Discussion of results

In our study, we investigated whether common genetic variations of *NPHS1* were associated with renal and T2DM-related traits.

We did not find any association of individual *NPHS1* SNPs and haplotypes with albuminuria and eGFR in the Chinese population. Previous studies investigating effects of individual *NPHS1* common genetic variations in Caucasian diabetic patients also reported a lack of *NPHS1* genetic involvement in DN [75-77]. Even though our study had a larger sample size, associations did not surface. This could be a reflection of true negative results in the local Chinese population. Despite this, the potential for *NPHS1* haplotype interactions with patient characteristics on renal traits were not overlooked.

Our most significant finding suggested that *NPHS1* haplotypes interacted significantly with age on eGFR. This was consistent with the predominant view that NPHS1 plays an important role in the kidney [40-48]. On the other hand, haplotypes did not interact with DM duration on eGFR. This may potentially be related to the fact that DM duration, unlike age, is not precisely known since many patients escape T2DM diagnosis for several years. Therefore, an underlying interaction of haplotypes with DM duration on eGFR may be harder to observe. Interestingly, although NPHS1 was initially implicated in the massive proteinuria in CNF, genetic variation in *NPHS1* was not associated with albuminuria, but only with eGFR. However, this eGFR association was in line with previous observations that nephrinuria was associated with renal function [73, 74].

Common genetic variations and haplotypes of *NPHS1* were not associated with T2DM and its related traits. This finding varied from that reported in the Japanese where a SNP rs437168 common to our study was significantly associated with T2DM [80]. We did not find a similar association in our Chinese population. This could be attributed to population specific genetic differences or a limited sample size of the Funagata Study. Nonetheless, our negative findings indirectly corroborated with the findings on albuminuria in the Caucasian study on T1 and T2DM subjects although they had studied a different set of SNPs [77].

There was some indication of the association of *NPHS1* common genetic variations with WHR after correction, although the biological plausibility is less well understood. This association was novel, the first reported to our knowledge. Although its statistical significance was not very strong, especially after correction, this finding clearly needs to be replicated in other populations. Indeed, in view of the studies implicating NPHS1 in β-cell function, this association with WHR is unexpected and its biological basis is not obvious. On the other hand, our results do strongly suggest that *NPHS1* does not modulate susceptibility to β-cell dysfunction.

Despite the positive findings, a few limitations of our study should be acknowledged. Firstly, this study is cross-sectional, hence conclusions regarding the change of eGFR over age is limited. Ideally, this finding should be replicated in a cohort study where changes of eGFR over time in individual patients can be monitored. Secondly, renal function was not directly measured but was estimated using the widely used MDRD formula. This was necessarily so, as obtaining direct GFR measurements are invasive and logistically difficult to carry out in such large numbers of patients. Thirdly, we cannot

exclude the possibility that reduced renal function and albuminuria may have a non-diabetic origin. However, previous studies in T2DM patients suggest that non-diabetic renal disease in T2DM patients is relatively uncommon [85]. Fourthly, our analysis cannot be readily extrapolated to the non-Chinese ethnicities because our analysis was confined to the Chinese patients at the time of our study as the number of Malays and Asian Indians from the NHS98 was relatively smaller compared to their Chinese counterparts.

On balance, our study does have a few strengths. Firstly, our positive findings were highly significant. This was evidenced by small *P*-values which remained significant after correction. This gave us some measures of confidence that our findings were true positives although replications in other independent populations are needed to confirm their validity. Secondly, our study was conducted in a substantial number of people especially in the Chinese ethnicity. This was made possible by leveraging on the existing cohort of SDCS and NHS98. Thirdly, our study surveyed the common genetic variations across the entire *NPHS1* gene. This approach extended beyond that of the previously reported studies which had focused on only a few SNPs. Our analysis was also comprehensive since we examined both SNPs and haplotypes with the traits of interest.

4.2 Further studies

4.2.1 Follow-up studies

Given that the SDCS was started in 2004, one intriguing study that can be performed would be to assess whether these common genetic variations of *NPHS1* have associations with renal function and albuminuria from baseline until the present moment. In this regard, we would be able to assess these changes of parameters over time, instead of talking about slopes of eGFR over age or DM duration.

4.2.2 Candidate genes

Current genome wide association study efforts to identify genes associated with DN have met with limited success. As such, it might be possible to identify new susceptible genes using the candidate gene approach. In this respect, the proteins that interact with NPHS1 at the SD have been well characterised and can be studied.

Our study suggests that *NPHS1* haplotypes may modulate renal function over time. To extend this finding, it may be possible to investigate if rare genetic variants of *NPHS1* may play even stronger roles in modulating these traits. Such a study can be performed using next generation sequencing although the cost of studying many patient samples may still be prohibitive.

4.2.3 Molecular characterisation of NPHS1

Urinary NPHS1 could be measured in T2DM patients using commercially available ELISA kits. With this additional data made available, the association of nephrinuria with common genetic variations of *NPHS1* could be further investigated. Intuitively, urinary NPHS1 should arise as a result of glomerular damage due to DN and may not be closely

linked to T2DM and its related traits as NPHS1 from the β-cells are potentially unlikely to surface in the urine.

A previous study reported that podocyte-specific deletion of insulin receptors in mice resulted in albuminuria along with histological features typical of DN. Thus, there may be an interesting relationship between insulin signaling and nephrinuria and vice versa [86, 87]. In addition, insulin was found to influence the *in vitro* actin reorganisation of human podocytes resulting in higher permeability of foot processes [87]. Therefore, the association of insulin signaling with nephrinuria could be a potential area for future research.

4.3 Conclusion

Our investigation has uncovered first evidence that *NPHS1* may be potentially involved in the modulation of eGFR over time in patients with T2DM. This may have significant implication in our understanding of DN and its treatment.

BIBLIOGRAPHY

[1] Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract (2010) 87: 4-14.

[2] World Health Organisation. *About Diabetes* (2012). Accessed on 26 Jun 2012. http://www.who.int/diabetes/action_online/basics/en/index.html.

[3] International Diabetes Federation. *IDF Diabetes Atlas*, 5th edn, Brussels, Belgium: International Diabetes Federation (2011). http://www.idf.org/diabetesatlas.

[4] Ministry of Health, Singapore. *Disease Burden* (9 Jan 2012). Accessed on 26 Jun 2012.http://www.moh.gov.sg/content/moh_web/home/statistics/Health_Facts_Singapore/Disease_Burden.html.

[5] Ritz E, Rychlik I, Locatelli F, Halimi S. End-stage renal failure in type 2 diabetes: a medical catastrophe of worldwide dimensions. Am J Kidney Dis (1999) 34: 795-808.

[6] Ritz E, Zeng XX, Rychlik I. Clinical manifestation and natural history of diabetic nephropathy. Contrib Nephrol (2011) 170: 19-27.

[7] Parving HH. Renoprotection in diabetes: genetic and non-genetic risk factors and treatment. Diabetologia (1998) 41: 745-759.

[8] Singapore Renal Registry. *Report of the Singapore Renal Registry 2007/2008*, Singapore: National Registry of Diseases Office (20 Jun 2012). Accessed on 26 Jun 2012. http://www.nrdo.gov.sg/page.aspx?id=78.

[9] Najafian B, Alpers CE, Fogo AB. Pathology of human diabetic nephropathy. Contrib Nephrol (2011) 140: 36-47.

[10] Jerums G, Premaratne E, Panagiotopoulos S, Clarke S, Power DA, MacIssac RJ. New and old markers of progression of diabetic nephropathy. Diabetes Res Clin Pract (2008) 82 Suppl 1: S30-37.

[11] Najafian B, Mauer M. Progression of diabetic nephropathy in type 1 diabetic patients. Diabetes Res Clin Pract (2009) 83: 1-8.

[12] National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis (2002) 39 Suppl 1: S1-S266.

[13] Stevens LA, Coresh J, Feldman HI *et al.* Evaluation of the modification of diet in renal disease study equation in a large diverse population. J Am Soc Nephrol (2007) 18: 2749-2757.

[14] Rigalleau V, Beauvieux MC, Gonzalez C *et al.* Estimation of renal function in patients with diabetes. Diabetes Metab (2011) 37: 359-366.

[15] Johnson CA, Levey AS, Coresh J, Levin A, Lau J, Eknoyan G. Clinical practice guidelines for chronic kidney disease in adults: part II. Glomerular filtration rate, proteinuria, and other markers. Am Fam Physician (2004) 70: 1091-1097.

[16] Levey AS, Stevens LA, Schmid CH *et al.* A new equation to estimate glomerular filtration rate. Ann Intern Med (2009) 150: 604-612.

[17] Mogensen CE. Twelve shifting paradigms in diabetic renal disease and hypertension. Diabetes Res Clin Pract (2008) 82 Suppl 1: S2-9.

[18] Frische S. Glomerular filtration rate in early diabetes: ongoing discussions of causes and mechanisms. J Nephrol (2011) 24: 537-540.

[19] Banerjee S, Ghosh US, Saha SJ. Role of GFR estimation in assessment of the status of nephropathy in type 2 diabetes mellitus. J Assoc Physicians India (2005) 53: 181-184.

[20] American Diabetes Association. Standards of medical care in diabetes--2012. Diabetes Care (2012) 35 Suppl 1:S11-63.

[21] Witte EC, LambersHeerspink HJ, de Zeeuw D, Bakker SJ, de Jong PE, Gansevoort R. First morning voids are more reliable than spot urine samples to assess microalbuminuria. J Am Soc Nephrol (2009) 20: 436-443.

[22] Eknoyan G, Hostetter T, Bakris GL *et al.* Proteinuria and other markers of chronic kidney disease: a position statement of the national kidney foundation (NKF) and the national institute of diabetes and digestive kidney diseases (NIDDK). Am J Kidney Dis (2003) 42: 617-622.

[23] Remuzzi G, Schiepppati A, Ruggenenti P. Clinical practice. Nephropathy in patients with type 2 diabetes. N Engl J Med (2002) 346: 1145-1151.

[24] Kriz W, LeHir M. Pathways to nephron loss starting from glomerular diseases-insights from animal models. Kidney Int (2005) 67: 404-419.

[25] Yamada T, Komatsu M, Komiya I *et al.* Development, progression, and regression of microalbuminuria in Japanese patients with type 2 diabetes under tight glycemic and blood pressure control: the Kashiwa study. Diabetes Care (2005) 28: 2733-2738.

[26] Hsieh MC, Hsieh YT, Cho TJ *et al.* Remission of diabetic nephropathy in type 2 diabetic Asian population: role of tight glucose and blood pressure control. Eur J Clin Invest (2011) 41: 870-878.

[27] Cooper ME, Frauman A, O'Brien RC, Seeman E, Murray RM, Jerums G. Progression of proteinuria in type 1 and type 2 diabetes. Diabet Med (1988) 5: 361-368.

[28] Hoefield RA, Kalra PA, Baker PG *et al.* The use of eGFR and ACR to predict decline in renal function in people with diabetes. Nephrol Dial Transplant (2011) 26: 887-892.

[29] Smoyer WE, Mundel P. Regulation of podocyte structure during the development of nephrotic syndrome. J Mol Med (1998) 76: 172-183.

[30] Rodewald R, Karnovsky MJ. Porous substructure of the glomerular slit diaphragm in the rat and mouse. J Cell Biol (1974) 60: 423-433.

[31] Liu XL, Kilpelainen P, Hellman U *et al.* Characterization of the interactions of the nephrin intracellular domain. FEBS J (2005) 272: 228-243.

[32] Tryggvason K, Ruotsalainen V, Wartiovaara J. Discovery of the congenital nephrotic syndrome gene discloses the structure of the mysterious molecular sieve of the kidney. Int J Dev Biol (1999) 43: 445-451.

[33] Boute N, Gribouval O, Roselli S *et al.* NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. Nat Genet (2000) 24: 349-354.

[34] Kaplan JM, Kim SH, North KN *et al.* Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. Nat Genet (2000) 24: 251-256.

[35] Donoviel DB, Freed DD, Vogel H *et al.* Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. Mol Cell Biol (2001) 21: 4829-4836.

[36] Shih NY, Li J, Cotran R, Mundel P, Miner JH, Shaw AS. CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. Am J Pathol (2001) 159: 2303-2308.

[37] Ng DP, Krolewski AS. Molecular genetic approaches for studying the etiology of diabetic nephropathy. Curr Mol Med (2005) 5: 209-525.

[38] Gabriel SB, Schaffner SF, Nguyen H *et al.* The structure of haplotype blocks in the human genome. Science (2002) 296: 2225-2229.

[39] Wall JD, Prichard JK. Haplotype blocks and linkage disequilibrium in the human genome. Nat Rev Genet (2003) 4: 587-597.

[40] Ahvenainen EK, Hallman N, Hjelt L. Nephrotic syndrome in newborn and young infants. Ann Paediatr Fenn (1956) 2: 227-241.

[41] Ruotsalainen V, Patrakka J, Tissari P *et al.* Role of nephrin in cell junction formation in human nephrogenesis. Am J Pathol (2000) 157: 1905-1916.

[42] Santin S, Garcia-Maset R, Ruiz P *et al.*Nephrin mutations cause childhood- and adult-onset focal segmental glomerulosclerosis. Kidney Int (2009) 76: 1268-1276.

[43] Philippe A, Nevo F, Esquivel EL *et al.*Nephrin mutations can cause childhood-onset steroid-resistant nephrotic syndrome. J Am Soc Nephrol (2008) 19: 1871-1878.

[44] Lahdenkari AT, Kestila M, Holmberg C, Koskimies O, Jalanko H. Nephrin gene (NPHS1) in patients with minimal change nephrotic syndrome (MCNS). Kidney Int (2004) 65: 1856-1863.

[45] Putaala H, Soininen R, Kilpeläinen P, Wartiovaara J, Tryggvason K. The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. Hum Mol Genet (2001) 10: 1-18.

[46] Hamano Y, Grunkemeyer JA, SudhakarA*et al.* Determinants of vascular permeability in the kidney glomerulus. J Biol Chem (2002) 277: 31154-31162.

[47] Rantanen M, Palmén T, Pätäri A *et al.* Nephrin TRAP mice lack slit diaphragms and show fibrotic glomeruli and cystic tubular lesions. J Am Soc Nephrol (2002) 13: 1586-1594.

[48] Tryggvason K. Unraveling the mechanisms of glomerular ultrafiltration: nephrin, a key component of the slit diaphragm. J Am Soc Nephrol (1999) 10: 2440-2445.

[49] Kestilä M, Lenkkeri U, Männikkö M *et al.*Positionally cloned gene for a novel glomerular protein --nephrin-- is mutated in congenital nephrotic syndrome. Mol Cell 1: 575-582.

[50] Lenkkeri U, Männikkö M, McCready P *et al.* Structure of the gene for congenital nephrotic syndrome of the Finnish type (NPHS1) and characterization of mutations. Am J Hum Genet 64: 51-61.

[51] Brummendorf T, Rathjen FG. Cell adhesion molecules. 1: immunoglobulin superfamily. Protein Profile (1994) 1: 951-1058.

[52] Ruotsalainen V, Ljungberg P, Wartiovaara J *et al.* Nephrin is specifically located at the slit diaphragm of glomerular podocytes. Proc Natl Acad Sci USA 96: 7962-7967.

[53] Holthöfer H, Ahola H, Solin ML *et al.* Nephrin localizes at the podocyte filtration slit area and is characteristically spliced in the human kidney. Am J Pathol (1999) 155: 1681-1687.

[54] Ovunc B, Asharf S, Vega-Warner V *et al.* Mutation analysis of NPHS1 in a worldwide cohort of congenital nephrotic syndrome patients. Nephron Clin Pract (2012) 120: c139-c146.

[55] Beltcheva O, Martin P, Lenkkeri U, Tryggvason K. Mutation spectrum in the nephrin gene (NPHS1) in congenital nephrotic syndrome. Hum Mutat (2001) 17: 368-373.

[56] Liu L, Done SC, Khoshnoodi J *et al.* Defective nephrin trafficking caused by missense mutations in the NPHS1 gene: insight into the mechanisms of congenital nephrotic syndrome. Hum Mol Genet (2001) 10: 2637-2644.

[57] Shono A, Tsukagushi H, Kitamura A *et al.* Predisposition to relapsing nephrotic syndrome by a nephrin mutation that interferes with assembly of functioning microdomains. Hum Mol Genet (2009) 18: 2943-2956.

[58] Liu L, Aya K, Tanaka H, Shimizu J, Ito S, Seino Y. Nephrin is an important component of the barrier system in the testis. Acta Med Okayama (2001) 55: 161-165.

[59] Wagner N, Morrison H, Pagnotta S *et al.* The podocyte protein nephrin is required for cardiac vessel formation. Hum Mol Genet (2011) 20: 2182-2194.

[60] Palmén T, Ahola H, Palgi J *et al.* Nephrin is expressed in the pancreatic beta cells. Diabetologia (2001) 44: 1274-1280.

[61] Zanone MM, Favaro E, Doublier S *et al.* Expression of nephrin by human pancreatic islet endothelial cells. Diabetologia (2005) 48: 1789-1797.

[62] Doublier S, Salvidio G, Lupia E *et al.*Nephrin expression is reduced in human diabetic nephropathy: evidence for a distinct role for glycated albumin and angiotensin II. Diabetes (2003) 52: 1023-1030.

[63] Bonnet F, Cooper ME, Kawachi H, Allen TJ, Boner G, Cao Z. Irbesartan normalizes the deficiency in glomerular nephrin expression in a model of diabetes and hypertension. Diabetologia (2001) 44: 874-877.

[64] Wu Y, Dong J, Yuan L *et al.* Nephrin and podocin loss is prevented by mycophenolatemofetil in early experimental diabetic nephropathy. Cytokine (2008) 44: 85-91.

[65] Kelly DJ, Aaltonen P, Cox AJ *et al.* Expression of the slit-diaphragm protein, nephrin, in experimental diabetic nephropathy: differing effects of anti-proteinuric therapies. Nephrol Dial Transplant (2002) 17: 1327-1332.

[66] Aaltonen P, Luimula P, Astrom E *et al.* Changes in the expression of nephrin gene and protein in experimental diabetic nephropathy. Lab Invest (2001) 81: 1185-1190

[67] Huwiler A, Ren S, Holthöfer H, Pavenstädt H, Pfeilschifter J. Inflammatory cytokines upregulatenephrin expression in human embryonic kidney epithelial cells and podocytes. Biochem Biophys Res Commun (2003) 305: 136-142.

[68] Langham RG, Kelly DJ, Cox AJ *et al.* Proteinuria and the expression of the podocyte slit diaphragm protein, nephrin, in diabetic nephropathy: effects of angiotensin converting enzyme inhibition. Diabetologia (2002) 45: 1572-1576.

[69] Toyoda M, Suzuki D, Umezono T *et al.* Expression of human nephrin mRNA in diabetic nephropathy. Nephrol Dial Transplant (2004) 19: 380-385.

[70] Baelde HJ, Eikmans M, Doran PP, Lappin DW, de Heer E, Bruijn JA. Gene expression profiling in glomeruli from human kidneys with diabetic nephropathy. Am J Kidney Dis (2004) 43: 636-650.

[71] Benigni A, Gagliardini E, Tomasoni S *et al.* Selective impairment of gene expression and assembly of nephrin in human diabetic nephropathy. Kidney Int (2004) 65: 2193-2200.

[72] Pätäri A, Forsblom C, Havana M, Taipale H, Groop PH, Holthöfer H. Nephrinuria in diabetic nephropathy of type 1 diabetes. Diabetes (2003) 52: 2969-2974.

[73] Ng DP, Tai BC, Tan E *et al.*Nephrinuria associates with multiple renal traits in type 2 diabetes. Nephrol Dial Transplant (2011) 26: 2508-2514.

[74] Jim B, Ghanta M, Qipo A *et al.* Dysregulated nephrin in diabetic nephropathy in type 2 diabetes: a cross sectional study. PLoS One (2012) 7: e36041.

[75] Pettersson-Fernholm K, Forsblom C, Perola M, Groop PH. Polymorphisms in the nephrin gene and diabetic nephropathy in type 1 diabetic patients. Kidney Int (2003) 63: 1205-1210.

[76] Gonzalez R, Tirado A, Balanda M *et al.* A pilot study on genetic variation in purine-rich elements in the nephrin gene promoter in type 2 diabetic patients. Biol Res (2007) 40: 357-364.

[77] Gonzalez R, Tirado A, Rojas LA *et al.* Analysis of the intronic single nucleotide polymorphism rs#466452 of the nephrin gene in patients with diabetic nephropathy. Biol Res (2009) 42: 189-198.

[78] Fornoni A, Jeon J, Varona Santos J *et al.*Nephrin is expressed on the surface of insulin vesicles and facilitates glucose-stimulated insulin release. Diabetes (2010) 59: 190-199.

[79] Patari A, Karhapaa P, Taipale H *et al.* A 100-kDa urinary protein is associated with insulin resistance in offspring of type 2 diabetic patients. Diabetologia (2005) 48: 1844-1850.

[80] Daimon M, Ji G, Oizumi T *et al.* Association of nephrin gene polymorphisms with type 2 diabetes in a Japanese population: the Funagata study. Diabetes Care (2006) 29: 1117-1119.

[81] Cutter J, Tan BY, Chew SK. Levels of cardiovascular disease risk factors in Singapore following a national intervention programme. Bull World Health Organ (2001) 79: 908-915.

[82] Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. 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: 461-470.

[83] Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. Diabetes Care (2004) 27: 1487-1495.

[84] Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics (2005) 21: 263-265.

[85] Olsen S, Mogensen CE. How often is NIDDM complicated with non-diabetic renal disease? An analysis of renal biopsies and the literature. Diabetologia (1996) 39: 1638-1645.

[86] Coward RJM, Welsh GI, Yang J *et al*. The human glomerular podocyte is a novel target for insulin action. Diabetes (2005) 54: 3095-3102.

[87] Fornoni A. Proteinuria, the podocyte, and insulin resistance. N Engl J Med (2010) 363: 2068-2069.

APPENDICES

Supplementary Table 1. HWE of *NPHS1* SNPs in SDCS patients.

SNP	SDCS	
	MAF	P_{HWE}
rs3814995	0.392	0.752
rs437168	0.153	0.534
rs10409299	0.257	0.774
rs2071327	0.359	0.623
rs460560	0.061	0.231
rs17777002	0.108	0.940

Supplementary Table 2. HWE of *NPHS1* SNPs in SDCS patients stratified by albuminuric status.

SNP	Genotype	CTRLS		MICRO		MACRO	
		N	P_{HWE}	N	P_{HWE}	N	P_{HWE}
rs3814995	*TT*	311	0.600	126	0.894	34	0.684
(T>C)	*CT*	399		159		47	
	CC	140		47		12	
rs437168	*GG*	613	0.736	244	0.826	72	0.408
(G>A)	*AG*	225		86		19	
	AA	25		7		3	
rs10409299	*AA*	469	0.870	189	0.695	50	1.000
(A>G)	*AG*	321		117		37	
	GG	57		21		7	
rs2071327	*TT*	340	0.813	144	0.374	39	1.000
(T>C)	*CT*	387		157		44	
	CC	115		33		11	
rs460560	*TT*	746	0.556	0	0.741	0	1.000
(T>C)	*CT*	108		36		8	
	CC	2		299		85	
rs17777002	*AA*	671	0.873	267	0.625	76	0.895
(A>G)	*AG*	167		61		17	
	GG	9		5		2	

Supplementary Table 3. LD of *NPHS1* SNPs in SDCS patients indicated by D' (top right triangle) and r^2 (bottom left triangle) values.

SNP	rs3814995	rs437168	rs10409299	rs2071327	rs460560	rs17777002
rs3814995	1.000	0.989	0.050	0.009	0.019	0.132
rs437168	0.273	1.000	0.109	0.132	0.594	0.144
rs10409299	0.001	0.006	1.000	0.982	1.000	0.391
rs2071327	0.000	0.006	0.605	1.000	1.000	0.499
rs460560	0.000	0.004	0.023	0.117	1.000	1.000
rs17777002	0.003	0.014	0.006	0.017	0.008	1.000

Supplementary Table 4. Association of *NPHS1* SNPs with stages of DN (dominant model).

SNP		CTRL (1)	MICRO (2)	MACRO (3)	(2) vs (1) OR (95% CI)	*P**	(3) vs (1) OR (95% CI)	*P**
rs3814995	*TT*	311 (36.6)	126 (38.0)	34 (36.6)				
	CT/CC	539 (63.4)	206 (62.0)	59 (63.4)	0.98 (0.79-1.31)	0.890	1.09 (0.64-1.87)	0.741
rs437168	*GG*	613 (71.0)	244 (72.4)	72 (76.6)				
	AG/AA	250 (29.0)	93 (27.6)	22 (23.4)	0.81 (0.58-1.11)	0.188	0.65 (0.35-1.19)	0.161
rs10409299	*AA*	469 (55.4)	189 (57.8)	50 (53.2)				
	AG/GG	378 (44.6)	138 (42.2)	44 (46.8)	0.92 (0.69-1.23)	0.589	1.20 (0.72-2.00)	0.473
rs2071327	*TT*	340 (40.4)	144 (43.1)	39 (41.5)				
	CT/CC	502 (59.6)	190 (56.9)	55 (58.5)	0.89 (0.66-1.18)	0.405	1.05 (0.62-1.77)	0.857
rs460560	*TT*	746 (87.1)	299 (89.3)	85 (91.4)				
	CT/CC	110 (12.9)	36 (10.7)	8 (8.6)	0.84 (0.54-1.29)	0.419	0.60 (0.24-1.47)	0.261
rs17777002	*AA*	671 (79.2)	267 (80.2)	76 (81.7)				
	AG/GG	176 (20.8)	66 (19.8)	17 (18.3)	0.93 (0.65-1.32)	0.687	0.84 (0.44-1.61)	0.599

*Adjusted for age, WHR, DM duration, HbA_{1C}%, SBP, triglycerides, cholesterol, LDL, eGFR, antihypertensive medication and diabetes medication.

Supplementary Table 5. Association of *NPHS1* SNPs with stages of DN (recessive model).

SNP		CTRL (1)	MICRO (2)	MACRO (3)	(2) vs (1) OR (95% CI)	P*	(3) vs (1) OR (95% CI)	P*
rs3814995	*TT/CT*	710 (83.5)	285 (85.8)	81 (87.1)	0.86 (0.57-1.28)	0.455	0.76 (0.36-1.62)	0.482
	CC	140 (16.5)	47 (14.2)	12 (12.9)				
rs437168	*GG/AG*	838 (97.1)	330 (97.9)	91 (96.8)	0.63 (0.23-1.72)	0.372	0.83 (0.15-4.65)	0.831
	AA	25 (2.9)	7 (2.1)	3 (3.2)				
rs10409299	*AA/AG*	790 (93.3)	306 (93.6)	87 (92.6)	1.07 (0.60-1.91)	0.824	1.27 (0.48-3.35)	0.623
	GG	57 (6.7)	21 (6.4)	7 (7.4)				
rs2071327	*TT/CT*	727 (86.3)	301 (90.1)	83 (88.3)	0.73 (0.46-1.16)	0.184	0.74 (0.32-1.70)	0.482
	CC	115 (16.7)	33 (9.9)	11 (11.7)				
rs460560	*TT/CT*	854 (99.8)	335 (100)	93 (100)	NA	NA	NA	NA
	CC	2 (0.2)	0 (0)	0 (0)				
rs17777002	*AA/AG*	838 (98.9)	328 (98.5)	93 (100)	1.17 (0.35-3.97)	0.798	NA	NA
	GG	9 (1.1)	5 (1.5)	0 (0)				

*Adjusted for age, WHR, DM duration, HbA_{1C}%, SBP, triglycerides, cholesterol, LDL, eGFR, antihypertensive medication and diabetes medication.

Supplementary Table 6. HWE of *NPHS1* SNPs in NHS98 subjects stratified by ethnicity.

SNP	Chinese		Malay		Asian Indian	
	MAF	P_{HWE}	MAF	P_{HWE}	MAF	P_{HWE}
rs3814995	0.366	0.006	0.473	0.659	0.284	0.165
rs437168	0.149	0.382	0.110	0.415	0.085	1.000
rs10409299	0.253	0.625	0.268	0.688	0.215	1.000
rs2071327	0.355	0.066	0.351	0.787	0.484	0.905
rs460560	0.069	0.526	0.064	0.733	0.190	0.471
rs17777002	0.128	0.531	0.073	0.454	0.174	0.443

Supplementary Table 7. LD of *NPHS1* SNPs in NHS98 subjects stratified by ethnicity indicated by D' (top right triangle) and r^2 (bottom left triangle) values.

	SNP	rs3814995	rs437168	rs10409299	rs2071327	rs460560	rs17777002
Chinese	rs3814995	1.000	0.933	0.044	0.011	0.044	0.124
	rs437168	0.260	1.000	0.067	0.128	0.172	0.088
	rs10409299	0.000	0.002	1.000	0.842	0.500	0.344
	rs2071327	0.000	0.005	0.437	1.000	0.968	0.462
	rs460560	0.000	0.000	0.006	0.128	1.000	0.875
	rs17777002	0.004	0.007	0.006	0.017	0.008	1.000
Malay	SNP	rs3814995	rs437168	rs10409299	rs2071327	rs460560	rs17777002
	rs3814995	1.000	0.886	0.239	0.128	0.284	0.004
	rs437168	0.107	1.000	0.354	0.024	0.289	0.082
	rs10409299	0.019	0.006	1.000	0.775	0.514	0.384
	rs2071327	0.008	0.000	0.410	1.000	0.858	0.042
	rs460560	0.006	0.001	0.007	0.096	1.000	0.007
	rs17777002	0.000	0.004	0.004	0.000	0.000	1.000
Asian Indian	SNP	rs3814995	rs437168	rs10409299	rs2071327	rs460560	rs17777002
	rs3814995	1.000	0.751	0.167	0.248	0.241	0.121
	rs437168	0.020	1.000	0.093	0.063	0.040	0.151
	rs10409299	0.003	0.000	1.000	0.791	0.933	0.616
	rs2071327	0.025	0.000	0.161	1.000	0.942	0.198
	rs460560	0.005	0.001	0.058	0.198	1.000	0.493
	rs17777002	0.001	0.010	0.022	0.009	0.012	1.000

Supplementary Table 8. Association of *NPHS1* SNPs with T2DM-related traits in NHS98 subjects excluding T2DM patients.

	Chinese			P*	Malay			P*	Asian Indian			P*
Number of risk alleles	**0**	**1**	**2**		**0**	**1**	**2**		**0**	**1**	**2**	
rs3814995 (T>C)												
BMI (kg/m^2)	22.6	22.4	22.2	0.035	25.3	25.1	24.9	0.470	23.9	24.4	24.9	0.107
WHR	0.821	0.818	0.815	0.077	0.825	0.825	0.824	0.803	0.836	0.84	0.844	0.280
Fasting glucose (mmol/l)†^	5.3	5.4	5.4	0.200	5.5	5.5	5.5	0.748	5.5	5.4	5.4	0.816
Fasting insulin (mmol/l)†^	5.9	6.0	6.0	0.775	7.0	6.9	6.8	0.763	8.3	8.2	8.1	0.718
HOMA-insulin resistance†^	1.4	1.4	1.4	0.650	1.7	1.7	1.7	0.817	2.0	2.0	2.0	0.708
HOMA-β-cell function (%)†^#	66.0	65.2	64.4	0.171	72.7	72.0	71.4	0.617	86.0	86.0	86.0	0.995
rs437168 (G>A)												
BMI (kg/m^2)	22.5	22.4	22.3	0.324	25	25.4	25.9	0.251	24.6	24.7	24.8	0.878
WHR	0.820	0.815	0.809	**0.002**	0.822	0.830	0.838	0.077	0.842	0.841	0.840	0.852
Fasting glucose (mmol/l)†^	5.4	5.4	5.4	0.984	5.5	5.5	5.5	0.473	5.5	5.4	5.4	0.521
Fasting insulin (mmol/l)†^	6.0	5.9	5.7	0.233	6.9	6.8	6.7	0.665	8.3	7.6	6.9	0.121
HOMA-insulin resistance†^	1.4	1.4	1.4	0.254	1.7	1.7	1.6	0.771	2.0	1.8	1.7	0.119
HOMA-β-cell function (%)†^#	65.5	65.1	64.6	0.542	72.4	70.9	69.5	0.483	86.2	85.6	84.9	0.827
rs10409299 (A>G)												
BMI (kg/m^2)	22.5	22.5	22.5	0.910	25.2	25	24.7	0.344	24.7	24.6	24.6	0.841
WHR	0.818	0.819	0.821	0.374	0.823	0.826	0.829	0.381	0.843	0.839	0.835	0.322
Fasting glucose (mmol/l)†^	5.4	5.4	5.4	0.544	5.5	5.5	5.5	0.759	5.4	5.4	5.5	0.820
Fasting insulin (mmol/l)†^	5.9	6.0	6.1	0.180	6.7	7.1	7.5	0.085	8.3	8.2	8.1	0.768
HOMA-insulin resistance†^	1.4	1.4	1.5	0.166	1.6	1.7	1.8	0.092	2.0	2.0	2.0	0.808
HOMA-β-cell function (%)†^#	65.7	65.5	65.3	0.785	71.9	72.6	73.2	0.663	87.1	86.4	85.8	0.724
rs2071327 (T>C)												
BMI (kg/m^2)	22.5	22.5	22.5	0.730	25.3	25	24.6	0.209	24.7	24.7	24.6	0.785
WHR	0.818	0.819	0.82	0.582	0.824	0.823	0.823	0.878	0.844	0.841	0.839	0.551
Fasting glucose (mmol/l)†^	5.4	5.4	5.4	0.592	5.5	5.5	5.5	1.000	5.5	5.4	5.4	0.787
Fasting insulin (mmol/l)†^	5.9	6.0	6.1	0.371	6.7	6.9	7.2	0.218	8.4	8.2	8.0	0.419
HOMA-insulin resistance†^	1.4	1.4	1.4	0.347	1.6	1.7	1.8	0.242	2.0	2.0	1.9	0.423
HOMA-β-cell function (%)†^#	65.7	65.5	65.3	0.711	71.7	72.1	72.6	0.758	85.8	86.1	86.3	0.868
rs460560 (T>C)												
BMI (kg/m^2)	22.5	22.5	22.5	0.973	25.1	25.4	25.8	0.461	24.8	24.4	24	0.241
WHR	0.819	0.817	0.816	0.579	0.825	0.825	0.826	0.923	0.843	0.837	0.832	0.180
Fasting glucose (mmol/l)†^	5.4	5.4	5.4	0.835	5.5	5.4	5.3	0.196	5.4	5.4	5.4	0.905
Fasting insulin (mmol/l)†^	6.0	5.8	5.7	0.383	6.9	7.0	7.2	0.709	8.3	8.1	7.9	0.466
HOMA-insulin resistance†^	1.4	1.4	1.4	0.385	1.7	1.7	1.7	0.892	2.0	2.0	1.9	0.504
HOMA-β-cell function (%)†^#	65.3	65.3	65.2	0.935	71.6	74.9	78.3	0.233	86.9	86.3	85.7	0.763
rs17777002 (A>G)												
BMI (kg/m^2)	22.4	22.7	23	0.034	25.1	25.1	23.1	0.982	24.5	24.9	25.3	0.320
WHR	0.818	0.819	0.82	0.612	0.824	0.826	0.828	0.716	0.836	0.850	0.864	**0.003**
Fasting glucose (mmol/l)†^	5.4	5.4	5.4	0.668	5.5	5.4	5.3	0.092	5.5	5.4	5.4	0.266
Fasting insulin (mmol/l)†^	6.0	6.0	5.9	0.892	6.9	7.0	7.1	0.789	8.2	8.2	8.3	0.827
HOMA-insulin resistance†^	1.4	1.4	1.4	0.943	1.7	1.7	1.7	0.971	2.0	2.0	2.0	0.969
HOMA-β-cell function (%)†^#	65.5	65.3	65.1	0.790	71.6	76.9	82.6	0.048	85.0	87.8	90.7	0.187

T2DM subjects were excluded from analyses; *all analyses were gender and age adjusted; †values were natural log transformed to improve normality in regression analysis, and adjusted means were subsequently back transformed; ^additionally adjusted for BMI and excluding subjects taking diabetic medication (59 Chinese, 35 Malays and 42 Asian Indians); $^{\#}$additionally adjusted for insulin resistance; bold-faced P-values are <0.05 after Bonferroni correction.

Supplementary Table 9. HWE test for *NPHS1* SNPs in Chinese SDCS and NHS98 subjects stratified by glucose tolerance status.

SNP		NGT		IGT/IFG		T2DM	
	Genotype	N	P_{HWE}	N	P_{HWE}	N	P_{HWE}
rs3814995	*TT*	810	0.0002	210	0.798	595	0.238
(T>C)	*CT*	1084		233		711	
	CC	256		60		243	
rs437168	*GG*	1548	0.997	380	0.096	1132	0.338
(G>A)	*AG*	556		107		386	
	AA	49		15		47	
rs10409299	*AA*	1204	0.786	290	1.000	847	1.000
(A>G)	*AG*	822		184		594	
	GG	135		30		103	
rs2071327	*TT*	921	0.123	210	0.203	631	0.589
(T>C)	*CT*	948		216		724	
	CC	283		72		195	
rs460560	*TT*	1895	0.875	436	0.840	1377	0.132
(T>C)	*CT*	279		74		185	
	CC	9		2		2	
rs17777002	*AA*	1667	0.821	368	0.067	1228	1.000
(A>G)	*AG*	469		135		305	
	GG	36		6		19	

Supplementary Table 10. Association of *NPHS1* SNPs with T2DM among Chinese (dominant model).

SNP		NGT (1)	IGT/IFG (2)	T2DM (3)	(2) vs (1) OR (95% CI)	*P**	(3) vs (1) OR (95% CI)	*P**
rs3814995	*TT*	810 (37.7)	210 (41.8)	595 (38.4)				
	CT/CC	1340 (62.3)	293 (58.2)	954 (61.6)	0.89 (0.72-1.10)	0.284	0.91 (0.69-1.20)	0.503
rs437168	*GG*	1548 (71.9)	380 (75.7)	1132 (71.9)				
	AG/AA	605 (28.1)	122 (24.3)	443 (28.1)	0.87 (0.68-1.11)	0.261	1.12 (0.83-1.51)	0.467
rs10409299	*AA*	1204 (55.7)	290 (57.5)	847 (54.9)				
	AG/GG	957 (44.3)	214 (42.5)	697 (45.1)	0.90 (0.73-1.12)	0.356	1.10 (0.84-1.44)	0.487
rs2071327	*TT*	921 (42.8)	210 (42.2)	631 (40.7)				
	CT/CC	1231 (57.2)	288 (57.8)	919 (59.3)	0.99 (0.80-1.23)	0.956	1.03 (0.79-1.36)	0.807
rs460560	*TT*	1895 (86.8)	436 (85.2)	1377 (88.0)				
	CT/CC	288 (13.2)	76 (14.8)	187 (12.0)	1.17 (0.87-1.57)	0.311	0.82 (0.55-1.22)	0.330
rs17777002	*AA*	1667 (76.8)	368 (72.3)	1228 (79.1)				
	AG/GG	505 (23.2)	141 (27.7)	324 (20.9)	1.26 (0.99-1.60)	0.056	0.97 (0.71-1.33)	0.855

*Adjusted for age, gender, BMI, WHR, SBP, DBP, total cholesterol, triglyceride, HDL, LDL and antihypertensive medication.

Supplementary Table 11. Association of *NPHS1* SNPs with T2DM among Chinese (recessive model)

SNP		NGT (1)	IGT/IFG (2)	T2DM (3)	(2) vs (1) OR (95% CI)	*P**	(3) vs (1) OR (95% CI)	*P**
rs3814995	*TT/CT*	1894 (88.1)	443 (88.1)	1306 (84.3)				
	CC	256 (11.9)	60 (11.9)	243 (15.7)	1.02 (0.73-1.41)	0.910	1.47 (0.99-2.20)	0.054
rs437168	*GG/AG*	2104 (97.7)	487 (97.0)	1528 (97.0)				
	AA	49 (2.3)	15 (3.0)	47 (3.0)	1.47 (0.78-2.76)	0.232	2.14 (0.93-4.91)	0.074
rs10409299	*AA/AG*	2026 (93.8)	474 (94.1)	1441 (93.3)				
	GG	135 (6.2)	30 (5.9)	103 (6.7)	0.94 (0.60-1.46)	0.777	1.01 (0.58-1.75)	0.982
rs2071327	*TT/CT*	1869 (86.9)	426 (85.5)	1355 (87.4)				
	CC	283 (13.1)	72 (14.5)	195 (12.6)	1.15 (0.85-1.56)	0.361	0.90 (0.60-1.35)	0.623
rs460560	*TT/CT*	2174 (99.6)	510 (99.6)	1562 (99.9)				
	CC	9 (0.4)	2 (0.4)	2 (0.1)	1.62 (0.33-7.91)	0.550	0.12 (0.003-4.47)	0.251
rs17777002	*AA/AG*	2136 (98.3)	503 (98.8)	1533 (98.8)				
	GG	36 (1.7)	6 (1.2)	19 (1.2)	0.51 (0.19-1.36)	0.180	0.64 (0.22-1.89)	0.418

*Adjusted for age, gender, BMI, WHR, SBP, DBP, total cholesterol, triglyceride, HDL, LDL and antihypertensive medication.