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Urinary biomarkers for the prediction of diabetic nephropathy, cardiovascular disease and mortality in individuals with type 1 diabetes

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ACADEMIC DISSERTATION

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"The future belongs to those who believe in the beauty of their dreams."

Eleanor Roosevelt

"Education is the most powerful weapon to change the world."

Nelson Mandela

To Miha,

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Panduru NM, Forsblom C, Saraheimo M, Thorn L, Bierhaus A, Humpert PM, Groop PH; FinnDiane Study Group. Urinary liver-type fatty acid-binding protein and progression of diabetic nephropathy in type 1 diabetes. Diabetes Care. 2013;36(7):2077-83. PubMed PMID: 3687279.

II Panduru NM, Saraheimo M, Forsblom C, Thorn LM, Gordin D, Wadén J, Tolonen N, Bierhaus A, Humpert PM, Groop PH; FinnDiane Study Group. Urinary adiponectin is an independent predictor of progression to end-stage renal disease in patients with type 1 diabetes and diabetic nephropathy. Diabetes Care. 2015;38(5):883-90. PubMed PMID: 25720601.

III Panduru NM, Sandholm N, Forsblom C, Saraheimo M, Dahlström EH, Thorn LM, Gordin D, Tolonen N, Wadén J, Harjutsalo V, Bierhaus A, Humpert PM, Groop PH; FinnDiane Study Group. Kidney injury molecule-1 and the loss of kidney function in diabetic nephropathy: a likely causal link in patients with type 1 diabetes. Diabetes Care. 2015;38(6):1130-7. PubMed PMID: 25784666.

IV Panduru NM, Forsblom C, Saraheimo M, Thorn LM, Gordin D, Elonen N, Harjusalo V, Bierhaus A, Humpert PM, Groop PH; FinnDiane Study Group. Urinary liver-type fatty acid binding protein is an independent predictor of stroke and mortality in individuals with type 1 diabetes. Diabetologia. 2017;60(9):1782-1790. PubMed PMID: 28601908.

The publications are referred to in the text by the corresponding Roman numerals as mentioned above.

ABBREVIATIONS

ACR	albumin to creatinine ratio
ADP	adiponectin
AER	albumin excretion rate
A1	normal AER (normal to mildly increased AER)
A2	microalbuminuria (moderately increased AER)
A3	macroalbuminuria (severely increased AER)
AUC	area under the curve
BM	basic model
B2M	Beta 2-microglobulin
BMI	body mass index
BTP	beta-trace protein
CAD	coronary artery disease
CD40	cluster of differentiation 40 protein
CKD	chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
cNRI	continuous net reclassification index for logistic model
CRP	C-reactive protein
CVD	cardiovascular disease
DBP	diastolic blood pressure
DC	diabetic cardiomyopathy
DCCT	Diabetes Control and Complications Trial
DKD	diabetic kidney disease
DN	diabetic nephropathy
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
eGFR	estimated glomerular filtration rate
eNOS	endothelial nitric oxide synthase
ESRD	end stage renal disease
GDM	gestational diabetes mellitus
GWAS	genome-wide association studies
HBA1C	glycated hemoglobin A1C
HDL-C	high-density lipoprotein cholesterol

IDI	integrated discrimination improvement
IDIS-5	integrated discrimination improvement calculated for survival data at 5 years survival
IDIS-10	integrated discrimination improvement calculated for survival data at 10 years
IV	instrumental variable
KIM-1	kidney injury molecule-1
LDL-C	density lipoprotein cholesterol
L-FABP	liver-type fatty acid binding protein
MAF	minor allele frequency
MR	Mendelian randomization
N/A	not applicable
NEFAs	non-esterified fatty acids
NO	nitric oxide
NRI	net reclassification index
NRIS-5	generalized net reclassification index calculated for survival data at 5 years survival
NRIS-10	generalized net reclassification index calculated for survival data at 10 years survival
NT	not tested
PCs	principal components
PVD	peripheral vascular disease
RAAS	renin-angiotensin-aldosterone system
RBP	Retinol-binding protein
ROC	receiver operating characteristic
SBP	systolic blood pressure
SD	standard deviation
SE	standard error
SNP	single nucleotide polymorphism
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TGF-β	transforming growth factor beta
WHR	waist to hip ratio

ABSTRACT

Introduction: Diabetic nephropathy (DN) is a devastating diabetes complication affecting 20 to 40% of individuals with type 1 diabetes mellitus (T1DM). DN is associated with a competitive risk of either progression to a worse stage of DN or premature mortality. Mortality is mainly due to cardiovascular events. DN progression as well as cardiovascular events can be predicted by either a high albumin excretion rate (AER), a low estimated glomerular filtration rate (eGFR) or both. These biomarkers, commonly employed in clinical practice, are mainly glomerular biomarkers and reflect various degrees of kidney damage, although they have limitations not only at the early stages of DN but also at later stages of the disease. The limitations arise mainly from the fact that in addition to the glomerular damage, tubular dysfunction also plays an important role in the pathogenesis of DN. It is worth mentioning, too, that eGFR is a rather insensitive marker of early dysfunction. However, at the later stages eGFR is a useful tool to assess the residual kidney function and the risk of further complications. Tubular dysfunction is present early in the course of T1DM and most likely even before there is an increase in the AER. Furthermore, tubular damage plays a major role in the final loss of kidney function. This aspect is highlighted by the fact that proximal tubular damage has a key role in the acute loss of kidney function (acute kidney injury [AKI]). Therefore, biomarkers that would be able to better reflect the precise nature of the tubular dysfunction both at the early and the later stages of DN could significantly bolster early identification of DN, prediction of DN progression as well as screening for risk of cardiovascular events.

Aims: The main aim of this study was to investigate, in individuals with T1DM, if three urinary biomarkers [liver-type fatty acid binding protein (L-FABP), kidney injury molecule 1 (KIM-1) and urinary adiponectin (ADP)] could outperform the currently available biomarkers for the prediction of DN development and progression, or even be causally related to the loss of kidney function. An additional objective was to examine whether these biomarkers could predict cardiovascular disease (CVD) Pand premature mortality, as also whether they are able to add clinical benefit to the biomarkers used in clinical practice for prediction of CVD and premature mortality.

Subjects and methods: All individuals with T1DM included in this research were enrolled between January 1998 and December 2002. The studies were part of the Finnish Diabetic Nephropathy Study (FinnDiane), a nationwide cohort of individuals with T1DM followed prospectively at more than 80 centers throughout Finland. The aim of the FinnDiane is to identify clinical, biochemical and genetic risk factors of

DN. For two of the four current studies, a group of individuals without diabetes and without any family history of kidney disease or diabetes were also enrolled. Blood and urine samples were collected at baseline and stored at −20°C until measured in 2008. The participants were followed for a median of 5.8 to 14.1 years, and clinical outcomes were evaluated prospectively. All studies were performed with the approval of the local ethics committees in accordance with the revised Declaration of Helsinki, and every participant signed an informed consent.

Results: In study I, L-FABP was shown to be an independent predictor of progression of DN, irrespective of the disease stage. However, L-FABP used alone or together with AER did not improve the risk prediction of DN progression, compared with actual biomarkers, in individuals with T1DM. In study II, urinary ADP was an independent predictor of progression to end-stage renal disease (ESRD) and performed even better than AER, and as well as eGFR. In addition, urinary ADP added significant predictive benefit when used together with either AER or eGFR. In study III, KIM-1 did not predict the progression to ESRD independently of AER and did not add any prognostic benefit to currently used biomarkers. However, the Mendelian randomization (MR) analysis showed that the inverse association of increased KIM-1 concentrations with lower eGFR is likely to represent a causal link. In study IV, L-FABP was an independent predictor of stroke and premature mortality but did not add any predictive benefit on top of AER and eGFR. In fact, L-FABP was not a predictor of other cardiovascular endpoints (coronary artery disease, peripheral vascular disease and overall CVD events) when adjusted for AER. It is noteworthy that urinary ADP and AER were common determinants of all the tested biomarkers, suggesting a complex interaction between tubular, glomerular and systemic mechanisms.

Discussion and conclusions: Through their ability to predict the progression of DN, the tested tubular biomarkers established that tubular dysfunction is an important part of DN progression. However, judging by the baseline determinants of their concentrations, the studied tubular biomarkers represent much more than tubular injury, capturing also glomerular damage as well as systemic factors. The fact that L-FABP was as good a predictor as eGFR or AER of stroke or premature mortality, while the other biomarkers predicted various other cardiovascular outcomes, confirms the potential role of these biomarkers for the prediction of CVD. A causal relationship between these biomarkers and loss of kidney function could be demonstrated only for KIM-1, but this particular observation confirms a causal role of tubular dysfunction in the DN progression. Future studies in individuals with T1DM are needed to explore the predictive potential and causal relationship between these biomarkers and loss of eGFR over time.

1 INTRODUCTION

Since the discovery of insulin, the development of chronic renal and cardiovascular complications due to diabetes has become the main challenge in individuals with T1DM. Compared with the general population, individuals living with T1DM have a higher risk of CVD (1, 2). In addition, the risk of premature death from cardiovascular causes is already higher early in the course of the disease (3). Furthermore, DN increases the risk of CVD and premature mortality along with the severity of kidney dysfunction (4, 5). DN and CVD thus progress in parallel and share a large number of common risk factors and pathogenic mechanisms (6).

Early screening and detection of DN is based on an increase of the AER or a lower eGFR. These commonly used biomarkers in clinical practice are mainly glomerular biomarkers. However, eGFR is a rather insensitive biomarker, as the eGFR may still be in the normal range despite advanced tissue damage. Thus, these biomarkers have inherent limitations both at the early and the late stages of DN. This is further highlighted by the fact that in addition to the glomerular damage, tubulointerstitial injury has also been demonstrated to play a major role in the pathogenesis of DN (7). In this context, it is an attractive approach to study the molecules linked to tubular dysfunction. They may serve as potential new biomarkers for the detection of the onset and progression of DN and may also provide additional information about the clinical course or the prognosis of individuals with T1DM. Such molecules may also enable an even earlier diagnosis than achieved by AER, and consequently provide an opportunity to tailor the treatment suitably at a stage when there may be a possibility of preventing further damage to the kidneys.

Despite their limitations, AER and eGFR are also strong predictors of DN in individuals living with T1DM (8). The same is true for the prediction of CVD. Given that DN and CVD progress in parallel and share risk factors and mechanisms, it is possible that the discovery of clinically useful biomarkers for the prediction of DN may also provide a tool for earlier prediction of risk of CVD. Therefore, this study aimed to examine the role of novel tubular biomarkers for the prediction of onset and progression of DN as well as of CVD.

2 REVIEW OF THE LITERATURE

2.1. Diabetes

2.1.1. Definition of diabetes

Diabetes is a syndrome comprised of a heterogeneous group of disorders with a common characteristic—an increase in the blood glucose concentration. Heterogeneity of diabetes arises from the considerably variable clinical presentation, disease progression and underlying causes. The main causes of diabetes are relative or absolute insulin deficiency, as well as resistance to the action of insulin.

2.1.2. Diagnosis of diabetes

A diabetes diagnosis can be made using fasting plasma glucose, 2-hour postprandial plasma glucose during a standardized oral glucose tolerance test, glycated hemoglobin A_{1C} (HbA_{1C}) or by random plasma glucose (in individuals with clear symptoms of hyperglycemia). The diagnostic criteria are presented in Table 1.

Table 1. The diagnostic criteria of diabetes (9).

Diagnostic test§	Diagnostic threshold
Fasting plasma glucose (FPG)*	≥ 126 mg/dl (7.0 mmol/l)
2-h postprandial plasma glucose (2-h PPG) ⁑	≥ 200 mg/dl (11.1 mmol/l)
HbA_{1C} ⁂	6.5 % (48 mmol/mol)
Random plasma glucose (RPG) ⁑⁑	200 mg/dl (11.1 mmol/l)

§ In the absence of unequivocal hyperglycemia, results should be confirmed by repeat testing.
* Fasting – no caloric intake for at least 8 h.
⁑ 2-h postprandial plasma glucose (2-h PG) is considered the plasma glucose measured at 2 hours during a standardized oral glucose tolerance test (OGTT) performed according to World Health Organization (WHO), using 75 g anhydrous glucose or an equivalent glucose load dissolved in water.
⁂ The method should be National Glycohemoglobin Standardization Program (NGSP) certified and Diabetes Control and Complications Trial (DCCT) standardized
⁑⁑ In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis

2.1.3. Classification of diabetes

Diabetes can be classified into four major categories: T1DM, type 2 diabetes mellitus (T2DM), gestational diabetes and other types of diabetes (10).

T1DM is the consequence of an autoimmune attack on the insulin producing pancreatic β-cells. The attack is associated with five types of autoantibodies that can be identified in the serum of individuals with T1DM: glutamic acid decarboxylase antibodies (GADA), islet cell antibodies (ICA), islet cell autoantibodies (IAA), protein tyrosine phosphatase antibodies (IA-2A) and zinc transporter 8 antibodies (ZnT8A). T1DM is considered to represent absolute insulin deficiency and based on the presence or absence of antibodies is classified into type 1A or type 1B (11). A special subtype of autoimmune mediated T1DM is the latent autoimmune diabetes in adults (LADA). LADA shares phenotypical features with T2DM but is characterized by the following general characteristics: presence of circulating islet autoantibodies; age of diabetes diagnosis > 30 years; no need for insulin treatment for at least 6 months after the diagnosis (12).

T2DM usually appears on a background of insulin resistance in genetically predisposed individuals who have impaired β-cell function and who fail to secrete sufficient amounts of insulin to keep the blood glucose levels within the normal range. T2DM is often associated with a relative decline in insulin secretion over time. These individuals may therefore require insulin treatment at some stage if the insulin secretion is greatly diminished (13).

Gestational diabetes mellitus (GDM) is defined as any degree of dysglycemia that is first recognized during pregnancy. If GDM is diagnosed during the first trimester it is considered to represent preexisting diabetes, while in those diagnosed during the second or third trimester of their pregnancy it represents more probably true gestational diabetes. GDM is associated with insulin resistance and hyperinsulinemia. In addition, GDM is associated with adverse neonatal and maternal outcomes during pregnancy and delivery. GDM is also associated with an increased maternal risk of diabetes after birth, as well as long term adverse health consequences for the infant (10).

Other specific types of diabetes may be due to monogenic defects of beta cell function, genetic defects of insulin action, other genetic syndromes sometimes associated with diabetes, diseases of the exocrine pancreas, endocrinopathies, infections, uncommon forms of immune-mediated diabetes or diabetes induced by drugs or chemicals (14).

Correct differentiation between T1DM, T2DM, LADA, monogenic forms of diabetes or other forms of diabetes has important implications for appropriate treatment and education, for appropriate follow up, as well as for the long-term prognosis of each individual diagnosed with diabetes. For a correct classification, additional diagnostic tools are necessary, on top of plasma glucose, HbA_{1C} or OGTT. Examples of such additional tests are: C-peptide concentration in serum or urine, homeostatic model assessment for insulin resistance (HOMA IR), HOMA β and autoantibodies (GADA, IAA, ICA, IA-2 or ZnT8) or gene sequencing. Based on these tests, insulin secretion or insulin resistance can be calculated, as well as the risk of a few monogenic forms of diabetes maturity onset diabetes of the young – (MODY). In addition, based on six variables (GADA, age at diagnosis, BMI, HbA_{1C}, and HOMA), five subgroups of diabetes have also been described.

2.1.4. T1DM definition and diagnosis

T1DM is an autoimmune disorder characterized by hyperglycemia due to destruction of beta cells of the Langerhans islets (15). Consequently, the autoimmune destruction of the beta cells (low/absent serum C peptide and the presence of autoantibodies) could be considered to confirm T1DM (type 1A) beyond clinical features (11). The absence of insulin treatment during the first 6 months after diagnosis in an individual without a negative C-peptide, but with the presence of autoantibodies, could be used to rule out T1DM (12).

However, the absence of autoantibodies together with the presence of clinical and metabolic features of overt hyperglycemia cannot rule out T1DM (type 1B), and is then considered idiopathic (11).

2.1.5. Epidemiology of T1DM

In 2015 the global prevalence of diabetes was 9.3%, with a projection of 10.9% by 2040 (16). It is estimated that 7 to 12% of all individuals with diabetes will have T1DM. In addition, 5 to 15% of the individuals with T2DM will in fact have either T1DM or LADA (17). There is an annual increase in T1DM incidence of approximately 3% (16). Notably, there are no sex differences in the incidence or prevalence of T1DM (13). However, there are regional differences both in Europe and across the world (18). One explanation for this variation could be ethnic differences, while another possible cause might be the geographical latitude gradient (19, 20).

2.1.6. Pathogenesis of T1DM

It is assumed that on a background of genetic predisposition to autoimmunity the action of an environmental factor may trigger the autoimmune process, leading to beta-cell destruction and a decline in insulin secretion (21).

Individuals with a family history of T1DM carry a higher risk of the disease compared with the general population. This risk is about 6 % in offspring, 5 % in siblings and 50 % in identical twins, while in subjects without family history the risk is about 0.4% (22). Consequently, the heritability of T1DM is maximum 40% in monozygotic twins, while other mechanisms (epigenetic, environmental, etc.) account for the remaining risk. The HLA (human leukocyte antigen) gene loci contribute with approximately 50% of the genetic risk of T1DM, while other non-HLA loci may be responsible for the other 50% of the genetic risk. In total, more than 40 genes have been identified that contribute to the genetic T1DM susceptibility (23).

The autoimmune attack is conducted by self-reactive B and T cells reactive to various antigens. While the role of the T cells is quite clear in the pathogenesis of T1DM, it seems that the B cells are not necessarily required for the development of the disease (24). The autoreactive T cells with epitopes derived from insulin and/or IGRP (islet-specific glucose-6-phosphatase catalytic subunit-related protein) or reactive with other antigens are critical for the initiation, maintenance and progression of the disease (25). Usually, IAA are the first antibodies detected in the serum of children with T1DM (26). Other important antibodies are: GADA, ICA, IA-2A and ZnT8A (27). Several additional autoantibodies could also be present in T1DM, being directed against the following antigens: ICA69 (islet cell autoantigen 69 kDa), IGRP, ChgA (chromogranin A), insulin receptor, heat shock proteins, jun-B antigen, peripherin and GFAP (glial fibrillary acidic protein) (28). A crucial organ implicated in the development of the T cell tolerance to tissue-restricted self-molecules is the thymus (29). However, the initiating factor of the tolerance dysregulation is not known. It is presumed that either late/low mRNAs expression of insulin and other islet cell autoantigens (GAD65, ICA69, IA-2) in human thymus or molecular mimicry between GAD65 and viral proteins could be responsible (30, 31).

Some pregnancy-related and perinatal factors are associated with T1DM: maternal age above 25 years, pre-eclampsia, neonatal respiratory disease, jaundice, birth weight (33, 34). In addition, several environmental factors such as bovine serum albumin from cow's milk, early exposure to cereals and nitrites have been associated with a higher risk of T1DM, while vitamin D and omega-3 fatty acids supplementation may be protective (35-39).

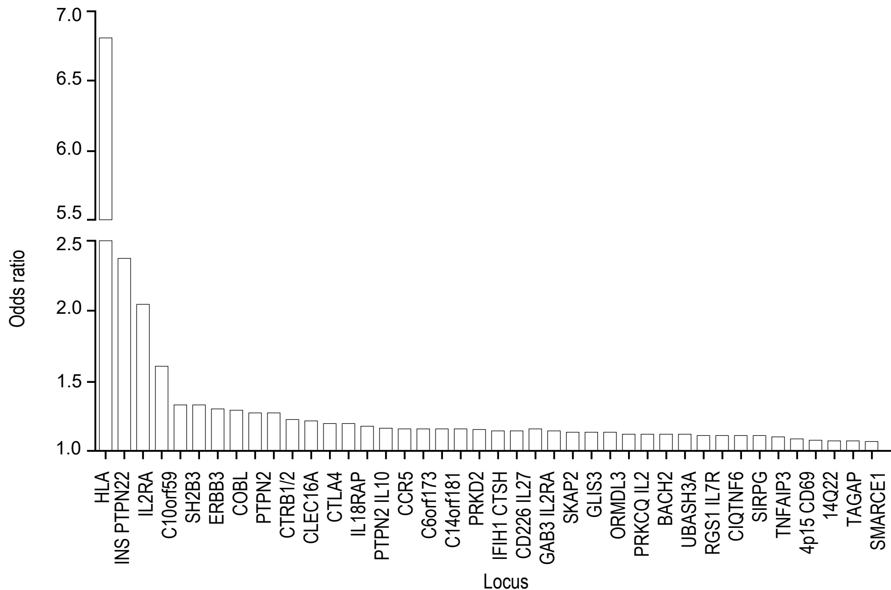

Figure 1. The genetic loci for T1DM.
On the Y axis, the effect size (odds ratio) of each locus on the T1DM risk is presented by using data from GWAS (genome-wide association studies). The effect size is presented for the SNP (single nucleotide polymorphism) most significantly associated with T1DM. The full names of all the presented genes can be found in the sources from which this figure was adapted (23, 32).

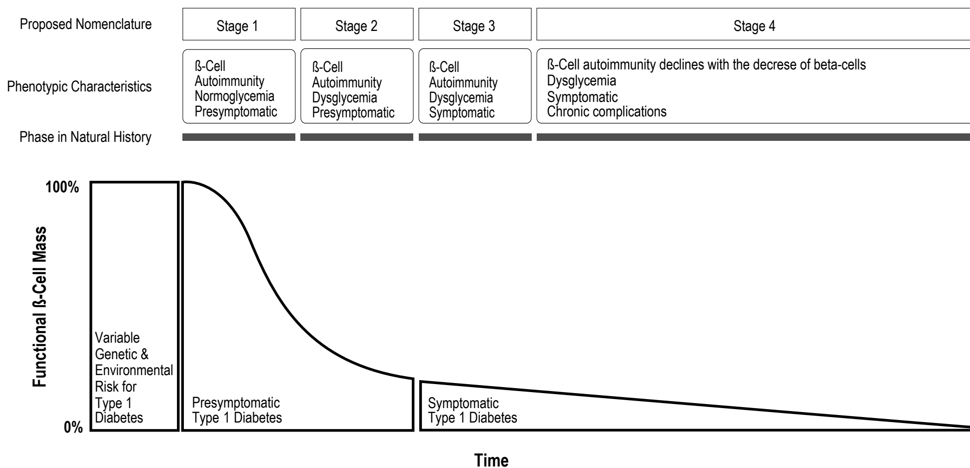

Figure 2. Early and late stages of T1DM. On the Y axis the % of the functional beta cell mass is presented. Adapted from (13, 40).

Based on these pathogenic features, the natural history of T1DM can be divided into four stages: presymptomatic T1DM with normoglycemia, presymptomatic T1DM with dysglycemia, symptomatic T1DM and T1DM with chronic complications. At the first stage, *"presymptomatic T1DM with normoglycemia,"* individuals with one or two antibodies present in the serum are still normoglycemic. At the second stage, *"presymptomatic T1DM with dysglycemia,"* the number of beta cells declines and

the glucose level starts to rise, but it is still below the diagnostic threshold for diabetes (40). At this stage, multiple antibodies are present, together with impaired fasting glucose and/or impaired glucose tolerance. The HbA_{1C} values are between 5.7 and 6.4%, or an increase in the HbA_{1C} of more than 10% can be diagnosed (13). However, the clinical symptoms are absent. At this stage, the 5-year probability of overt diabetes is 75% (41). At the third stage the affected subjects become symptomatic and T1DM is diagnosed according to biochemical criteria. At this stage, the individuals encounter polyuria, polydipsia, weight loss and fatigue, while DKA (diabetic ketoacidosis) is usually present at diagnosis (13, 40). In general, after 5 or more years from diabetes onset chronic complications start to appear and the individuals with T1DM may enter the fourth stage — *"T1DM with chronic complications"* (Figure 2).

2.2. Diabetic complications

2.2.1. Definition and classification

Diabetic complications are those long-term diseases that appear only in individuals with diabetes or which appear much faster than in the general population. A potential risk estimation of the most important chronic outcomes in T1DM can be performed using the Archimedes model or the EURODIAB model (42, 43). Other models of risk estimation for chronic complications have also been proposed (44, 45).

The chronic complications are mainly triggered and/or accelerated by poor glycemic control. Poor glycemic control primarily affects the blood vessels independently of their caliber. Other factors such as high blood pressure, dyslipidemia or the prothrombotic status may also contribute. The diabetes complications are divided into microvascular complications (when the small blood vessels are damaged) and macrovascular (when the larger blood vessels are injured).

Microvascular diabetic complications include damage to the eyes (diabetic eye disease or diabetic retinopathy), the kidneys (diabetic kidney disease or DN) and the nerves (diabetic neuropathy). *Diabetic eye disease* leads to retinopathy, cataract and glaucoma which are the leading causes of blindness (46). *DN* involves glomerular and tubular damage that leads to a relentless decline in the kidney function and ultimately renal failure.

Diabetic neuropathy can affect somatic neurons (peripheral and/or central) as well as vegetative neurons (sympathetic and/or parasympathetic). Thus, diabetic neuropathy can affect any somatic neuron, leading to distal symmetric polyneuropathy, mononeuropathy or radiculopathy, frequently leading to diabetic foot disorders and to toe/limb amputations. In addition, by affecting the autonomic nervous system, it can lead to autonomic neuropathy, which can affect any organ with sympathetic or parasympathetic innervation such as the heart, stomach, bowel and urogenital organs, among others. The clinical consequences of autonomic neuropathy are usually represented by hypotension or sudden death, diarrhea/constipation, genital organ dysfunction, neurogenic bladder, sudomotor dysfunction or hypoglycemia unawareness (47).

Other emerging complications presumed to be linked to microvascular damage are diabetic cardiomyopathy and diabetic encephalopathy. *Diabetic cardiomyopathy* (DC) is "*a distinct entity characterized by the presence of abnormal myocardial performance or structure in the absence of epicardial coronary artery disease, hypertension, and significant valvular disease*"(48). DC was first reported in 1972 in individuals with T2DM but is also present in those with T1DM. An understanding of its complex mechanisms is still elusive (49). The main clinical consequence of DC is left ventricular hypertrophy (LVH), together with a variable degree of diastolic dysfunction, leading to heart failure with preserved ejection fraction (50). *Diabetic encephalopathy (diabetic cognitive dysfunction/impairment)* (DE) refers to the deleterious long-term effects of diabetes on central nervous system function and cognition. The clinical consequence is a more rapid decline in the cognitive function compared with the general population, due to structural and functional changes in the brain (51, 52). The mechanisms behind the cognitive impairment in diabetes are far from being clear, but it seems that both hyper- and hypoglycemia have a triggering and/or accelerating effect (53, 54).

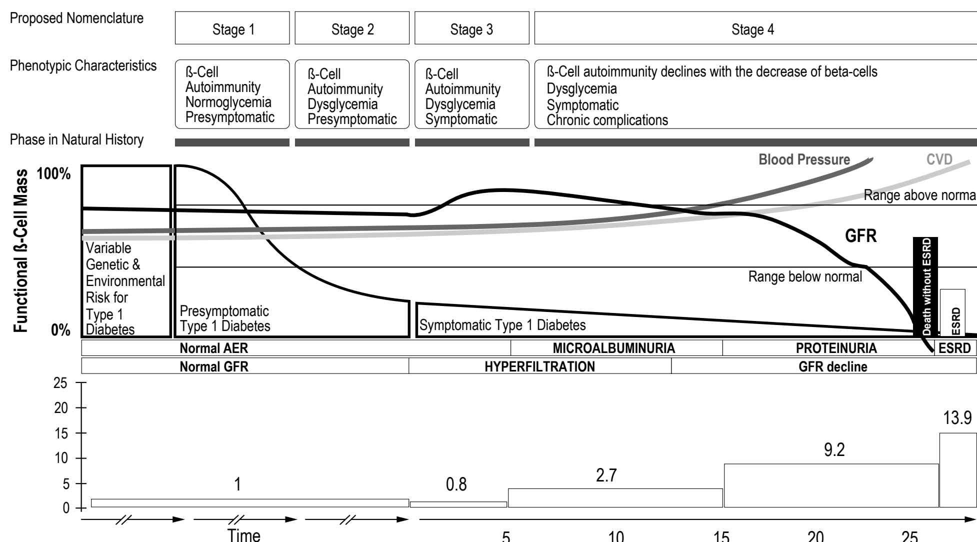

Figure 3. Principal stages of type 1 diabetes mellitus and its natural history in regard to blood pressure, GFR, CVD, DN and mortality. Adapted from (13, 40).

Macrovascular complications include cardiovascular diseases such as coronary artery disease (CAD), cerebrovascular disease or stroke and peripheral vascular disease (PVD). *CAD* is defined as coronary artery damage followed by inadequate blood supply of oxygen and nutrients to the myocardium. The pathogenic mechanism involves cholesterol containing deposits (atherosclerotic plaque) in the coronary walls, while the clinical consequence of ischemia may vary from stable to unstable angina, myocardial infarction and sudden death (55). *Stroke* is defined as a neurological deficit attributable to vascular damage that leads to a focal injury in the central nervous system (CNS). Such CNS injuries are represented by cerebral infarction, intracerebral hemorrhage or subarachnoidal hemorrhage (56). Both in the general population and in individuals with diabetes, stroke is a major cause of

disability and death (57). *PVD* is defined as chronic obstructive arterial disease of the lower extremities. Arterial occlusion is due to atherosclerosis and leads to decreased lower extremity arterial perfusion. The clinical consequence of chronic ischemia is intermittent or permanent pain at the lower limbs during walking or at rest (58). Individuals with diabetes have a 3.5 to 6 times higher risk of PVD (3.6 for women and 6.1 for men) compared to the general population (59).

Other likely long term diabetic complications are periodontal disease, infertility, diabetic myonecrosis, altered immune responses, diabetic pneumopathy (respiratory infections and restrictive lung disease), dermatologic manifestations, insulin treatment related lipohypertrophy, frozen shoulder, tunnel carpal syndrome, etc. (60-68).

2.2.2. Diabetic nephropathy

2.2.2.1. Definition and classification

DN is defined by structural and functional changes in the kidney triggered and sustained by diabetes, followed by severe clinical manifestations at the late stages of the disease.

Typical *structural changes* are represented by mesangial expansion, podocyte injury, glomerular basement membrane thickening and at the late stages by glomerular sclerosis. In addition, atypical tubular atrophy, advanced glomerular arteriolar hyalinosis and global glomerular sclerosis with mild or absent mesangial expansion could be present (69). Based on the histological findings, at least two classifications have been published (70, 71).

The most important *functional changes* are represented by glomerular endothelial cell dysfunction, glomerular hemodynamic changes, podocyte and basement membrane injury, proximal tubular dysfunction and altered tubulo-glomerular feedback (72).

The *clinical consequences* of these functional changes are a persistent and gradual increase in AER together with a progressive decline of the kidney function assessed by eGFR, a progressive increase in the blood urea nitrogen (BUN) and the development of anemia. Finally, when the kidney function is severely impaired, the blood pressure becomes very difficult to manage, the need for insulin or other antidiabetic medications to control the glucose concentration starts to decline, and symptoms like morning sickness, nausea and/or vomiting, weakness, paleness or itching start to appear.

The first consequence of diabetic kidney damage is progressive increase in a*lbuminuria,* which can be measured in a 24 hour or overnight urine collection (AER) as well as in a spot urine sample [albumin to creatinine ratio (ACR)]. The upper limit of normal urinary AER rate is 30 mg/24h or 20 µg/min, while a normal ACR is up to 30 mg/g (73). Microalbuminuria (moderately increased albuminuria) is defined as an AER of 30 to 300 mg/24h (20 – 200 µg/min or an ACR of 30 – 300 mg/g). Macroalbuminuria (severely increased albuminuria or proteinuria) represents a urinary AER above 300 mg/24h or 200 µg/min or an ACR >300 mg/g.

Because the AER has a coefficient of variation (CV) of approximately 40% in the same individual, 2 out of 3 consecutive urine samples with elevated AER, over 3 to 6 months, are necessary to conclude that the AER is persistently abnormal (74).

The second clinical consequence of DN is the progressive decline of kidney function. Assessment of kidney function by GFR can be measured directly or estimated. Direct measurement of GFR can be performed by diverse methods that use filtration markers such as inulin, iohexol, ^{125}I-iothalamate, ^{99m}Tc-diethylenethiaminepenta-acetic acid (DTPA) or ^{51}Cr-ethylenediaminetetra-acetic acid (EDTA) (75, 76). However, these methods are cumbersome and difficult to use in clinical practice and therefore the most popular method to assess kidney function is an estimation of the GFR (eGFR). The GFR estimation can be done by various mathematical formulas that are based on the serum/plasma creatinine or cystatin C values. The most commonly used are the MDRD (Modification of Diet in Renal Disease Study Equation) and the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formulas (77-79). eGFR has a coefficient of variation over 2 to 4 months of 6.3% to 16%, according to the method or the formula used for the estimation (80-82). These variations are due to physiological variations in the urinary creatinine excretion and creatinine production (81, 83). In addition, in acute situations these eGFR variations may be even higher for short periods. Consequently, to make a definitive diagnosis of chronic change in the kidney function, two creatinine measurements are necessary over 3 months (73).

				Persistent albuminuria categories, description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				< 30 mg/g < 3 mg/mmol	30-300 mg/g 3-30 mg/mmol	>300 mg/g >30 mg/mmol
GFR categories (ml/min/1.73m²), description and range	G1	Normal or high	≥90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	59-45			
	G3b	Moderately to severly decreased	40-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Figure 4. Kidney function classification into five stages according to Kidney Disease Improving Global Outcomes (KDIGO).

Based on AER, *DN* could "classically" be diagnosed in an individual with diabetes based on "*persistent albuminuria of >300 mg per 24h or a ACR >300 mg/g, the presence of diabetic retinopathy, and the absence of any clinical or laboratory evidence of other kidney or renal tract diseases*" (84). Importantly, other potential extrarenal causes of increased AER should be excluded, such as poor glycemic control,

strenuous physical exercise, elevated blood pressure, heart failure, pregnancy, menstruation or acute illnesses with fever, before a definitive diagnosis is made (85). However, in the mid-1980s microalbuminuria was already considered “incipient nephropathy” (86). This classical definition was independent of any other kidney damage markers of chronic kidney disease (CKD) or of eGFR, while the appearance of persistent macroalbuminuria was considered the point of ‘no return’ for disease progression (87).

Based on the clinical and structural renal alterations, DN in T1DM can be divided into five stages: 1) early renal hypertrophy—hyperfunction, 2) renal lesions without clinical signs, 3) incipient DN (microalbuminuria), 4) clinical overt DN (macroalbuminuria), 5) ESRD (88). The main clinical feature mirroring the progression of DN in this classification is urinary AER. In addition, at the very early stages hyperfiltration is present.

Based on the eGFR values, kidney function also is divided into five stages: 1) $\geq$90 ml/min/1.73m^2 — normal GFR or hyperfiltration, 2) 60–89 ml/min/1.73m^2 — mild renal dysfunction, 3) 30–59 ml/min/1.73m^2—moderate renal dysfunction, 4) 15–29 ml/min/1.73m^2 — severe renal dysfunction, 5) $\leq$15 ml/min/1.73m^2 or dialysis — renal insufficiency (89, 90).

Even if AER and eGFR both mirror the progression of DN, the use of just one of them for the risk stratification could miss out individuals progressing towards ESRD. It is worthy of note that the true significance and independent existence of hyperfiltration has been debated (91, 92). Others have supported the idea of an early decline in kidney function, even within the normal GFR range, as a better clinical predictor of progression (93). Furthermore, the presence of a long asymptomatic period with normal eGFR, but with clear histological kidney abnormalities, has been widely accepted (88). Last but not the least, 2 to 4% of individuals with T1DM have low eGFR and normal AER, while up to 23% of individuals with T2DM have non-albuminuric chronic kidney disease (94-96). Therefore, screening and diagnosis of renal complications in diabetes should include both eGFR and AER (Fig.4) (73). According to the latest KDIGO guideline, in most individuals with diabetes CKD should be attributable to diabetes if: *macroalbuminuria* is present; or *microalbuminuria* is present in the presence of diabetic retinopathy or in *T1DM of at least 10 years’ duration* (97).

2.2.2.2. Epidemiology

Before discussing the epidemiology of DN, it is important to understand the differences in the diagnostic methods. Until the beginning of the 1980s, DN was diagnosed based on the presence of proteinuria, since there was no other diagnostic method available (98). The concepts of microalbuminuria and incipient DN appeared in 1982 (99-101). Because of this novel and early biomarker of kidney damage, the diagnosis could be made even before the “point of no return,” and with newer treatments (angiotensin converting enzyme inhibitors, lipid lowering treatment, blood pressure control) the progression could be slowed down considerably.

Early studies showed that 40% of the individuals with T1DM developed proteinuria with an incidence peak at 15 years of diabetes duration and a prevalence peak at

25 years of diabetes duration (102). Newer studies have shown similar cumulative incidence rates for proteinuria, but with 5 to 15 years delay, suggesting the beneficial effects of early diagnosis and treatment (103-105). In addition, similar trends in the cumulative incidence are observed when looking at the progression to ESRD, which nowadays also occurs at older ages (5, 106, 107).

In T2DM, the prevalence of diabetic kidney disease (DKD) ranges between 38 and 50% at 15 years of diabetes duration (108, 109). The number of individuals with T2DM receiving renal replacement therapy (RRT) has increased five-fold since 1993. Notably, today T2DM is the leading cause of ESRD, representing 1/3 of individuals with ESRD (110).

2.2.2.3. Clinical risk factors

The most important treatable clinical risk factors for DN include an unfavorable glycemic control, high blood pressure and dyslipidemia. In addition, several other factors could also play a role in the pathogenesis of DN, e.g. smoking (111), lack of physical exercise (112) and genetic propensity (113).

Glycemic control

Glycemic control can be assessed by using its two main components—the average glycemic control and its variability. In addition, other factors responsible for the biological variation in protein/hemoglobin glycosylation could also play a role in its estimation.

The average glycemic control can in the short term be evaluated by using self-monitoring of blood glucose (SMBG) or continuous glucose monitoring (CGM) systems. With these methods the blood glucose values can be translated into an estimated average glucose (eAG) and subsequently into HbA_{1C} values to estimate the long-term average glycemic control (114). A satisfactory short term glycemic control means fasting glycemia of 70 to 130 mg/dl (3.8 – 7.2 mmol/l) and postprandial glycemia below 180 mg/dl (10 mmol/l), which can be translated into an average glucose of 154 mg/dl (8.5 mmol/l) or a HbA_{1C} of less than 7% (53 mmol/mol) (115). Glycemic control evaluated by its average is the main risk factor for DN in individuals with T1DM and for DKD in individuals with T2DM (116, 117). Other important metrics of average glycemic control derived from ambulatory glucose profiles or CGM are target range and time in range. In individuals with diabetes if the glycemic values are in target range for more than 50% of the time HbA_{1C} will be less than 7% (118).

Short-term glycemic variability can be measured by multiple indexes. There is a consistent association between short-term glycemic variability and the microvascular complications in individuals with T2DM. However, the association of short-term glycemic variability with chronic complications in individuals with T1DM is less clear (119).

Long-term glycemic variability refers to fluctuations in the glycemic control from one visit to another and is most commonly evaluated by the HbA_{1C} variability (120). HbA_{1C} variability can be assessed by its standard deviation (SD) or using the HbA_{1C} variability score (121, 122). In T1DM, long-term variability of HbA_{1C} is a risk factor for microalbuminuria and DN progression (123, 124). Also in individuals with T2DM the long-term HbA_{1C} variability is associated with DKD (125). Other factors

associated with the biological variation in protein/hemoglobin glycosylation are the hemoglobin glycosylation index (HGI) and the glycation gap (GG). Both these indexes predict DN in individuals with T1DM and DKD in T2DM (126, 127).

Blood pressure

Blood pressure is another important risk factor for DN. The treatment targets for an optimal blood pressure control in most individuals with diabetes are a *systolic blood pressure* <140 mmHg and a *diastolic blood pressure* <90 mmHg (128). In addition to the systolic and the diastolic blood pressure, other components of the blood pressure also have been associated with DN. One component is the *visit-to-visit blood pressure variability*, which was shown to be an independent risk factor for microvascular complications in diabetes (129-131). An altered circadian rhythm, and in particular an *elevated nocturnal blood pressure*, has also been associated with the onset and the progression of DN (132-134).

Lipid profile

The relationship between the lipid profile and risk of DN is often complex (135). In individuals with T1DM, there are conflicting reports that usually show an association between single components of the lipid profile and DN, while the other components have either not been reported or have not been associated with DN (136-139). However, a low *high-density lipoprotein cholesterol (HDL-C)* concentration has constantly been associated with the onset and progression of DN (140). In addition, serum *triglycerides* and *apolipoprotein C3 (ApoC-III)* have also been associated with DN progression (141-143).

In individuals with T2DM, higher triglycerides and lower *HDL-C*, though not *low-density lipoprotein cholesterol (LDL-C)*, have been associated with DKD (144). Other prospective studies have demonstrated that triglycerides and LDL-C predict not only eGFR decline but also the onset of albuminuria (145).

Other factors

In addition to the above-mentioned well-known risk factors, age, age of onset of diabetes, sex, BMI, smoking, family history and ethnicity, genetic background, low vitamin D level, uric acid, puberty, pregnancy, hormonal status, hyperfiltration, oxidative stress and subclinical inflammation have also been associated with DN (135, 146, 147).

2.2.2.4. Pathogenesis

Biochemical mechanisms

Biochemical mechanisms involved in the pathogenesis of DN are classically associated with *oxidative stress*, which triggers a range of molecular signaling pathways that first lead to functional changes and then to structural changes in the kidneys.

Oxidative stress reflects an imbalance between the systemic manifestation of *reactive oxidative species (ROS)* and the ability to detoxify the reactive intermediates or to repair the resulting damage. During hyperglycemic conditions there is overproduction of mitochondrial superoxide (ROS), which suppresses the antioxidant systems and induces oxidative stress and subsequent damage to the

nuclear deoxyribonucleic acid (DNA). Such DNA damage leads to activation of the DNA repair enzyme poly-ADP-ribose polymerase-1 (PARP-1), which is an enzyme that inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the glycolysis. The inhibition results in increased levels of glyceraldehyde-3-phosphate (GAP) and other glycolytic intermediates and glucose. Accumulation of these molecules in the cell stimulates pro-oxidative pathways like the advanced glycation end product (AGE) and protein kinase C (PKC) pathways due to increased levels of GAP, but also the hexosamine and polyol pathways due to increased levels of fructose-6-phosphate and glucose. All four pathways are involved in the pathogenesis of diabetic complications (148). The mitochondrial ROS overproduction is a consequence of intracellular hyperglycemia in the microvasculature, but in contrast a consequence of increased oxidation of fatty acids in the large blood vessels and the heart (149).

Another source of ROS is the activation of the renin-angiotensin-aldosterone system (RAAS) that could also contribute to the increased oxidative stress alongside the hyperglycemia (150). Increased amounts of ROS and oxidative stress lead to uncoupling of eNOS (endothelial nitric oxide synthase), which in turn decreases nitric oxide production and results in endothelial dysfunction (vasoconstriction and leucocyte adherence). In addition, oxidative stress promotes glomerular cells' apoptosis (151).

Oxidative stress activates several intracellular signaling pathways such as mitogen activated protein-kinase (MAPK), Janus kinase-signal transducer and activator of transcription (JAK-STAT), phosphatidylinositol 3-kinase (PIK3) and the Protein Kinase B (Akt/PKB) pathway (152-154). Then, through activation of several transcription factors such as nuclear factor kB (NF-kB), activator protein 1 (AP1), signal transducer and activator of transcription (STAT), the production of growth factors is enhanced [mainly transforming growth factor beta (TGF-β), connective tissue growth factor (CTGF)] (155). Finally, increased expression of extracellular matrix proteins (type IV collagen, laminin and fibronectin), secondary hypertrophy of the mesangial cells and decreased expression of matrix metalloproteinases leads to deposition of extracellular matrix (155-157). Mesangial cell expansion and deposition of extracellular matrix are the first structural changes that appear in DN.

In addition, through multiple signaling pathways induced by oxidative stress, the podocytes suffer cytoskeleton rearrangement and foot process effacement with subsequent loss of the slit diaphragm (158). One important step in this process is also the loss of nephrin, an important protein of the slit diaphragm (157). Moreover, increased ROS production has been implicated in podocyte apoptosis (159). Podocyte foot process effacement and apoptosis together with nephrin and slit diaphragm loss are the most important changes leading to proteinuria.

At the tubular level, hyperglycemia and oxidative stress trigger activation of RAAS, hypoxia, apoptosis and fibrosis (160). Hyperglycemia increases the production of citric acid intermediates such as alpha-ketoglutarate, which in turn activates G-protein-coupled receptor 91(GPR91) (161). Activation of GPR91 in endothelial juxtaglomerular cells triggers changes in cytosolic Ca2+, nitric oxide (NO) and prostaglandin E2 (PGE2), and increases the release of renin. Renin further increases the angiotensin I, which finally leads to high levels of angiotensin II (162). Binding of angiotensin II to its angiotensin II receptor type 1 (AT1) receptors in the

endothelial glomerular cells, mesangial cells, podocytes and endothelial tubular cells triggers vasoconstriction, increased intraglomerular pressure, hypoxia, increased production of TGF-β and extracellular matrix proteins (163).

Kidney hypoxia as a consequence of hyperglycemia by itself induces oxidative stress, a change in the oxygen-hemoglobin dissociation curve, impaired stabilization of hypoxia-inducible factor 1-alpha (HIF-1α) and growth factor production, abnormalities in red blood cells as well as tubular cell apoptosis. An immediate effect of tubular and peritubular cell apoptosis is a decrease in the activation of vitamin D and production of erythropoetin. Low vitamin D also activates the renin production, while low erythropoetin exacerbates the hypoxia (164, 165).

Tubular cell exposure to growth factors leads to "tubular cell activation" with subsequent secretion of chemokines [monocyte chemoattractant protein-1 (MCP-1), regulated upon activation normal T cell expressed and presumably secreted (RANTES)] and growth factor peptides [platelet derived growth factor (PDGF-B)] into the interstitium as well as the attraction of monocyte and macrophages, with interstitial microinflammation and fibrosis as the net result (166). Finally, extensive remodeling of the mesangium and tubulointerstitial fibrosis will appear due to sustained TGF-β activity and exacerbation of oxidative stress secondary to AT1 receptor activation (167, 168). Tubular atrophy and interstitial fibrosis are the leading tubulointerstitial lesions in DN.

Both glomerular and tubular cell damage can also be triggered and sustained by high intracellular fatty acid (FA) concentrations that lead to lipotoxicity through complex mechanisms (169).

Finally, the tubulo-glomerular feedback is one of the several mechanisms by which the kidney regulates the renal blood flow and the glomerular filtration rate. Sodium and glucose are reabsorbed by the sodium-glucose co-transporter 2 (SGLT2) in the proximal tubule, and during hyperglycemic conditions there is a maladaptive increase in the glucose and sodium reabsorption, leading to less sodium reaching the macula densa, a collection of densely packed epithelial cells at the junction of the thick ascending limb and distal convoluted tubule. Sodium chloride is sensed by the apical Na-K-2Cl cotransporter in the macula densa, and an increase in the distal tubular sodium chloride concentration causes basolateral release of adenosine from the cells in the macula densa. Adenosine is a strong vasoconstrictor in the afferent arteriole and leads to reduced renal blood flow, increase in the renal vascular resistance and ultimately a decrease in the GFR (170).

Mechanical stress

Mechanical stress on the mesangial cells activates the expression of GLUT-1 (glucose transporter 1) and results in increased glucose uptake, oxidative stress and excess TGF-β production (171). In addition, mechanical stress contributes to podocyte foot process effacement and podocyte detachment (172).

In conclusion, the diabetic milieu (high glucose, oxidative stress, glycated proteins) together with lipid accumulation and hypertension-induced mechanical stress triggers angiotensin II, inflammatory cytokines and growth factors production. Therefore, glomerular and tubular injury develops as a consequence of complex remodeling of the glomerular and tubular structures, with proteinuria and renal function decline as the net result.

2.2.3. Cardiovascular disease

CVD is a major chronic comorbidity of diabetes, and individuals with diabetes have more than 10-fold increase in the risk of CAD compared with the non-diabetic population (173). CVD comprises the following disorders: CAD, DC, heart failure, stroke and PVD. Compared with T2DM, heart failure and DC are much less prevalent in T1DM, this being the reason why I will mainly discuss CAD, PVD and CVD or stroke below (174, 175).

2.2.3.1. Definition

CAD is usually defined as coronary artery damage followed by inadequate blood supply of oxygen and nutrients to the myocardium (55). *Stroke* is defined as a neurological deficit attributable to cerebrovascular damage leading to a focal injury of the CNS that could be represented by cerebral infarction, intracerebral hemorrhage or subarachnoidal hemorrhage (56). *PVD* is defined as a chronic obstructive arterial disease affecting the lower extremities, with decreased lower extremity arterial perfusion leading to pain, ulcers, gangrene and ultimately amputations (58).

An estimation of the cardiovascular risk can be performed by using the United Kingdom Prospective Diabetes Study (UKPDS) cardiovascular risk calculator for individuals with T2DM or using either the Swedish National Diabetes Register (NDR) model or the Steno T1 Risk Engine for individuals with T1DM (44, 176, 177).

2.2.3.2. Epidemiology

In individuals with T1DM, CVD events occur earlier compared with nondiabetic subjects (178). In addition, individuals with T1DM have a 10 times higher risk of CVD compared with the general population (179).

The annual incidence of CAD is reported to be on average around 1% per year in those with T1DM, with a maximum cumulative incidence of 19% after 15 years of follow-up (180). Age-adjusted incidence rates for major CAD events in individuals with T2DM are 1.5% for women and 1.6% for men, with differences according to age, geographical area and presence of other diabetic complications (181).

The exact incidence and prevalence of PVD is very difficult to estimate, since it varies according to the diagnostic criteria used [symptoms of claudication, palpation of peripheral pulses or ankle-brachial index (ABI) calculation], age, type of diabetes and country. Consequently, the PVD incidence varies between 1.3% and 2.1%, while the prevalence also varies between 8 and 38% (182).

The annual incidence of stroke in individuals with T1DM varies between 0.3% and 0.74% per year depending on the population studied (180, 183). The incidence rate in T2DM starts from 5% and rises up to 15% in those with a previous history of CVD (184). These rates are much higher than in the general population, which are usually between 0.2 and 0.3% per year (185).

2.2.3.3. Clinical risk factors for CVD

Similar to DN, glycemic control, lipids and blood pressure are important risk factors for CVD.

Glycemic control

In the UKPD Study, after a median follow up of 10.4 years, each 1% reduction of the HbA_{1C}, a measure of the *average of glycemic control*, was associated with a risk reduction of 21% for any end point related to diabetes, of 21% for deaths related to diabetes, of 14% for myocardial infarction, of 12% for fatal and non-fatal stroke and a 43% decrease in the risk of amputations. No threshold of risk was observed for any of the endpoints (186). Later studies in individuals with long standing T2DM and high cardiovascular risk suggested a non-linear relationship between mean HbA_{1C} and all-cause mortality or cardiovascular events, with a suggested threshold of 7% (187). Further studies in T2DM showed that at the same HbA_{1C} level the risk reduction was dependent on the drug classes used to achieve the mean glycemic control, irrespective of the diabetes duration (188-193).

In T1DM, the HbA_{1C} values are also an important risk factor for CVD. In the DCCT Trial, intensive treatment reduced the risk of CVD endpoint by 42 to 57 %, with durable effects at 30 years of follow up (173, 194).

Short-term glycemic variability assessed by fasting plasma glucose has been associated with cardiovascular mortality. Although studies exploring the impact of postprandial glucose are very few, they do point in the same direction (195). In addition, daily glycemic variability was positively associated with cardiovascular autonomic neuropathy, subclinical atherosclerosis and central blood pressure (196-198). Hypoglycemia, another part of the glycemic variability, has also been linked to cardiovascular outcomes both in T1DM and T2DM (190, 199, 200). Furthermore, hypoglycemia was demonstrated to be linked not only with cardiovascular events but also with mortality, especially in T1DM (201).

Long-term glycemic variability estimated as a visit-to-visit variation of fasting glycemia or HbA_{1C} (SDs and coefficients of variation) was positively associated with micro- and macrovascular complications independently of the HbA_{1C} level, both in T1DM and T2DM (121, 202).

Blood pressure

The best-known components and therapeutic targets of blood pressure (*systolic* and *diastolic blood pressure*) are well established risk factors for CVD in individuals with diabetes (203, 204). However, blood pressure is much more complex than these two components (205). Some of the other components of the blood pressure such as the *pulse pressure* and the *arterial stiffness* are also predictive measures of CVD (206, 207). It is worth mentioning that these measures are also influenced by the glycemic control (208, 209).

In addition to long-term HbA_{1c} variability, *visit-to-visit systolic blood pressure variability* was associated with mortality in individuals with T1DM (210). Furthermore, long-term systolic and diastolic blood pressure variability was also associated with cardiovascular disease in individuals with T2DM (211, 212).

Other components of blood pressure such as *orthostatic hypertension* may also contribute to the risk of CVD (213).

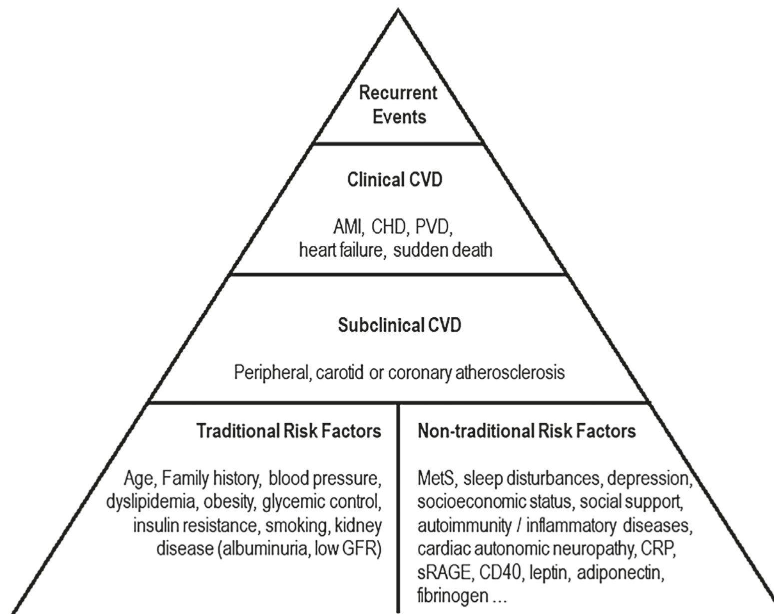

Figure 5. Cardiovascular risk pyramid and risk factors.
Adapted for T1DM after Khambhati J and de Ferranti SD (180, 223). AMI — acute myocardial infarction, CAD — coronary artery disease, PVD — peripheral vascular disease, Srage — soluble form of receptor for advanced glycation end products, MetS — metabolic syndrome, CD40 — cluster of differentiation 40 protein.

Lipid profile

Similar to the general population, dyslipidemia is a risk factor for CVD in individuals with diabetes (180, 214). However, which fraction of the lipids and the lipoproteins may contribute to the higher risk in individuals with T1DM is still debated. Furthermore, better glycemic control may significantly improve the lipid values, thereby complicating the role of the lipids in the CVD prediction, especially in individuals with T1DM (215).

The *HDL-C* metabolism is altered in individuals with T1DM due to abnormal hepatic lipase and vascular lipoprotein lipase (216, 217). Although a linear decrease in the incidence of CAD was observed with increasing HDL-C concentration in men, the incidence increased in women, when the HDL-C concentration was either below 47 mg/dl (1.22 mmol/l) or above 80 mg/dl (2.07 mmol/l) (218). However, not all HDL-C subclasses are associated with the same risk of CVD (219).

LDL-C was strongly associated with CVD in individuals with T2DM, but its relationship with CVD in T1DM is less clear (220-222). However, different subfractions of LDL, such as oxidized LDL (Ox-LDL), advanced glycation end products LDL (AGE-LDL) or lipopolysaccharides-derived LDL (LSP-derived LDL) particles seem to have a major impact on the progression of subclinical CVD in T1DM (137, 220).

Triglycerides were also demonstrated to be strong predictors of CVD in type 2 diabetes, but in T1DM their role in CVD prediction is still debated (224).

Other lipid fractions or indexes such as non-HDL cholesterol, non-HDL/non-LDL cholesterol, Ox-LDL, AGE-LDL, atherogenic index of plasma, apoC3 and lipoprotein B:C3 (LpB:C3), apolipoprotein B, lipoprotein(a) and apolipoprotein B/ apolipoprotein A-I ratio have also been studied (220, 224-230).

Visit-to-visit lipids variability of fasting HDL-cholesterol, triglycerides and LDL-cholesterol has been demonstrated to predict cardiovascular events, especially in T2DM (231, 232).

Other risk factors

Other traditional risk factors for CVD are sex, diabetes duration, obesity and insulin resistance, smoking, kidney disease and probably ethnicity (180). The association of microalbuminuria, inflammation and smoking with CVD is relatively similar in T1DM and T2DM, while obesity, insulin resistance and lipids are less strongly associated with CVD in T1DM compared with T2DM (180). The gender effect on CVD is clear in T2DM, while in T1DM the protective role of the female sex seems to be lost, especially in those with DN (233, 234). Finally, family history of T2DM seems to play a role in the CVD risk generation in T1DM (235). Other non-traditional risk factors are presented in Figure 5.

2.2.3.4. Pathogenesis

Similar to what is seen in DN, risk factors such as hypertension, lipids, hyperglycemia or cigarette smoking seem to trigger increased ROS production in the vascular wall, leading to oxidative stress (236, 237). ROS are produced from molecular oxygen triggered by the above-mentioned risk factors by multiple enzymes: nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase, xanthine oxidase, enzymes of the mitochondrial respiratory chain and inducible endothelial NO synthase (238).

Enzymes that detoxify ROS include are superoxide dismutase, catalase, glutathione peroxidase, etc. (239). The disequilibrium between the production and neutralization of ROS leads to oxidative stress and activation of different pathways leading to accelerated atherosclerosis in the vascular wall (148). Hyperglycemia leads to ROS overproduction and activation of the hexosamine pathway, which further inhibits more than 60% of the endothelial NO synthase activity (240). Furthermore, hyperglycemia activates PKC, which in turn inhibits the insulin-stimulated eNOS activity and stimulates the endotelin-1 that has vasoconstrictor properties (241). Finally, high glucose exposure of endothelial cells triggers eNOS uncoupling through the Nox4-NADPH subunit with subsequently enhanced superoxide production and decrease of the NO production, thereby causing vasoconstriction and blood flow abnormalities (242).

Endothelial cells exposed to *LDL cholesterol* also show eNOS uncoupling (243, 244). High glucose levels accelerate the lipid oxidation, with formation of small, dense LDL particles and ox-LDL, especially in individuals with hypertriglyceridemia (245, 246). Some other key players besides glucose also influence lipid peroxidation and eNOS uncoupling, e.g., free fatty acids, leucocyte myeloperoxidase and smoking (247, 248). Ox-LDL activates NADPH and xanthine oxidase, with a boost of oxidative stress generation (249). Activation of NAPH further enhances eNOS uncoupling, on top of the native LDL effect (242-244). Lipid peroxidation in the

arterial wall together with leucocyte/macrophage chemoattraction and oxidative stress favors LDL deposition in the vessel walls that in turn leads to atherosclerotic lesions (248). Multiple dysfunctionalities of the HDL-C metabolism further favor the atherosclerotic process (219). Vasoconstriction and atherosclerotic plaque are the main mechanisms of cardiovascular disease.

Hyperglycemia activates the *RAAS* through multiple mechanisms, leading to exacerbated oxidative stress, endothelial dysfunction and hypertension (162, 250). Hyperglycemia further induces the release of renin (162). Furthermore, hyperglycemia increases the activity of the angiotensin converting enzyme, upregulates the AT1 receptors and downregulates the angiotensin receptors type 2 (AT2)(250). Activation of AT1 receptors by angiotensin II leads to activation of NAD(P)H oxidase and xanthine oxidase with oxidative stress exacerbation, downstream activation of other pathways, endothelial dysfunction and vasoconstriction, hypoxia, inflammation and vascular remodeling (250, 251). All these mechanisms lead to hypertension and blood flow abnormalities. The resulting hemodynamic shear stress accelerates the plaque formation and the plaque rupture with subsequent thrombosis – the major culprits in CVD (252).

Hypoglycemia is also involved in CVD and cardiovascular mortality (253). The vascular consequences of acute hypoglycemia reside in an increase in the diastolic blood pressure "*with a relatively late reduction of the diastolic blood pressure,*" "*and a reduction of the plasma volume, reflected by an increase in the haematocrit*" by resetting the baroreflex working range (254). In addition, activation of alpha adrenoreceptors leads to an increase in the number of lymphocytes and platelets together with platelet activation. Furthermore, acute hypoglycemia leads to a marked increase in C reactive protein, coagulation factor VIII activity as well as the von Willebrand factor and thrombin generation (255, 256). Thus, by multiple mechanisms (capillary closure, thrombosis and atherogenesis) hypoglycemia promotes cardiovascular complications (257). Furthermore, hypoglycemia has important effects on the heart such as increased heart rate, prolonged QT interval and cardiac arrhythmia, potentially leading to sudden death (258-260).

In conclusion, hyperglycemia and lipid abnormalities trigger hypertension and together cooperate to produce the atherosclerotic background, while hypoglycemia may lead to acute cardiovascular events in individuals with diabetes.

2.3. Link between renal complications, cardiovascular disease and mortality

There are multiple links between the renal complications, CVD and premature mortality, which are clearly highlighted by their common risk factors and pathogenic mechanisms. The common risk factors, among others, are hyperglycemia, hypoglycemia, unfavorable lipid and lipoprotein profiles, high blood pressure, smoking, endothelial dysfunction and hypoxia. The clinical proofs of the links are: 1) diabetes increases the risk of CVD; 2) the presence of DN (either a low eGFR or increased AER or both) enhances the risk of CVD and premature death; 3) treatment of both DN and CVD targets the hyperglycemia, lipid levels, hypertension, smoking and endothelial dysfunction.

2.4. Glomerular biomarkers for DN and CVD in T1DM

The glomerular wall has 3 layers: endothelial cells (fenestrated endothelium), glomerular basement membrane and epithelial cells (podocyte slit diaphragm) (261). These 3 layers together with the glycocalyx (syndecan, heparin sulphate, chondroitin sulphate, hyaluronan) and a few other podocyte proteins (nephrin, podocalyxin) are responsible for the electrical charge and the selectivity of the glomerular filtration barrier (262). This barrier is damaged in DN, and plasma proteins that are not normally filtered at the glomerular level (e.g., albumin) show up in the urine (263). The main protein present in the urine is albumin. As a consequence, albuminuria does not reflect only kidney (glomerular basement membrane and epithelial cells) damage but also endothelial damage (264-267). By reflecting the kidney and the general endothelial status in individuals with diabetes, albuminuria (proteinuria) may predict not only kidney disease, but also CVD (180).

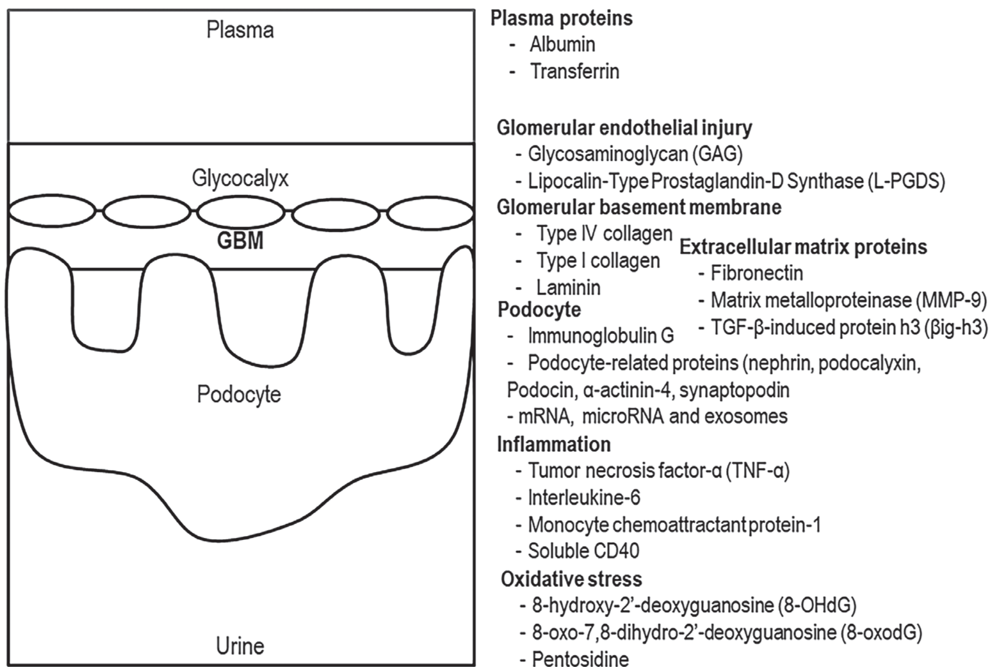

Figure 6. Glomerular biomarkers of DN.
Adapted after Moresco RN (270) and Lee YS (271).

Serum albumin (65 kDa) is filtered at the glomerular level and then reabsorbed at the tubular level and represents the major source of urinary albumin. *Albuminuria* may reflect also the tubular reabsorptive capacity, apart from the glomerular and endothelial function. Therefore, albumin in the urine is one of the first asymptomatic clinical signs of microvascular complications. Based on the urinary albumin concentration in relation to the time the urine was collected (either overnight or 24 hours), the AER can be calculated. According to the AER, DN has been divided into: microalbuminuria (moderately increased albuminuria) and macroalbuminuria

(severely increased albuminuria) (268). Persistent microalbuminuria appears usually 5 or more years after the diabetes diagnosis and predicts, in more than 80% of cases, progression to overt proteinuria and ESRD in individuals with T1DM, but also in T2DM (99, 269). In addition, it is an important predictor of cardiovascular outcomes both in T1DM and T2DM (270).

Transferrin (76.5 kDa) has a similar molecular weight as albumin but is less anionic and may be filtered at the glomerular level more easily than albumin (272). Cross-sectional studies in T1DM have shown that transferrin may be an early marker of glomerular damage (273). However, no prospective studies have been performed to evaluate the predictive value of transferrin in T1DM, although in T2DM urinary transferrin was shown to predict the onset of microalbuminuria (274). There are no studies on transferrin and CVD.

Glycosaminoglycans (GAG) are important components of the endothelial glycocalyx. These GAGs play an important role in the endothelial dysfunction in diabetes (275). However, the use of urinary GAG as biomarkers for the diagnostics or the prediction of DN has not shown any promising results so far (271, 276).

Lipocalin-type prostaglandin D synthase/β-trace (L-PGDS/BTP) (26kDa) is an enzyme secreting prostaglandin D_2 (PGD_2). It is secreted by the choroid plexus into the cerebrospinal fluid and then into the blood stream (277). Due to its low molecular weight, L-PGDS is normally filtered at the glomerular level and an increase in its urinary concentration is considered a marker of increased endothelial permeability (278). No study on its predictive properties in individuals with T1DM has been performed so far, but in T2DM L-PGDS was shown to predict the onset and progression of DKD (279). Furthermore, urinary L-PGDS was associated with essential hypertension in the general population and with vascular injury in individuals with T2DM, though no data are available for T1DM (280, 281).

Type IV collagen (~ 540 kDa) is a major component of the mesangial matrix, the glomerular and tubular basal membranes and is minimally affected by its serum levels (282, 283). Urinary type IV collagen was proposed to be an indicator of the matrix turnover rate and to serve as a biomarker for early DN in T1DM (284). Furthermore, the urinary type IV collagen to albumin ratio has been suggested to be a useful tool to differentiate DN from non-diabetic kidney damage in individuals with diabetes (285). Type IV collagen was associated with kidney function decline in individuals with T1DM, as well as in T2DM (286, 287). In addition, type I collagen fragments predicted the progression to macroalbuminuria 3 to 5 years in advance in individuals with T1DM (288). Finally, the panels of biomarkers based on collagen fragments have also been associated with CAD (289).

Laminin is a large glycoprotein (~ 900 kDa), without glomerular passage, present in the glomerular and tubular basement membranes and acting as a cellular adhesion molecule (290). Its presence in urine is suggested to represent glomerular basement membrane remodeling and laminin turnover (291). The L-P1 fragment of laminin was found to be increased in individuals with diabetes, while the LG1M [a specific MMP-9-generated neo-epitope fragment of LAMC1 (laminin γ1 chain)] predicted the progression of CKD in non-diabetic individuals (291, 292). No studies in individuals with T1DM with DN or CVD are available.

Urinary immunoglobulins (Ig), and especially immunoglobulin G and IgG4 (~ 150 kDa), have been proposed as biomarkers of early loss of glomerular charge selectivity in individuals with normal AER (293, 294). Other studies have shown different patterns of immunoglobulins in normoalbuminuric and microalbuminuric individuals with T1DM (295). Their utility as indicators of glomerular membrane selectivity was questioned when IgG expression in podocytes was demonstrated (296). However, their clinical importance as a predictor of microalbuminuria was reconfirmed (274, 295, 297). With regard to CVD, so far, only IgM has been shown to be predictive of cardiovascular events in individuals with T1DM, independently of AER (297).

Nephrin (~180 kDa) is a podocyte trans-membrane protein and its presence in urine was suggested to represent podocyte injury (298). In individuals with diabetes, nephrinuria has been associated with albuminuria and lower eGFR even in normoalbuminuric individuals (299). Messenger RNA expression of other podocyte related proteins such as *podocin*, *synaptopodin*, *WT-1* (*Wilms' tumor suppressor protein)* and *alpha-actinin-4* is higher in individuals with DN than in healthy controls (300).

MicroRNAs (miRNAs) are short (~22 nucleotide long), antisense, non-coding RNAs present in cells with an important post-transcriptional regulatory role of gene expression (301). Different miRNAs have been found to be up/downregulated in the urine of individuals with DN compared with those without DN or have been shown to be predictive of the development of DN (302-305).

Extracellular matrix proteins such as *fibronectin*, *matrix metalloproteinases* or *TGF-β-induced protein h3* have also been proposed as biomarkers for DN. Fibronectin (~440 kDa) is a structural protein of the glomerular extracellular matrix. In cross-sectional studies it was found to be increased in individuals with T1DM and macroalbuminuria, as well as in those with T2DM and microalbuminuria (306). Matrix metalloproteinases (MMP-2 and MMP-9) were correlated with albuminuria in individuals with T1DM (307, 308). Finally, urinary MMP-2 and MMP-9 were increased in individuals in the general population with atherosclerosis or CAD, but no data are available for T1DM (309).

People with T1DM have been shown to have higher urinary levels of the profibrotic growth factors and inflammatory chemokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10). However, only the urinary TNF-α was independently associated with the severity of albuminuria (310). In addition, the levels of soluble tumor necrosis factor-α receptor 1 (sTNFR1) are strongly independent predictors of progression to ESRD (311). Monocyte chemoattractant protein-1 (MCP-1) was predictive of changes in kidney interstitial volume (312). MCP-1 also correlated with the eGFR decline at the late stages of DN (313).

Individuals with T1DM with poor glycemic control have been demonstrated to have higher plasma levels of C-reactive protein (CRP), IL-6, MMP-9 and soluble CD40 (cluster of differentiation 40 protein). However, these levels were not elevated due to a decreased excretion by the kidneys, since their urinary levels were also elevated, suggesting increased production (314). There are no studies on urinary CD40 and DN progression, despite the fact that CD40 alters the glomerular permeability (315). Urinary sTNFR1, sTNFR2 and sIL-6R were associated with the severity of heart

failure in the general population (316). However, no studies were found on sTNFR1, sTNFR2, sIL-6R, MCP-1 or CD40 and CVD in individuals with T1DM.

Hyperglycemia has been associated with oxidative stress and subsequent endothelial dysfunction (317). Markers of oxidative stress were therefore proposed as predictors of chronic complications in T2DM, but no systematic studies have been performed in individuals with T1DM and DN (318, 319). However, in some studies free urinary *pentosidine* predicted the progression of DN (320, 321). A few urinary markers of oxidative stress have been associated with CHD, cardiovascular and all-cause mortality in people with diabetes (322, 323).

Based on the two endogenous biomarkers *serum creatinine* and *cystatin C*, a GFR can be estimated by using different equations. During the last 50 years, more than 70 mathematical equations have been developed to estimate the GFR (324).

2.5. Limitations of glomerular biomarkers and need for other biomarkers

Albuminuria is the only glomerular urinary biomarker that has made it into clinical practice as a predictor of DN. Despite its prediction of DN progression towards ESRD or its prediction of CVD, albuminuria also has several limitations (325), as presented in Table 2.

Table 2. Main characteristics and limitations of albuminuria relating to DN and CVD.
Modified after Tuttle RK (325)

Major advantages
Diabetic nephropathy
Higher AER is associated with faster eGFR decline
Lowering of AER by treatment lowers the risk of clinical events and DN progression
Cardiovascular disease
Independently predicts events and mortality
Regression reduces the risk
Major limitations
Diabetic nephropathy
Low eGFR can be present without increased AER
Cardiovascular disease
Categorical nomenclature not reflecting the continuous nature of CVD or DN
General
Non-standardized measurement and reporting
- Assay variability approximately 40%
- Reporting variability - concentrations, timed excretion, ratio to creatinine
Individual variability
- Day to day variability approximately 40%
- Influenced by: high protein diet, glycemic control, blood pressure, heart failure, exercise, urinary tract infections, fever, etc.

The gold standard of kidney function assessment is the measurement of GFR. GFR measurement can be performed by using several methods with administration of different substances such as: inulin, chromium-51 labeled ethylenediamine tetraacetic acid (^{51}Cr-EDTA), ^{99}Tc-pentetic acid (DTPA), iohexol or iothalamate (324). To avoid the administration of such substances, the glomerular filtration rate in clinical practice has been measured by 24-hour endogenous creatinine clearance. In this case, urine was collected over 24 or 12 hours and the serum creatinine was measured at the beginning and at the end of the collection. Based on these determinations the creatinine clearance could be calculated (326). When considered

for implementation in routine clinical practice, all methods designed to measure GFR have been impractical, difficult to perform, burdensome and time-consuming. The main advantages and disadvantages of the GFR measurement methods are summarized in Table 3.

Table 3. Main advantages and disadvantages of GFR measurement methods.
Modified after Schaeffner E et al. (334)

Major advantages
Inulin
“Gold standard among Gold standards”
No side effects
Iothalamate
Inexpensive assay
Long half-life period
No urinary clearance needed
Iohexol
Inexpensive and sensitive assay
It allows a low dose administration
More precise than iothalamate clearance and similar to inulin clearance (if multiple samples are taken)
No urinary clearance needed
Single-sample technique possible
Simplified by dried blood spot testing
^{51}Cr-EDTA
Reliable alternative to inulin
Widely available in Europe
^{99}Tc- DTPA
Widely available in Europe
Major limitations
Inulin
Expensive
Urinary clearance needed
Complex procedure
Iothalamate
Expensive
Contains iodine
Potential tubular secretion
Complex procedure
Iohexol
Contains iodine
^{51}Cr-EDTA
Radiolabeled substance
Complex procedure
^{99}Tc-DTPA
Radiolabeled substance
Complex procedure

^{51}Cr-EDTA – chromium ethylenediaminetetraacetate;
^{99}Tc-DTPA – diethylenetriaminepentaacetic acid

The “true” kidney function can also be estimated by using different equations (327). In 20 to 80% of individuals with diabetes, the eGFR error was more than ± 30% of measured GFR (328). The problem of imprecise GFR estimation appears both at the low and the high ends of the range. Imprecision at high eGFRs is of particular concern at the early stages of kidney damage in diabetes, which can be associated with an elevated eGFR (potential hyperfiltration). However, the lack of

precision is also important at the late stages (329). Imprecise estimation lies less in the mathematical methods and more in the performance of the two biomarkers creatinine and/or cystatin C used in these equations (328).

Resulting from the non-enzymatic degradation of muscle creatine, *creatinine* is a small metabolite (113 Da) (330). Creatinine is not bound to any proteins, and it is freely filtered at the glomerular level (331). These features overlap with the characteristics of an ideal filtration marker. Despite these common characteristics, however, creatinine is not an ideal filtration marker, since it suffers from tubular secretion and reabsorption, while its production rate is variable depending on the muscle mass and protein intake (331). Creatinine's tubular secretion is less than 10% of the urinary creatinine secretion under normal conditions. However, when kidney function declines the tubular secretion increases up to 80 – 100% at late stages of kidney disease. Thus, in advanced CKD this massive tubular secretion masks the true GFR reduction, making creatinine a less appropriate marker for GFR estimation (332). Furthermore, extra-renal clearance and recycling of creatinine are also present when the renal function declines, contributing to an overestimation of the kidney function (333).

Cystatin C is a small protein (13 kDa) freely filtered at the glomerular level (335). In contrast to creatinine, cystatin C is present in all nucleate cells being produced by a "housekeeping" gene (CST3) at an approximately constant rate (336). In addition, it has no tubular secretion, but is largely reabsorbed and metabolized by the proximal tubular cells (337). These characteristics make cystatin C a better filtration marker than creatinine, but even so it is far from being an ideal filtration marker. In contrast to ideal filtration markers, though, cystatin C may reflect other factors in addition to the kidney function. Even if produced at an approximately constant rate, its levels are higher in individuals with hypertension, obesity, metabolic syndrome or T2DM (338). Also unlike an ideal marker is its significant tubular degradation, which prevents its urinary clearance measurement (335, 336). Finally, its small coefficient of variation and its curvilinear relation with the measured GFR may seriously influence the Cystatin C based eGFR (339).

2.6. Tubular biomarkers for DN in T1DM

Since the 1980s, tubular dysfunction has been accepted as an important early mechanism of DN and many tubular biomarkers have been studied (340). Important evidence suggests that tubular dysfunction is indeed as important as glomerular damage for the DN progression to ESRD (341-343).

2.6.1. Distal tubular biomarkers

In almost all kidney disorders the last step towards ESRD is hypoxia, acidosis and loss of kidney function, which are all associated with distal tubular damage (344, 345). Thus, urinary biomarkers reflecting injury in the distal tubules have also been studied as potential biomarkers for the progression of DN.

Tamm-Horsfall protein (THP), also known as *uromodulin (UMOD)*, is a glycoprotein encoded by the UMOD gene, and derived from the thick ascending limb of Henle. This glycoprotein is the most abundant protein excreted into the urine,

and a specific biomarker for the distal tubule. Studies have shown increased urinary excretion of this protein from the first year after onset of T1DM up to 14 years of diabetes duration (346). Furthermore, THP proteins also increased in those with microalbuminuria and hyperfiltration (347).

Epidermal growth factor (EGF) is a protein that stimulates cell growth and differentiation by binding to its receptor EGFR. The proteins of the *EGF*-family bind to *ErbB* (EGFR)-family receptors that play an important role in the regulation of various fundamental cell processes in many organs including the kidney, where they are suggested to play important para- or autocrine roles in tubular repair (348). Major EGF production sites in the kidney are the thick ascending limb of Henle's loop and the distal convoluted tubule (348). *EGF* was shown to predict CKD progression in a renal biopsy transcriptome-driven approach from the European Renal cDNA Bank (ERCB) (349). Its clinical utility was assessed in more than 600 participants of the Edinburgh Type 2 Diabetes Study, who had a normal AER and preserved kidney function at baseline. A lower urinary *EGF* to creatinine ratio was associated with new-onset eGFR less than 60 ml/min/1.73 m^2, a rapid decline in renal function or the composite of both outcomes (350). Whether *EGF* would be a useful biomarker for the identification of new-onset and progression of DN in individuals with T1DM is not known. However, the data from T2DM are interesting and suggest the need for further studies in T1DM.

Urinary *heart-type fatty acid binding protein (H-FABP)* was suggested as a distal tubular biomarker. In a study on approximately 100 individuals with diabetes, urinary H-FABP concentrations were significantly elevated in individuals with diabetes and normal AER, compared with healthy subjects. In addition, H-FABP was associated with eGFR independently of AER and other clinical risk factors. Therefore, urinary H-FABP may be a promising marker of distal tubular damage and kidney function (351).

2.6.2. Proximal tubular biomarkers

Even though the distal tubular dysfunction and acidosis may be the last step towards ESRD, in early DN other tubular biomarkers have also been shown to be elevated (352). That is because in DN the proximal tubule is affected first and then triggers the dysfunction of the distal tubule (353). Indeed, initial reports in the 1980s suggested that proximal tubular biomarkers are present in early T1DM, when albuminuria is still in the normal range (346). In a diabetic milieu the proximal tubules suffer structural changes (peritubular capillary rarefaction, interstitial fibrosis or tubular atrophy) by many mechanisms, in parallel with the eGFR decline (354). Thus, quite a few proximal tubular biomarkers have been studied in order to reflect these pathogenic mechanisms.

Alpha 1-microglobulin is a protein that was first identified in the urine of persons with tubular proteinuria (356). It is freely filtered at the glomerular level and 99% is reabsorbed by the proximal tubular cells, thus being a potential marker of proximal tubular injury. It is interesting that an elevation of urinary alpha 1-microglobulin was one of the first observations suggesting that tubular injury is present in people with T1DM (340, 357). Alpha 1-microglobulin showed 89.0% accuracy, 86.3% sensitivity and 94.2% specificity in distinguishing between normoalbuminuric people with

diabetes and people without diabetes (358). Its role in DN was studied in a urinary discovery-phase proteomics study in individuals with T1DM (359). Whether it can predict onset and progression of DN in individuals with T1DM is still not known.

Beta 2-microglobulin (B2M) is a small protein normally present on the cell membranes of all nucleated cells and released into the circulation at a constant rate (360). It is filtered and is almost fully reabsorbed by the proximal tubular cells (361). In T2DM, urinary *B2M* did not improve the prediction of DN progression (362), although some studies in T2DM have reported higher urinary levels in individuals with T2DM and albuminuria (363). Expression of *B2M* in cells of the urinary sediment of 51 individuals with T1DM was higher in those with DN compared to healthy controls (364). Interestingly, in normoalbuminuric T1DM individuals an increased excretion of χ light chains was found despite normal excretion of *B2M* (365). Thus, it is still not known whether urinary excretion of *B2M* is an early or late phenomenon of DN, or whether it could serve as a true predictor of the onset and progression of DN.

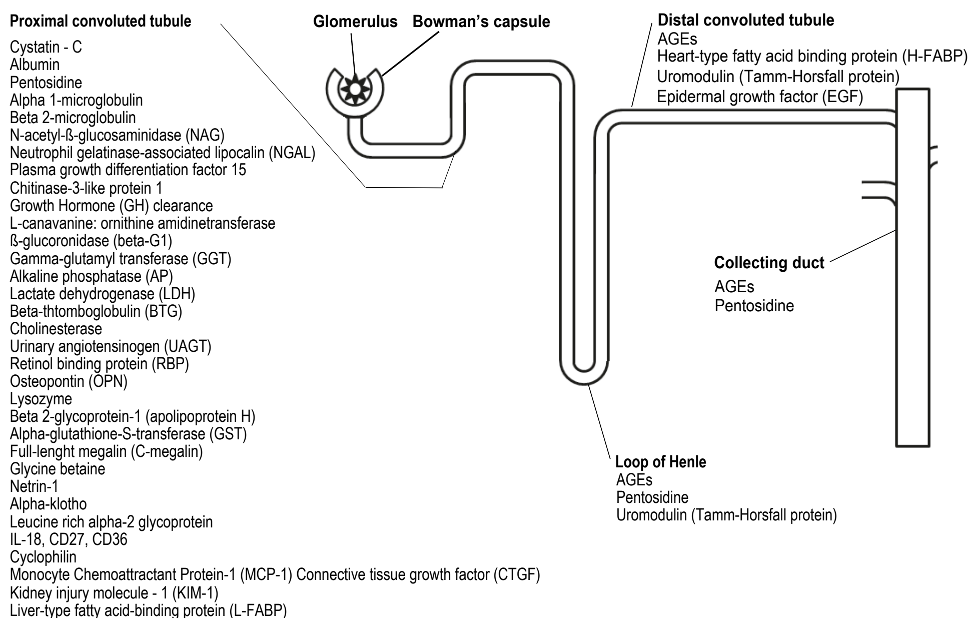

Figure 7. Tubular biomarkers for DN studied using a candidate biomarker approach.

Adapted for DN after (355)

Urinary *N-acetyl-*β*-(D)-glucosaminidase (NAG)* is a large protein present in the lysosomes of the proximal tubule epithelial cells. Due to its high molecular weight (130 kDa), it is considered to be present in the urine exclusively because of secretion from the proximal tubular cells (366). Urinary NAG was shown to be elevated in individuals with T1DM when compared with healthy control subjects, and the amount correlated with the glycemic control (367). Further increase was found in people with microalbuminuria, while lower *NAG* concentrations were associated with the regression of microalbuminuria (368, 369). Despite these promising results, its ability to predict DN progression is still debated (370).

Neutrophil gelatinase-associated lipocalin (NGAL) is a 22 kDa protein that was initially identified in mature neutrophil granules but has since been described in many other cell types. The binding of *NGAL* to siderophores-small, high-affinity iron chelating compounds secreted by microorganisms is important for the innate immune response to bacterial infections. When encountering invading bacteria, toll-like receptors on the immune cells stimulate the synthesis and secretion of *NGAL*. The reason why *NGAL* is used as a biomarker of renal injury is because it is rapidly released into urine in response to tubular damage. In addition, it is stable and resistant to proteases and as a consequence is the biomarker of choice for the diagnosis of acute kidney injury (371). In T1DM urinary *NGAL* was not associated with the decline of renal function, while in T2DM its role is still debated (372, 373).

Growth Hormone (GH) and *Insulin-Like Growth Factor -1 (IGF-1)* are two small proteins with molecular weights of 22 and 28 kDa, respectively. The major sites of their production are the pituitary gland and the liver, although the kidneys too produce these proteins. It appears that glomerular filtration and tubular reabsorption contribute to their urinary concentrations. Both molecules play an important role in the pathogenesis of glomerular lesions in individuals with kidney disease but may also reflect proximal tubular damage (374). Urinary *IGF-1* was shown to be associated with kidney volume, a marker of glomerular hypertrophy (375), and urinary *GH* with tubular function and glycemic control (376). Both urinary IGF-1 and urinary GH were associated with microalbuminuria in individuals with T1DM (377). Whether they predict the onset or progression of DN has not been studied.

Retinol-binding protein (RBP), a member of the lipocalin super-family, which carries vitamin A precursors, is a low molecular weight protein (21 kDa). Like all small proteins, *RBP* is filtered, but to limit its filtration it circulates in plasma bound to another protein – *transthyretin (TTR)* (378). Filtered *RBP* undergoes subsequent catabolism in the proximal renal tubules. Thus, the urinary level of *RBP* may reflect tubular function (379). In individuals with T1DM but without microalbuminuria, *RBP* was increased in comparison with healthy individuals, suggesting that this molecule may be an early marker of DN (380). More recent studies have shown that *RBP* is a marker of progression of DKD in individuals with T2DM and macroalbuminuria (381). However, its predictive value if any in T1DM is not known.

L-PGDS, also named *beta-trace protein (BTP)*, is a low molecular weight protein (23–29 kDa) (please see also page 32). *L-PGDS/BTP* transports retinoids and other molecules and catalyzes the transformation of prostaglandin H_2 to prostaglandin D_2. It is produced at constant rates, is stable and not influenced by acute phase reactions, is almost completely filtered and reabsorbed by the proximal tubular cells (382, 383). These characteristics makes *L-PGDS/BTP* an ideal tubular marker (384). Indeed, *L-PGDS/BTP* showed similar accuracy as creatinine but was no better than cystatin C for the prediction of early GFR decline in people without diabetes, while in Pima Indians with T2DM this protein was associated with ESRD (385, 386). Furthermore, increased urinary *L-PGDS/BTP* excretion was associated with renal injury in a cross-sectional analysis of more than 600 individuals with T2DM and predicted renal injury during follow up (279). Significantly, it is not only a kidney function biomarker, but also a predictor of CVD (387). However, no data from people with T1DM are available at this moment.

The role of *RAAS* in the onset and progression of DN is well established. The triggering factor in the proximal tubular cells is glucose, which via increased oxidative stress increases angiotensinogen production (388). Thus, urinary *angiotensinogen (AGT)* may be a useful tubular biomarker. Indeed, *AGT* was associated with *B2M*, albuminuria and annual decline of eGFR in individuals with T2DM with or without DKD. *AGT* was also predictive of cardiovascular events in individuals with microalbuminuria (389, 390). In T1DM the urinary angiotensinogen/creatinine ratio was greater in 28 individuals with T1DM than in 21 control subjects and preceded the increase in albuminuria (391). However, these data need to be confirmed in larger populations.

The concept of proximal tubule injury as a major player in the onset and progression of kidney disease has led to a large number of studies testing specific tubular biomarkers for early detection of DN and their predictive potential regarding DN progression (Table 4). As shown above, these studies have involved a large number of molecules holding potential as biomarkers and also various populations with T1DM or T2DM. However, despite promising findings, none of the above have emerged as top candidates for use in clinical practice. This is of course to a large extent due to inconclusive and insufficient data, and further studies are needed to arrive at any conclusions regarding these biomarkers.

2.6.3. Novel candidates

Some new candidates have emerged from studies on acute kidney injury. Two of these novel biomarkers, the *KIM-1* and the *L-FABP*, have drawn particular attention due to their pathogenic implications and their clinical relevance for the assessment of AKI. It is also worth mentioning that DN, CKD and AKI are all considered a continuum, sharing similar risk factors and mechanisms, and therefore biomarkers that work in the diagnosis of AKI may also work for DN and CKD. Accordingly, these two novel candidates are a major focus of the current thesis.

L-FABP is a low molecular weight protein (15 kDa) member of the FABP family of intracytoplasmatic lipid chaperones that coordinate lipid responses in cells and are strongly linked to metabolic and inflammatory pathways (392, 393). L-FABP expression is particularly increased in cells exposed to non-esterified fatty acids (NEFAs) or hypoxia, and promotes fatty acid metabolism and acts as an endogenous antioxidant that mitigates the effects of hypoxia and high lipid levels (394, 395).

L-FABP shows high expression levels in the liver, too. Thus, similar to the other low molecular weight proteins, L-FABP may be filtered at the glomerular level and reabsorbed at the tubular level. Interestingly, in CKD the urinary L-FABP concentrations were not influenced by its serum concentrations, probably because the urinary L-FABP originates mainly from the tubular cells (396).

In the tubular cells, L-FABP's main intracellular function is to carry FAs – the main energy source for the proximal tubular cells (397). The FFAs are bound to albumin, and when massive albuminuria is present they are filtered at the glomerular level and reabsorbed by the proximal tubular cells (398, 399). As a consequence, in individuals with albuminuria, L-FABP's expression in the proximal tubule cells is triggered by the FFA overload. Such FFA overload in the proximal tubular cells has also been reported in relation to hyperglycemia, hypertension, hypoxia and toxins (400-402).

Initially, urinary L-FABP was shown to predict acute kidney injury and non-diabetic kidney disease progression (403, 404). In individuals with diabetes and normal AER, hyperglycemia leads to kidney hypoxia (a very early event), which triggers L-FABP expression and increases the urinary L-FABP concentrations (165). The urinary concentrations are also influenced by lipid lowering and antihypertensive medications, and are increased early in the course of diabetes (405, 406). In early T1DM and T2DM, urinary L-FABP predicted the progression to microalbuminuria and was further associated with DN (407, 408). However, no well-powered studies have been performed in individuals with T1DM, investigating the ability of L-FABP to predict DN progression or elucidating whether there is a causal relationship between increased L-FABP concentrations and new-onset DN. Furthermore, its role in cardiovascular risk prediction in individuals with T1DM is also unclear.

KIM-1 is a small transmembrane glycoprotein (14-kD membrane-bound fragment of KIM-1), also known as hepatitis A virus cellular receptor 1 (HAVcr-1) or as T-cell immunoglobulin and mucin domain 1 (TIM-1). KIM-1 is expressed mainly in the liver and the proximal tubules, but also in the glomerular epithelial cells. In the tubular cells the expression goes normally undetected but is increased in AKI (409). However, an increase in the urinary concentrations of KIM-1 was triggered by a variety of causes, one of them being hypoxia (410-412). At the early stages of AKI, urinary KIM-1 was demonstrated to be a valuable biomarker of proximal tubular damage (413). In addition to AKI, KIM-1 was also considered to be a potential biomarker of CKD (414). In individuals with diabetes, urinary KIM-1 was elevated compared with healthy controls and correlated with other biomarkers of kidney function (351, 415-417). It is of note that in some prospective studies, KIM-1's predictive value with respect to DN was studied, but the data are contradictory (369, 418, 419). Furthermore, its causal relationship, if any, with the loss of kidney function has not been studied in individuals with T1DM. Finally, its predictive abilities for the prediction of cardiovascular events have not been evaluated, either. Given the strong evidence of KIM-1 as a biomarker for AKI and CKD, the potential for its use to predict DN is still there and deserves further studies.

Adiponectin (ADP) is a small protein primarily expressed in adipocytes with no apparent expression in the kidneys (420). Serum ADP has three molecular isoforms: low molecular weight (LMW – 90 kDa), medium molecular weight (MMW – 180 kDa) and high molecular weight (HMW – 300 kDa). The ADP molecule has a wide range of well-known protective effects against insulin resistance, vascular dysfunction, atherosclerosis and inflammation (421). Importantly, serum ADP was inversely correlated with eGFR (422). In T1DM, serum ADP was further shown to be increased and to predict the progression to ESRD (423). Notably, ADP was abundantly present in biopsy specimens of both nondiabetic and diabetic human kidneys (422, 424), and was suggested to be a regulator of albuminuria and podocyte function (425). Being a protein molecule, ADP may be filtered at the glomerular level and excreted into the urine, thereby reflecting glomerular damage. Indeed, various ADP isoforms can be detected in the urine (424, 425). In addition, urinary ADP has also been linked to renal tubular injury (426). Thus, *urinary ADP* may reflect both glomerular and tubular damage. In T1DM, urinary ADP excretion increases with increasing albuminuria (427). Despite all this evidence, there are no large prospective studies on the predictive abilities of urinary ADP, or whether ADP can

provide added benefit on top of commonly used biomarkers or if there is a causal relationship between increased urinary ADP concentrations and loss of kidney function in T1DM. Neither is there any information on whether urinary ADP can predict CVD events or mortality.

There is mounting evidence that diabetes increases cardiovascular risk. In addition, the risk of cardiovascular events and death increases with the decline of GFR (428). Therefore, a better reflection of kidney function could lead to a more accurate assessment of CVD risk. Glomerular biomarkers reflect progression of DN, but also the CVD risk. Despite their exceptional qualities, actual glomerular biomarkers are still not perfect. One explanation could be that in DN tubular dysfunction may also be present in addition to glomerular damage. Therefore, having novel biomarkers that also reflect tubular injury may be one way of improving prediction of both DN and CVD.

3. AIMS

The main aims of this study were to investigate, in individuals with T1DM, the following aspects of three common tubular urinary biomarkers, namely, L-FABP, KIM-1 and ADP:

I. Their ability to predict AER based progression of DN
II. Whether they add any benefit to actual clinically available biomarkers in predicting AER based progression of DN
III. Whether they play a causal role in the loss of kidney function in individuals with T1DM
IV. Their ability to predict CVD and premature mortality
V. Whether they add any benefit to actual clinically available biomarkers in predicting CVD and premature mortality.

4. MATERIAL AND METHODS

4.1. Subjects and study design

These studies were part of the Finnish Diabetic Nephropathy Study (FinnDiane) – a prospective, ongoing study with the eventual goal of unraveling the clinical, biochemical, environmental and genetic risk factors for DN and other chronic diabetic complications, in individuals with T1DM. The FinnDiane Study is a national multicenter study performed in approximately 80 centers across Finland. The study has collected data from more than 20% of all Finnish individuals with T1DM. The complete study protocol was approved by the local ethics committees and the Ethical Committee of the Helsinki and Uusimaa Hospital District. All participants signed an informed consent at enrolment and the study was performed in accordance with the revised Helsinki Declaration (429).

Participants included in the FinnDiane Study fulfilled the following criteria: presence of T1DM and age above 18 years at study baseline. T1DM was defined as disease onset before the age of 40 years with permanent insulin treatment initiated within the first year after the diagnosis. In addition, the diagnosis was confirmed by a fasting C-peptide level below 0.3 nmol/l.

The *first cross-sectional phase of the study* was conducted between 1997 and 2006. During this phase, participants were enrolled according to a baseline visit protocol.

The *second phase comprised an* investigation of parents and siblings of the study participants. However, no data from this phase has been used in the studies presented here.

The *third phase* is still ongoing and comprises follow up of existing participants combined with the continued baseline enrolment of additional participants. Two approaches are used for the follow up. The first approach, especially for participants living in the Helsinki area, is to have follow-up visits that are almost identical to the enrolment visits, thereby collecting similar data as well as blood and urine samples. For participants residing outside the Helsinki area an alternative approach is used and consists of collection of all existing follow-up data from their medical files. This approach includes visits by the FinnDiane investigators to all medical centers that had originally enrolled participants to the FinnDiane Study as well as a comprehensive review of the individual's medical files at the hospital archives. For the studies reported in this thesis all available follow-up data at the time of each study were used.

4.2. Baseline visit

At the baseline visit, comprehensive data were collected in a uniform fashion. Each participant's attending physician completed a standardized questionnaire with data based on the participant's medical file and reviewed and verified in detail by the attending physician. The questionnaire comprised questions on detailed patient

history, medication, chronic diabetic complications and comorbidities as well as on potential risk factors for complications (e.g., smoking). A clinical examination was performed on each participant, including anthropometric and blood pressure measurements. In addition, fasting blood samples were drawn for routine laboratory tests (creatinine, HbA_{1c}, lipids, etc.), and at least three timed, overnight and/or 24-hour urine collections were performed for baseline determination of renal status. The blood and urine samples collected at the baseline visit were stored at -20°C for the determination of various biomarkers.

4.2.1. Smoking status

According to their smoking habits the participants were considered a) current smokers, b) having a history of smoking or c) non-smokers. A current smoker was an individual who had smoked at least one cigarette per day at the time of the baseline visit. A history of smoking was considered in those individuals who had quit smoking, but had been smoking at least one cigarette per day for at least 3 months at one point in their life before enrolment to the FinnDiane Study. Non-smokers were those who had never smoked.

4.2.2. Anthropometric measures

The anthropometric parameters were represented by height, weight, waist-to-hip ratio (WHR) and BMI. Height was measured using a stadiometer with an accuracy of less than 1 cm. Weight was determined using a standardized scale with an error of less than 0.1 kg. Waist was considered the abdominal circumference at the mid-distance between the lowest rib and the iliac crest. Hip was the longest measured circumference in the gluteal region. WHR was calculated by dividing the waist by the hip circumference, measured in centimeters. BMI was calculated by dividing the weight (kg) with the square of the height (m^2).

4.2.3. Blood pressure

Blood pressure was measured twice, at 2-minute intervals. The measurements were performed in the sitting position, after an initial 10 minutes of rest. The average of the two measurements was used for all analyses. Participants were considered to be under antihypertensive treatment if it was prescribed and the participant currently used at least one antihypertensive medication.

4.2.4. Fasting blood measurements

In fasting blood samples obtained at the baseline visit, the following measurements were performed from serum and plasma.

HbA_{1C} was measured locally by a standardized assay at the FinnDiane centers throughout Finland.

The serum *lipid profile* (total cholesterol, HDL-cholesterol, triglycerides) was measured by a Cobas Mira analyzer (Hoffman-La Roche, Basel, Switzerland) at the Helsinki University Hospital. LDL cholesterol was calculated using the Friedewald formula.

Serum creatinine was measured until January 2002 by a kinetic Jaffé reaction using a Hitachi 911 E analyzer (Boehringer Mannheim, Mannheim, Germany). Since 2002 a photometric, enzymatic (isotope dilution mass spectrometry – IDMS) method was used. The new method was performed using a Hitachi 917 analyzer (Boehringer Mannheim/Roche Diagnostics, Basel, Switzerland). The inter-method correlation coefficient was 0.98. eGFR was calculated by the creatinine based CKD-EPI formula (78).

4.2.5. Urinary measurements

The 24-hour urine samples collected were used for the measurements of AER and urinary creatinine. AER was determined in the 24-hour urine collections using a radioimmunoassay (Pharmacia, Uppsala, Sweden). Urinary creatinine was measured using a Cobas Mira analyzer.

4.3. Biomarker measurements

The three urinary biomarkers L-FABP, KIM-1 and ADP were measured from a single 24-hour urine sample collected at the initial visit and then frozen at -20°C until the actual measurement. All three urinary biomarkers were measured in 2009.

4.3.1. Urinary L-FABP

Urinary L-FABP was quantified in a single, frozen 24-h urine collection using a research L-FABP Elecsys assay on a Cobas Elecsys 411 Immunoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). The complete description of the urinary L-FABP determination method is provided elsewhere (430). The lower detection limit of the assay was < 0.1 ng/ml, and no cross-reactivity was observed for other FABP types. For the statistical analyses, the urinary L-FABP values were normalized with urinary creatinine and presented as urinary L-FABP to creatinine ratio.

4.3.2. Urinary adiponectin

We measured ADP in a single 24-h urine collection using an ALPCO Diagnostic kit (Salem, NH, USA) for the quantitative measurement of multimeric ADP. The protocol was modified for urine samples, without protease pre-treatment. The ADP levels were normalized for urinary creatinine and presented as ADP to creatinine ratio.

4.3.3. Urinary KIM-1

Urinary KIM-1 was measured using a DuoSet ELISA Development kit from R&D Systems from frozen 24-h urine samples. The same Cobas Elecsys 411 Immunoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany) was used for the measurement process. A complete description of the detection technique is provided elsewhere (431). Urinary KIM-1 levels were normalized with urinary creatinine and depicted as urinary KIM-1 to creatinine ratio.

4.4. Genotypes

Genotyping was performed in 3,651 samples using an Illumina 610Quad assay. After quality control, genotypic data for 3,546 individuals at 549,530 SNPs across the autosomal genome were available. Imputation was performed with the HapMapII CEU samples as reference panel and resulted in ~2.4×10^6 SNPs.

4.5. Follow-up time

The median follow-up time differed between the studies. For the studies on DN progression the follow-up was 6.0 years (IQR 4.45 – 6.88). For the study that investigated the prediction abilities of the studied biomarkers on cardiovascular events and mortality the median observation period was 9.1 years (IQR 6.2 – 12.4). During follow-up, all participants were managed by their own physicians, without any intervention from the FinnDiane Study group members.

4.6. Definition of outcomes

4.6.1. Renal outcomes

The baseline *renal status* was defined based on several AER values. For the classification of each individual, we used at least two out of three urine samples collected. Accordingly, the participants were divided into one of the following three AER categories: normal AER (<30 mg/24-h or <20 µg/min), microalbuminuria or moderately increased albuminuria (30–300 mg/24-h or 20-200 µg/min) and macroalbuminuria or severely increased albuminuria (>300 mg/24-h or >200 µg/min). ESRD was considered if the participants were undergoing dialysis or had received a kidney transplant. In all studies the participants with ESRD at the initial visit were excluded.

Progression of DN was defined as the passage from one AER category to the next one, according to the above-mentioned thresholds. Progression of DN was an outcome in studies I, II and III.

Progression to ESRD was defined as de novo requirement of dialysis or kidney transplantation. Information about dialysis and kidney transplantation was identified from the participant's medical file and cross-checked with the Finnish Registry for Kidney Diseases and the Finnish Hospital Discharge Register (FHDR) as well as the center databases. Progression to ESRD was an outcome in studies I, II and III.

Kidney function in the Mendelian Randomization study was defined as the eGFR at the study enrolment (baseline visit).

4.6.2. Cardiovascular outcomes

For the cardiovascular endpoints, all data regarding cardiovascular events present as of September 18th, 2011 in the Finnish Hospital Discharge Registry (HDR) on all the FinnDiane participants were retrieved (22, 23). The cardiovascular outcomes were

documented from the HDR by using the participants' individual hospital admission, treatment procedures and discharge diagnoses based on the International Statistical Classification of Diseases and Related Health Problems – the 9th and 10th revision together with the Nordic Classifications of Surgical Procedures.

History of either ischemic or hemorrhagic stroke was considered if the corresponding ICD codes (ICD8 or ICD9: 430 - 438 and ICD10: I60 – I64) were recorded in the HDR.

CAD was defined as a history of acute myocardial infarction (ICD8 or ICD9 code 410; ICD10 codes I21, I22) or a coronary artery procedure (bypass grafting surgery or angioplasty).

PVD was considered if a limb amputation or if a peripheral artery procedure (bypass grafting surgery or angioplasty) had been performed.

CVD events were defined as an outcome that included any of the three endpoints: stroke, CAD and PVD.

The full description of the cardiovascular outcomes used in the FinnDiane Study is presented elsewhere (23). All individuals with cardiovascular events at baseline were excluded from the analysis of study IV.

4.6.3. Mortality

The ascertainment of all-cause mortality was performed by linking the FinnDiane data with all the data present at September 18th, 2011 in the Finnish Cause of Death Registry (CDR) as previously described (22, 23).

4.7. Data collection and management system

For all five studies, we used the clinical data collected at the baseline visit as well as prospective clinical data regarding the progression of DN, eGFR loss, cardiovascular outcomes or mortality. All data were stored in the BC/SNPmax database system (Biocomputing Platforms Ltd, Espoo, Finland) developed by FinnDiane together with the commercial company. All collected data were centralized in BC/SNPmax, integrating each individual's genetic, phenotypic and pedigree data into one database. Doctors and researchers can access the database by user and password codes through a user-friendly web-browser interface. The web-browser based platform can be connected to a local network and then be used from any workstation (PC, UNIX or Mac etc.) without the need to install any local software. In addition, the platform can be used in conjunction with various types of analysis tools and programs. The data security is "bank quality" and all web traffic is encrypted and cannot be "eavesdropped".

4.8. Statistical analysis

4.8.1. Descriptive statistics

Descriptive statistics were similar in all studies in this thesis, as described below. All continuous variables were tested for normal distribution-based skewness, kurtosis,

normality tests and histogram of their values. Skewness and kurtosis values between -3 and 3 were considered sufficient for a normal distribution. For all the tests for normal distribution, a Shapiro-Wilk *p-value* greater than 0.05 suggested normal distribution. Graphical inspection of the distribution of the variable values was also performed by using histograms (432). A generalized extreme Studentized deviate test ($\alpha = 0.05$ and no outliers < 15) was used for outlier detection and exclusion.

Continuous variables that were normally distributed are presented as mean ± SD. Non-normally distributed variables are presented as median and interquartile range. Normally distributed variables were compared between groups using independent two-sample t-test or one-way ANOVA, while for non-normally distributed variables group comparison was performed using Mann-Whitney U test or Kruskal-Wallis test for non-parametric distributions.

Categorical variables are presented as percentages, while the comparisons between groups of categorical variables were done by χ^2 test.

For all the statistical tests, the urinary biomarker to creatinine ratio was transformed to the natural logarithm and presented as ln(biomarker), except in the descriptive statistics tables, in which the raw urinary biomarker to creatinine ratios are presented. In addition, all non-normally distributed variables were logarithmically transformed and then used in the statistical analyses if needed, while the normally distributed variables were used as raw values.

For all tests in the descriptive statistics section a *p-value* < 0.05 was considered statistically significant.

4.8.2. Determinants of each urinary biomarker's level

At first, for each stage of DN, the clinical and biochemical determinants of each biomarker's levels were assessed using a linear regression model with stepwise selection of covariates.

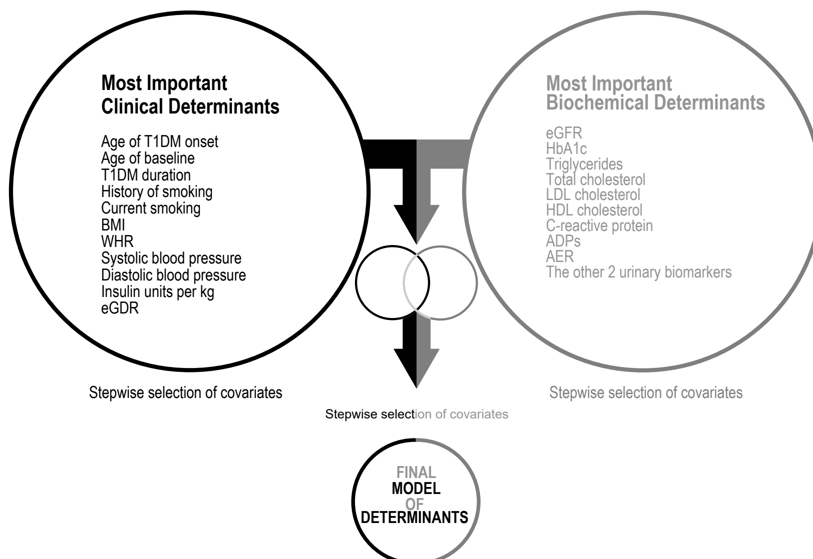

Figure 8. Assessment strategy for most important determinants of each biomarker's level

For the clinical determinants, at each stage of DN, the following variables were tested: age of diabetes onset, age at baseline, diabetes duration, history of smoking, current smoking, BMI, WHR, systolic and diastolic blood pressure, insulin per kilogram and estimated glucose disposal rate (eGDR).

Then, for each DN stage, the biochemical determinants of each biomarker's level were tested by using another linear regression model with stepwise selection of covariates. This model included the following variables: eGFR, HbA_{1C}, serum triglycerides, serum total cholesterol, HDL-cholesterol LDL-cholesterol, C reactive protein, serum ADP, urinary biomarkers AER, L-FABP, KIM-1 and ADP.

Finally, the two models were combined and followed by a stepwise selection of variables, resulting in a list of determinants for each urinary biomarker and at each stage of DN.

4.8.3. Evaluation of each biomarker's ability to predict the outcomes

Assessment of the biomarkers' ability to predict each study outcome was performed using Cox proportional hazard models, with each biomarker as an explanatory variable. The dependent variables used in the Cox proportional hazard models were as follows:

- Progression from normo- to microalbuminuria: Studies I, II and III
- Progression of DN from micro- to macroalbuminuria: Studies I, II and III
- Progression of DN from macroalbuminuria to ESRD: Studies I, II and III
- CVD, CAD, PVD, stroke and mortality: Study IV

For all studies, a test of the biomarkers' prediction of outcomes was performed by using simple Cox proportional hazard models without any adjustment. Thereafter, adjustment models for each study were built as described below.

For studies I, II and III all variables present in the database were tested for their prediction of DN in simple Cox proportional hazard models. All variables with $p<0.25$ after individual testing were later introduced into the Cox regression models with backward selection of covariates. The variables retained in the models after this backward selection represented the basic models (BM) for the progression at various stages of DN. As a result, different basic Cox models for the progression were built for each stage of DN. Further adjustments for AER and/or eGFR were also used, apart from the basic model of progression.

In study IV a different approach was used and included the building of a single model from traditional cardiovascular risk factors (TRF) for all endpoints (CVD, CAD, PVD, stroke and mortality). The traditional Cox proportional hazard models for the prediction of cardiovascular outcomes and mortality comprised the following variables: age, sex, diabetes duration, HDL-C, triglycerides, HbA_{1C}, history of smoking, mean systolic blood pressure, BMI and proliferative retinopathy. Besides these traditional risk factors, further adjustments for eGFR and AER were also performed.

To test the independent predictive potential of the biomarkers for studies I, II and III, we adjusted the initial Cox proportional hazard models with the basic models

of progression at each stage. Finally, these models were also adjusted for AER to test the biomarker's independence of AER. In study IV the same approach was used to test the independent predictive abilities for each cardiovascular endpoint (CVD, CAD, PVD and stroke) by adjusting the model for traditional risk factors and finally for AER and eGFR.

All available interactions between the variables included in the models were tested. Cox's models fit was assessed by cumulative Cox–Snell residuals to (-log) Kaplan–Meier estimates. The validity of the model assumption was tested by checking the normal distribution of the model's residuals by using the D'Agostino–Pearson test. The potential for multiple co-linearity was also evaluated based on the variance inflation factor (VIF) and tolerance. Values of VIF less than 10 and of tolerance higher than 0.5 were considered acceptable (433). The validity of each model's assumption was tested by evaluating the normal distribution of the residuals (434).

To test the potential influence of the competing risk between death and each outcome on the results we also performed for each study a Fine and Gray regression analysis. This analysis extends the Cox proportional hazard models to competing risk data by consideration of the sub-distribution hazard (435, 436). In the competing risk analysis, the same sequential analysis and models used in the Cox regression analysis were employed.

For all these tests a *p-value* of less than 0.05 was considered statistically significant.

4.8.4. Correction for multiple testing

In study IV, since urinary L-FABP was tested regarding the prediction of multiple cardiovascular outcomes and premature mortality, the most stringent Bonferroni correction was applied even if less than 20 outcomes were tested. The *p-value* for the prediction of outcomes was considered significant if < 0.01 (αBonferroni = $\alpha/m = 0.05/5$, where m = the number of tested hypotheses).

4.8.5. Assessment of each biomarker's predictive clinical benefit as to progression of DN

The clinical predictive value of the tested biomarkers was assessed using the following methods: ROC curve analysis, net reclassification improvement (NRI), the integrated discrimination improvement (IDI).

4.8.5.1. Diagnostic abilities for prediction

At first, we tested the average *diagnostic abilities* for prediction using an *ROC curve analysis* with estimation of the area under the curve (AUC) for every biomarker and each studied outcome, also considering the follow-up time. Then, we compared the diagnostic abilities by comparing the AUCs of each urinary biomarker with AER used alone. We then compared the AUCs of the models comprising both the urinary

biomarker and AER with the AUC of either the urinary biomarker alone or AER alone. Finally, we compared the variation of AUCs at different time points during follow up using a time dependent ROC curve analysis with 5 time points. Evaluation of the standard errors (SE) of the AUC, confidence intervals of AUCs (95% CI) and of the differences between AUCs was performed by the method described by Delong et al. (1988) (437).

4.8.5.2. Improvement of prediction

The improvement of prediction obtained by the addition of each biomarker to either AER alone or to the basic progression models plus AER was assessed by calculating NRI and IDI (438, 439).

NRI is the difference in percent moving up and down risk strata (i.e., reclassified) after the inclusion of the investigated urinary biomarker in the above-mentioned multivariable models. In this way the NRI differentiates the movement in the correct direction, (i.e., the proportion of subjects being reclassified to a higher risk category among those reaching the outcomes or to a lower risk category among those without events). Since no well-established risk categories existed at the time of the analyses (studies I, II, III), it was considered more prudent to use a version of the NRI that does not require categories, rather than trying to create them. Consequently, in these studies the category-less or continuous NRI (NRI (>0) or cNRI) was used, which is the relative increase in the predicted probabilities for subjects who experienced events, and the decrease for subjects who did not, when a biomarker was added to the models. cNRI and IDI were obtained by 10-fold cross-validation using 1000 bootstrap repetitions of the whole data set. In study IV, three relevant thresholds (5%, 10% and 20%) for cardiovascular risk decision making were used (440), and the same cut-offs were used also for the mortality analysis. In addition, in all studies we calculated the NRI generalized for survival data at 5 (NRI_{S-5}) and 10 (NRI_{S-10}) years of follow up. All types of NRIs were presented as a percentage (%) (438, 439).

IDI is the increase of the difference in average predicted probabilities between cases and controls, when one urinary biomarker was added to the previously presented models, without considering any risk thresholds. In addition, in all studies we calculated IDI generalized for survival data at 5 (IDI_{S-5}) and 10 (IDI_{S-10}) years of follow up. IDI was also presented as a percentage (%) (439).

For all tests regarding improvement of prediction, a *p-value* less than 0.05 was considered statistically significant.

4.8.6. Causality between biomarkers and eGFR – a Mendelian randomization (MR) approach or instrumental variable (IV) analysis

The Mendelian randomization approach was used to test the causality between biomarkers and the eGFR loss during lifetime. This method is a type of instrumental variable analysis, which explores the causality between the biomarker's levels ("modifiable exposure") and an outcome (eGFR) (Figure 9) (441, 442).

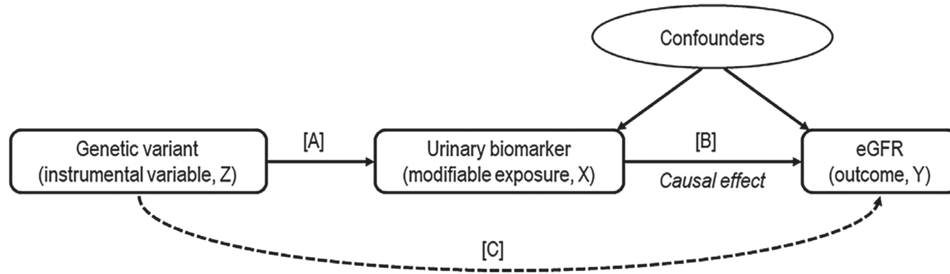

Figure 9. Investigation of the causal link between a biomarker and eGFR – MR analysis or IV analysis.

The IV analysis has three assumptions: 1) the IV is strongly associated with the modifiable exposure [A]; 2) the IV is independent of confounding factors that may confound the association between the modifiable exposure and the outcome [B]; 3) the IV affects the outcome only through the modifiable exposure and not by other biological pathways (Figure 9). It is expected that no other pathway is found. However, if there is a possible direct association between the IV (genetic variant) and the outcome (eGFR) [C], it should disappear when adjusted for the modifiable exposure.

At first, all three assumptions of the MR analysis were tested to see if they were fulfilled. After the fulfilment of these assumptions the effect sizes were estimated by two methods: a conventional linear regression and a two stage least squares method (the IV analysis). Finally, the endogeneity was tested to see if there is any difference between the two estimates (the conventional estimate from the linear regression and the IV estimate from the IV analysis).

4.8.6.1. First assumption – identification of the IV by genome-wide association analysis

In the MR approach, the IV is either a SNP or a genetic score comprising multiple SNPs. To identify the most suitable IV for these studies a genome-wide association study (GWAS) was performed to detect the genetic variants associated with the modifiable exposure (X or urinary biomarker's level) according to the previous description (Figure 9) (113). For each biomarker, the GWAS data for all subjects that had measurements for the urinary biomarker available were utilized. The association analysis between each biomarker's levels and the imputed allele dosage data was carried out using linear regression, assuming an additive association model, as implemented in Plink (v1.07) (443). The models were adjusted for sex, diabetes duration and the two first principal components (PCs) to fulfil the first assumption. Further analyses were performed only for the signals with genome-wide significance (*p-value* $< 5 \times 10^{-8}$). The imputed allele dosage data were converted to the most likely genotypes, accepting genotype calls with >0.9 genotype likelihood as estimated by the MACH imputation software (444).

4.8.6.2. Second assumption

Since genetic variants are assumed to be randomly distributed before birth, MR assumes that the IV (Z or SNP genotype in this case) is regarded as independent of confounders, a priori fulfilling the second assumption.

4.8.6.3. Third assumption

To test if the association between the eGFR (outcome, Y) and the genetic variant/ genetic score (IV, Z) is mediated through the biomarker's levels, a multiple regression between the SNP and eGFR was performed. If mediated through the biomarker, this association should disappear completely after adjustment for the biomarker's levels. To test the existence of other pathways, all variables in the database were evaluated to see if they were associated with the IV (SNP genotype) by simple linear regressions or χ^2 test. The distributions of all residuals were tested for normality.

4.8.6.4. Effect size estimation for the association between biomarker's levels and GFR

Linear regression or the conventional estimate that evaluated the association between the biomarker's levels and eGFR was then adjusted for diabetes duration, and additionally for AER. Since age and sex are included in the eGFR formula, they were not used as covariates in the models.

Two-stage least squares method (2SLS) or *the IV estimate (the causal estimate)* was used for the IV analysis. At first the raw estimators were obtained and then they were adjusted for diabetes duration. Finally, the estimates were also adjusted for AER. F-statistics from the first-stage regression of the 2SLS was used to evaluate whether the combination of instrument strength and sample size is adequate. A value of $F \geq 10$ was considered sufficient to ensure the validity of the IV analysis (445).

4.8.6.5. Endogeneity testing

Finally, the endogeneity was tested to see if there is any difference between the two estimates (the conventional estimate from the linear regression and the IV estimate from the 2SLS analysis)(446, 447). A *p-value* <0.05 was considered statistically significant.

4.8.7. Statistical software

For prediction analyses as well as for the evaluation of clinical diagnostic abilities and prediction benefit, MedCalc 12.1.3.0 (MedCalc Software BVBA, Mariakerke, Belgium) and Stata/MP2 software (Version 13, StataCorp LP, College Station, Tx) were used. The ROC curve analysis was performed using MedCalc 12.1.3.0 (MedCalc Software BVBA, Mariakerke, Belgium) and Stata/MP2 software (Version 13, StataCorp LP, College Station, Tx) and the 'timeROC' package implemented in RStudio (version 1.0.136, Boston, MA, USA). cNRI and IDI were tested via the 'incrisk' Stata module (Studies I, II, III and IDI in study IV). Estimation of NRI in relation

to the cardiovascular outcomes was performed using ‘nri’ Stata module (440). In addition, the NRI and IDI generalized for survival data at 5 and 10 years of follow up were calculated using the ‘nricens’ and ‘survIDINRI’ packages implemented in RStudio (version 1.0.136, Boston, MA, USA).

For evaluation of the first assumption of the MR approach using the genetic data analyses the following software was used: Plink (v1.07) EIGENSTRAT software (EIGENSOFT v. 3.0) and MACH imputation software (443, 444, 448). For evaluation of causality by MR analyses the two-stage 2SLS or the IV estimate (the causal estimate) was employed using the IV analysis, implemented in Stata/MP2 software (Version 13, StataCorp LP, College Station, Tx).

5. RESULTS

5.1. Study I – Urinary L-FABP and progression of DN

The baseline clinical features of the individuals with T1DM and the healthy control subjects included in study I are presented in Table 4. The average follow-up time in this study was 5.8 years (95% CI 5.7 - 5.9) and during this period there were 112 new cases of microalbuminuria, 46 new cases of macroalbuminuria and 78 new cases of ESRD.

Table 4. Clinical baseline data for individuals enrolled in study I.

Variable		Healthy controls	Normoalbuminuric individuals	Microalbuminuric individuals	Macroalbuminuric individuals
Number of individuals (M/F)		208 (106/102)	1549 (732/817)	334 (195/139)	363 (199/164)
Age (years)		35.9 ± 11.3	36.2 ± 12.3	38.8 ± 12.7	41.8 ± 10.5
Age of onset (years)		-	17.4 ± 9.3	13.0 ± 9.1	12.5 ± 8.5
Duration (years)		-	18.8 ± 11.7	25.7 ± 11.1	29.3 ± 8.1
BMI (kg/m^2)		24.0 ± 3.0	24.9 ± 3.5	25.6 ± 3.6	26.2 ± 4.1
WHR	Men	0.92 ± 0.06	0.89 ± 0.07	0.92 ± 0.07	0.94 ± 0.07
	Women	0.83 ± 0.05	0.80 ± 0.06	0.83 ± 0.07	0.84 ± 0.07
History of Smoking (%)		22.3	41.2	52.4	60.4
SBP (mmHg)		126 ± 15	130 ± 16	136 ± 17	143 ± 20
DBP (mmHg)		77 ± 9	78 ± 9	81 ± 10	83 ± 10
HbA_{1C} (%)		5.5 ± 0.4	8.2 ± 1.4	8.8 ± 1.5	9.0 ± 1.6
Total cholesterol (mmol/l)		4.75 ± 0.88	4.80 ± 0.90	4.97 ± 0.88	5.39 ± 1.09
HDL cholesterol (mmol/l)		1.55 ± 0.33	1.35 ± 0.37	1.30 ± 0.39	1.21 ± 0.37
LDL cholesterol (mmol/l)		2.76 ± 0.82	2.95 ± 0.81	3.08 ± 0.80	3.39 ± 0.89
Triglycerides (mmol/l)		0.90 (0.84 - 0.97)	0.94 (0.92 - 0.97)	1.08 (1.02 - 1.14)	1.36 (1.27 - 1.46)
AER (mg/24h)		3 (2 - 3)	8 (7 - 8)	50 (43 - 58)	453(371 - 584)
eGFR (ml/min/1.73 m^2)		111 ± 36	101 ± 24	90 ± 24	60 ± 40
Urinary L-FABP (µg/µmol)		0.014 (0.008 - 0.020)	0.039 (0.036 - 0.044)	0.091 (0.074 - 0.107)	0.504 (0.426 - 0.643)

M/F – male / female

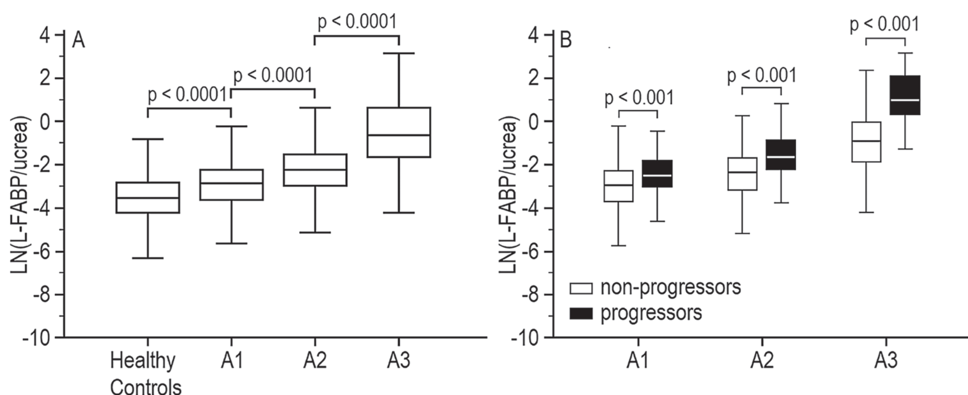

Figure 10. A. Urinary L-FABP levels in non-diabetic subjects as well as in individuals with T1DM at the study baseline. **B.** Urinary L-FABP levels according to baseline DN stage and progression status.

Compared with any other group of individuals with T1DM, healthy control subjects presented with significantly lower urinary L-FABP ($p < 0.001$). Urinary L-FABP increased by worsening stage of DN and compared with each previous DN stage ($p < 0.001$). Regardless of their initial DN stage, when compared with non-progressors, all progressors had significantly higher levels of urinary L-FABP ($p < 0.001$) (Figure 10).

5.1.1. Baseline determinants of urinary L-FABP at each stage of DN

A 3-step methodology was used to assess the most important determinants of each biomarker's baseline level at each stage of DN (please see the Material and Methods section 4.8.2). In the first step, the most important clinical determinants of urinary L-FABP levels were identified. Then, the most important biochemical determinants were evaluated. In the third step, the two models were joined, and the final determinants were selected through a stepwise selection process (please see the Material and Methods section, Figure 8). In individuals with normal AER, the most important clinical determinants of urinary L-FABP were: eGDR (estimated glucose disposal rate), age of diabetes onset, BMI and current smoking (data not shown). The most important biochemical determinants, at this stage, were HbA_{1C}, CRP, AER and urinary ADP (data not shown). When both clinical and biochemical variables were combined into a single model the most important determinants of urinary L-FABP were: AER, HbA_{1C}, urinary ADP, CRP, BMI and age of diabetes onset (Table 5).

Table 5. Baseline determinants of urinary L-FABP levels at each stage of DN in Study I.

Variable	Beta	SE	p
Normal AER			
AER (mg/24h)	0.001	0.0001	0.0001
HbA_{1C} (%)	0.007	0.003	0.01
Urinary ADP (μg/g)	0.04	0.007	0.0001
CRP (mg/l)	0.002	0.001	0.002
BMI (kg/m^2)	-0.003	0.001	0.005
Age of onset (years)	-0.001	0.0004	0.006
Microalbuminuria			
AER (mg/24h)	0.001	0.0001	0.0001
Urinary ADP (μg/g)	0.236	0.018	0.0001
History of smoking	0.066	0.03	0.02
Macroalbuminuria			
HbA_{1C} (%)	0.305	0.083	0.0001
Triglycerides (mmol/l)	0.370	0.124	0.003
Total cholesterol (mmol/l)	0.334	0.137	0.01
Serum ADP (mg/l)	-0.023	0.013	0.08
eGFR (ml/min)	-0.022	0.005	0.0001
AER (mg/24h)	0.001	0.005	0.0001
Urinary ADP (μg/g)	0.142	0.021	0.0001

Similarly, in individuals with microalbuminuria at baseline, the most important clinical and biochemical determinants associated with the urinary L-FABP values were identified. The most important clinical determinants, at this stage, were WHR and current smoking (data not shown). The most important biochemical

determinants were HDL cholesterol, AER and urinary ADP (data not shown). When the two models were combined and a step-wise selection of variables was applied, the most important determinants associated with urinary L-FABP were AER, urinary ADP and history of smoking (Table 5).

Finally, in the individuals with macroalbuminuria at baseline the most important clinical determinants of urinary L-FABP were: SBP, DBP and insulin sensitivity (eGDR) (data not shown). The most important biochemical variables and final model associated with urinary L-FABP, at this stage, were: HbA_{1C}, serum triglycerides, serum total cholesterol, serum ADP, eGFR, AER and urinary ADP (Table 5).

It is worth mentioning that both AER and urinary ADP were selected among the most important determinants of urinary L-FABP levels across all stages of DN. In addition, HbA_{1C} was also a significant determinant of urinary L-FABP in individuals with normal AER and macroalbuminuria (Table 5).

5.1.2. Prediction of DN progression by urinary L-FABP

Urinary L-FABP predicted progression of DN at all stages when tested using Cox models.

When the adjusted models were built the variables selected in the BM for progression to microalbuminuria were: WHR, history of smoking, HbA_{1C} and total cholesterol. The BM of progression to macroalbuminuria comprised of WHR, HbA_{1C} and triglycerides. The BM of progression to ESRD included eGFR and triglycerides (data not shown).

Table 6. Prediction of DN progression by urinary L-FABP

	Unadjusted			Adjusted for BM			Adjusted for BM and AER		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Normal AER									
AER (mg/24h)	1.02	1.01 - 1.02	< 0.0001	1.02	1.01 - 1.02	< 0.0001	1.01	1.01 - 1.02	< 0.0001
Urinary L-FABP (μg/μmol)	4.10	2.31 - 7.29	< 0.0001	3.22	1.74 - 5.96	0.0002	2.97	1.50 - 5.90	0.002
Microalbuminuria									
AER (mg/24h)	1.01	1.01 - 1.02	< 0.0001	1.01	1.01 - 1.02	< 0.0001	1.01	1.01 - 1.02	< 0.0001
Urinary L-FABP (μg/μmol)	1.49	1.20 - 1.85	0.0003	1.41	1.10 - 1.79	0.006	0.67	0.48 - 0.95	0.03
Macroalbuminuria									
AER (mg/24h)	1.01	1.01 - 1.02	< 0.0001	1.01	1.01 - 1.02	< 0.0001	1.01	1.01 - 1.02	0.02
Urinary L-FABP (μg/μmol)	1.24	1.20 - 1.29	< 0.0001	1.20	1.14 - 1.26	< 0.0001	1.17	1.10 - 1.24	< 0.0001

At each stage, after adjustments with the BM of progression, urinary L-FABP was still an independent predictor of progression. Finally, even when AER was added to each model, urinary L-FABP was still an independent predictor of progression to the next DN stage, including the progression to ESRD (Table 6). After adjustments with the risk factors from the basic risk factor models, one logarithmic unit increase in urinary L-FABP was associated with a 50% higher risk of progression from

micro- to macroalbuminuria as well as a 24% increase in the risk of progression from macroalbuminuria to ESRD. Surprisingly, after adjustment for AER in the individuals with microalbuminuria at baseline, one logarithmic unit increase in urinary L-FABP was associated with a 33% decrease in the risk of progression to macroalbuminuria (Table 6).

The results did not change when the competing risk analysis was performed, with death as a competing event for progression at any stage.

5.1.3. Urinary L-FABP's diagnostic performance and added clinical benefit for prediction of DN progression

When the average diagnostic performance for prediction of DN progression over time was considered, urinary L-FABP was no better than AER when adjusted for the basic model of progression to microalbuminuria.

Table 7. Average diagnostic performance over time using ROC curves analysis for prediction of DN progression

	ROC	AUC	95%CI
Normo-albuminuria	AER	0.772	0.750 - 0.793
	L-FABP	0.669	0.645 - 0.693
	L-FABP+AER	0.770	0.749 - 0.791
	BM+AER	0.778	0.756 - 0.799
	BM+L-FABP	0.735	0.711 - 0.757
	BM+L-FABP+AER	0.786	0.765 - 0.807
Micro-albuminuria	AER	0.839	0.795 - 0.877
	L-FABP	0.720	0.668 - 0.767
	L-FABP+AER	0.839	0.795 - 0.877
	BM+AER	0.847	0.803 - 0.884
	BM+L-FABP	0.777	0.728 - 0.821
	BM+L-FABP+AER	0.841	0.797 - 0.879
Macro-albuminuria	AER	0.793	0.748 - 0.833
	L-FABP	0.822	0.779 - 0.860
	L-FABP+AER	0.851	0.810 - 0.886
	BM+AER	0.862	0.818 - 0.898
	BM+L-FABP	0.850	0.806 - 0.888
	BM+L-FABP+AER	0.863	0.820 - 0.900

L-FABP+AER – Cox regression model with urinary L-FABP and AER; BM+L-FABP – Urinary L-FABP adjusted with the BM; BM+AER – AER adjusted with the BM; BM+L-FABP+AER – Cox regression model with urinary L-FABP plus AER and BM.

However, when both urinary biomarkers were included in addition to the basic model of progression to microalbuminuria, the AUC of urinary L-FABP used together with AER was not significantly larger ($\Delta_{AUCs} = 0.008$, $p = 0.09$) than the AUC of AER (Table 7). In individuals with baseline micro- or macroalbuminuria, when the AUCs of urinary L-FABP and AER were compared regarding progression to the next stage, again AER performed better than urinary L-FABP (Table 7).

Table 8. ROC curves analysis at different time points (quintiles of follow-up time) for the main progression models in study I.

Progression from normo- to microalbuminuria										
Time points (years)	3.75		4.37		5.40		6.30		6.83	
Cases / controls (number)	72/1186		78/1038		92/741		100/444		101/297	
ROC	AUC	SE	AUC	SE	AUC	SE	AUC	SE	AUC	SE
AER	81.01	2.89	80.02	2.76	76.09	2.86	74.59	2.89	73.92	3.01
L-FABP	68.53	3.18	69.73	3.06	66.58	2.91	64.60	3.17	63.61	3.29
L- FABP+AER	81.19	2.64	81.41	2.52	76.67	2.84	74.29	2.93	73.64	3.06
BM+L-FABP	74.77	3.10	76.55	2.92	75.88	2.88	75.17	2.99	75.38	3.03
BM+AER	80.32	2.63	81.08	2.46	80.18	2.42	78.72	2.76	79.07	2.79
BM+L-LFABP+AER	81.33	2.57	82.30	2.40	80.99	2.38	79.27	2.69	79.31	2.74
Progression from micro- to macroalbuminuria										
Time points (years)	3.75		4.52		5.71		6.65		7.16	
Cases / controls (number)	22/262		27/228		37/164		42/99		43/66	
ROC	AUC	SE	AUC	SE	AUC	SE	AUC	SE	AUC	SE
AER	82.99	5.45	78.92	5.23	82.41	3.94	86.41	3.33	87.20	3.36
L-FABP	75.49	4.76	71.77	5.11	68.32	4.94	70.52	4.81	71.90	5.05
L-FABP+AER	82.97	5.45	78.89	5.24	82.45	3.94	86.43	3.33	87.06	3.39
BM+L-FABP	76.42	6.09	73.63	5.62	73.82	4.64	75.96	4.49	81.38	4.30
BM+AER	84.97	4.67	80.94	5.13	80.98	4.53	84.89	4.02	88.93	3.74
BM+L-LFABP+AER	84.53	4.42	80.62	4.93	81.15	4.52	84.76	4.00	89.23	3.73
Progression from macroalbuminuria to ESRD										
Time points (years)	3.42		4.48		6.23		6.98		7.37	
Cases / Controls (number)	30/224		40/196		51/140		54/84		56/56	
ROC	AUC	SE	AUC	SE	AUC	SE	AUC	SE	AUC	SE
AER	84.05	3.59	78.01	4.49	74.83	4.46	75.04	4.50	75.20	4.77
L-FABP	88.44	4.03	82.46	4.22	81.29	4.06	80.08	4.14	81.52	4.21
L-FABP+AER	90.58	2.97	84.12	3.71	83.56	3.51	82.74	3.71	83.41	3.91
BM+AER	91.79	2.89	91.02	3.53	89.98	2.97	85.81	4.19	82.96	5.04
BM+L-FABP	92.51	3.52	90.94	3.03	89.86	2.84	85.25	4.05	83.13	4.78
BM+L-LFABP+AER	92.62	3.40	90.99	2.91	90.64	2.67	86.30	3.98	83.98	4.78

L-FABP+AER – Cox regression model with L-FABP and AER; BM+L-FABP – Urinary L-FABP adjusted with the BM; BM+AER – AER adjusted with the BM; BM+L-FABP+AER – Cox regression model with urinary L-FABP plus AER and BM

When the diagnostic performance and added clinical benefit of urinary L-FABP for the prediction of progression to the next DN stage using time dependent ROC curve analysis by quintiles of follow-up time was assessed, the AUC for urinary L-FABP was no better than AER at any time point or at any stage (Table 8). Moreover, when urinary L-FABP and AER were added to basic models for progression to the next stage, the difference was not statistically significant (Table 8 and Figure 11). Variation in the AUC over time in individuals with normal AER at baseline showed that AUCs of the BM for progression to next stage together with either AER or AER$_+$LFABP are almost constant in individuals with normal AER. In individuals with microalbuminuria at baseline, the discrimination of progressors starts to increase from 4.5 years, while in individuals with macroalbuminuria at baseline the diagnostic ability drops significantly after 6 years for both models (Figure 11).

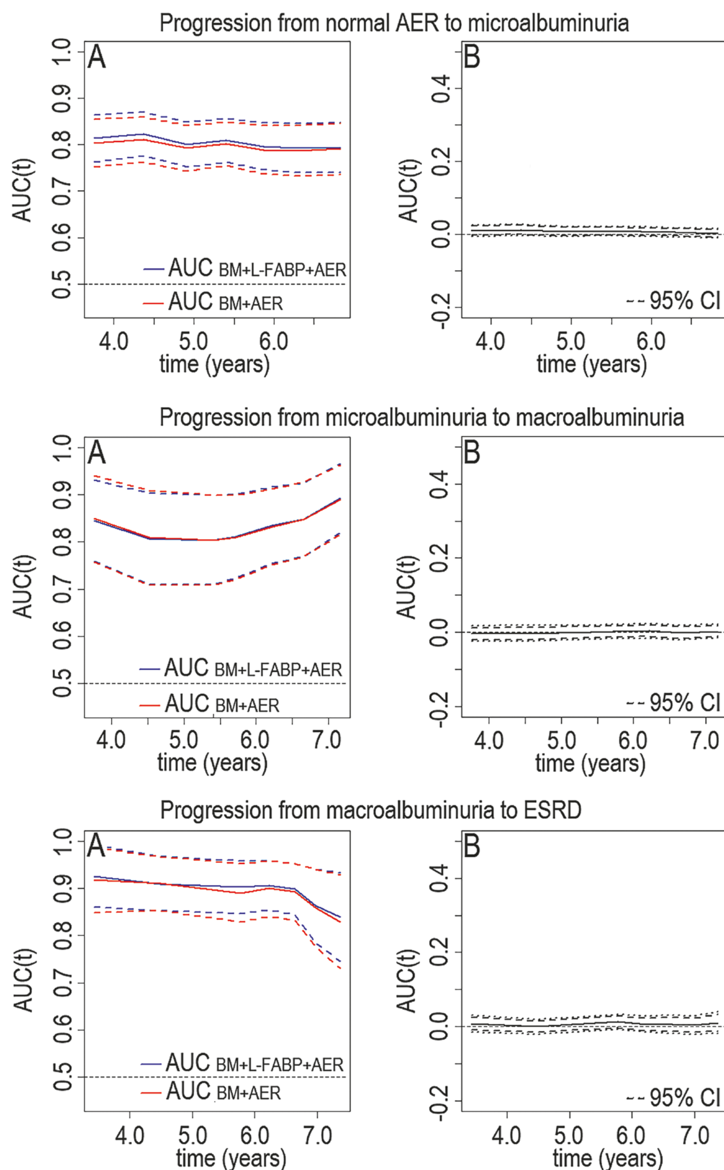

Figure 11. Variation over time of diagnostic performance (AUC) for identification of progressors (A), as well as variation of the difference between AUCs (added clinical benefit) (B) for the following two models: BM+AER and BM+AER+L-FABP. BM+AER – basic models for progression to next stage plus AER, BM+AER+LFABP – basic models for progression to next stage plus AER and urinary. L-FABP. Continuous lines represent the mean difference. Dotted lines represent the 95% CI.

Reclassification analysis is a different way to estimate the added clinical benefit compared with the AUCs comparison and uses NRI and IDI (please see the Material and methods section). When NRI was calculated in individuals with normal AER at baseline, urinary L-FABP added significant benefit to basic progression models and

AER at five years follow up. When IDI was analyzed, an added predictive benefit was observed also in individuals with baseline normal AER or macroalbuminuria, at 5 years follow up (Table 9).

Table 9. Evaluation of added clinical benefit using reclassification analysis for the main comparisons between progression models.

	$cNRI_{lm}$	NRI_{S-5}	NRI_{S-10}	IDI_{lm}	IDI_{S-5}	IDI_{S-10}
Progression to microalbuminuria						
L-FABP+AER *vs* AER	0.245*	0.383*	0.166	0.005	0.012*	0.005
AER+BM *vs* BM	0.747***	0.104	0.052	0.101***	0.028*	0.030
L-FABP+BM *vs* BM	0.300***	0.251*	0.192	0.009	0.006*	0.007
L-FABP+AER+BM *vs* AER+BM	0.254**	0.295*	0.216	0.007	0.007	0.012
Progression to macroalbuminuria						
L-FABP+AER *vs* AER	0.453	0.045*	-0.213	-0.004	0	-0.001
AER+BM *vs* BM	0.899***	-0.021	-0.665	0.197***	0	0
L-FABP+BM *vs* BM	0.197	0.022	-0.996	0.009	0	0
L-FABP+AER+BM *vs* AER+BM	0.601	0.041	-0.649	-0.003	0	0
Progression to ESRD						
L-FABP+AER *vs* AER	0.800***	0.928*	NA	0.081**	0.111*	NA
AER+BM *vs* BM	0.822***	-0.415	NA	0.122***	0.110*	NA
L-FABP+BM *vs* BM	0.652***	-0.159	NA	0.118***	0.201*	NA
L-FABP+AER+BM *vs* AER+BM	0.304	0.127	NA	0.048*	0.110*	NA

BM – basic model for progression; L-FABP+AER – Cox regression model with urinary L-FABP and AER; BM+L-FABP – model with urinary L-FABP added to BM; BM+AER – model with AER added to BM; L-FABP+AER – model with urinary L-FABP plus AER and BM, * - significant p-value ($p < 0.05$), ** - significant p-value ($p < 0.01$), *** - significant p-value ($p < 0.001$).

5.1.4. Urinary L-FABP and causality for DN progression

Causality, using the Mendelian randomization approach, could not be investigated since no SNP or genetic score was sufficiently strongly associated ($p < 5 \times 10^{-8}$) with the modifiable exposure (urinary L-FABP levels) in our GWAS (Table 10). Thus, the first assumption of the Mendelian randomization approach was not fulfilled and no further analysis could be performed.

Table 10. Top SNPs associated with urinary L-FABP values

CHR	SNP	BP	A1	A2	MAF	β	95% CI	P
1	rs2811982	23817438	G	-	0.236	0.15	0.08 – 0.21	7.81×10^{-6}
5	rs13172069	8184880	G	-	0.175	0.13	0.08 – 0.19	4.18×10^{-6}
21	rs7281691	29104983	T	-	0.151	0.34	0.19 – 0.49	5.55×10^{-6}
22	rs137885	48805358	C	-	0.388	0.12	0.07 – 0.16	3.45×10^{-6}
22	rs137888	48806623	G	-	0.362	0.13	0.08 – 0.17	2.23×10^{-7}

Models were adjusted for diabetes, gender, duration and two first PCs. A significant association is considered if $p < 5 \times 10^{-8}$.

5.2. Study II – Urinary adiponectin and progression of DN

For this study, the following individuals had the necessary data and samples at enrolment visit: 1451 individuals with no albuminuria, 319 individuals with microalbuminuria and 320 with macroalbuminuria. In addition, 111 urine samples from healthy subjects were available for urinary ADP and urinary creatinine measurements. Urinary ADP values were expressed as values of urinary ADP normalized for urinary creatinine (please see the Material and methods section).

The baseline characteristics of individuals included in Study II are shown in Table 11. The median follow-up was 5.8 (IQR: 4.4 – 6.9) years. During follow-up, 214 individuals progressed to a higher stage: 101 to microalbuminuria, 42 to macroalbuminuria and 71 to ESRD. With each higher DN stage as well as with progression of DN, individuals presented longer diabetes duration as well as higher age, BMI, WHR, SBP, DBP, total cholesterol, triglycerides, HBA_{1C}, AER, CRP, serum and urinary ADP (Table 11).

Table 11. Clinical baseline data for individuals enrolled in Study II.

Variable		Healthy controls	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
Number of individuals (M/F)		111 (41/70)	1451 (688/763)	319 (185/134)	320 (178/142)
Age (years)		39.6 ± 11.9	37.0 ± 12.3	39.1 ± 12.6	42.1 ± 10.5
Age of onset (years)		-	17.4 ± 9.4	13.7 ± 9.4	12.8 ± 8.3
Diabetes duration (years)		-	19.6 ± 11.7	25.4 ± 10.8	29.3 ± 7.8
BMI (kg/m^2)		23.8 ± 2.8	24.9 ± 0.14	25.7 ± 3.7	26.2 ± 4.1
WHR	Men	0.94 ± 0.05	0.89 ± 0.07	0.92 ± 0.07	0.94 ± 0.07
	Women	0.84 ± 0.04	0.80 ± 0.06	0.83 ± 0.07	0.84 ± 0.07
History of Smoking (%)		27.0	42.5	53.7	60.6
SBP (mmHg)		126 ± 14	130 ± 16	137 ± 17	144 ± 20
DBP (mmHg)		77 ± 9	78 ± 9	81 ± 10	83 ± 10
HbA_{1C} (%)		5.6 ± 0.3	8.3 ± 1.4	8.8 ± 1.5	9.1 ± 1.6
Total cholesterol (mmol/l)		4.82 ± 0.93	4.83 ± 0.90	4.97 ± 0.90	5.39 ± 1.10
HDL cholesterol (mmol/l)		1.56 ± 0.32	1.36 ± 0.38	1.30 ± 0.38	1.21 ± 0.37
LDL cholesterol (mmol/l)		2.80 ± 0.84	2.96 ± 0.81	3.07 ± 0.82	3.39 ± 0.87
Triglycerides (mmol/l)		0.90 (0.69 - 1.17)	0.94 (0.73 - 1.29)	1.06 (0.81 - 1.52)	1.37 (1.02 - 2.05)
AER (mg/24h)		3 (1- 4)	7 (5 - 12)	51 (25 - 100)	440 (176 - 1207)
eGFR (ml/min/1.73 m^2)		92 (76 - 111)	87 (72 - 107)	81 (64 - 101)	46 (28 - 69)
CRP(mg/l)		1.04 (0.53 - 2.37)	1.87 (1.13 - 3.55)	2.16 (1.27 - 4.70)	2.68 (1.66 - 5.81)
Serum ADP (mg/l)	All	9.69 (7.33 - 12.17)	10.69 (7.96 - 14.82)	10.78 (7.92 - 15.08)	14.7 (10.26 - 22.00)
	Males	7.60 (5.26 – 9.65)	8.80 (6.67 - 11.53)	10.01 (7.24 - 13.19)	12.45 (9.05 - 15.87)
	Females	10.80 (8.37 - 13.40)	13.14 (9.71 - 16.70)	12.55 (9.04 - 19.07)	18.68 (12.67 - 26.27)
Urinary ADP (µg/g)	All	0.34 (0.21 - 0.66)	0.56 (0.26 - 1.31)	0.97 (0.39 - 2.42)	5.52 (1.53 - 22.9)
	Males	0.23 (0.15 - 0.50)	0.43 (0.21 - 0.99)	0.82 (0.32 - 2.23)	5.03 (1.51 - 20.14)
	Females	0.42 (0.26 - 0.83)	0.72 (0.32 - 1.54)	1.07 (0.50 - 2.62)	5.95 (1.77 - 24.67)
Urinary L-FABP (µg/µmol)		0.01 (0.00 - 0.04)	0.04 (0.01 - 0.09)	0.09 (0.03 - 0.18)	0.52 (0.19 - 1.97)
Urinary KIM-1 (ŋg/mmol)		37.3 (18.6 - 58.3)	26.2 (12.2 - 48.7)	34.5 (16.4 - 62.0)	48.5 (27.3 - 88.7)

M/F – male / female

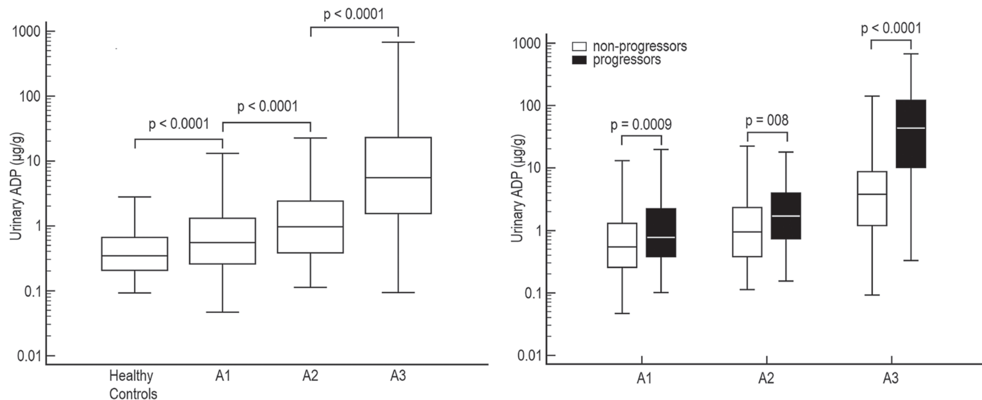

Figure 12. Urinary ADP levels across the study groups at the baseline. A – Urinary ADP levels across baseline DN stages. B – Urinary ADP levels according to baseline DN stage and progression status.

Urinary ADP was higher in individuals with diabetes without albuminuria compared to non-diabetic subjects ($p < 0.0001$). In addition, urinary ADP increased with progression of DN stage ($p < 0.0001$). Moreover, urinary ADP was always higher in progressors to a higher stage of DN compared with non-progressors (Figure 12).

5.2.1. Baseline urinary ADP determinants at each stage

To investigate the most important determinants associated with baseline urinary ADP at each baseline stage of DN, we tested clinical and biochemical parameters present in our database (please see the Material and methods section).

In individuals with baseline normal AER the most important variables associated with urinary ADP after combining models and stepwise selection were: age at diabetes onset, BMI and sex together with AER, urinary KIM-1, urinary L-FABP, serum ADP and HbA_{1C} (Table 12). Of these variables, gender, urinary L-FABP and HbA_{1C} explained most of the urinary ADP variation (65%), while the rest of the variables in the model explained another 1% of urinary ADP variation.

In individuals with baseline microalbuminuria the following variables were finally associated with urinary ADP: gender, BMI as well as urinary KIM-1, urinary L-FABP, AER and HbA_{1C} (Table 12). At this stage, $HbA_{1C,}$ urinary L-FABP and gender explained most of the urinary ADP variation.

Finally, in individuals with baseline macroalbuminuria the most important determinants of baseline urinary ADP were: age of diabetes onset, BMI together with urinary KIM-1, urinary L-FABP, AER, GFR, HbA_{1C}, LDL cholesterol and serum ADP. Out of these variables, LDL, HbA_{1C} and urinary L-FABP explained most of the urinary ADP variability (Table 12).

The common urinary ADP determinants across all stages were BMI, urinary KIM-1, urinary L-FABP, AER and $HbA_{1C,}$ while gender was a determinant only for individuals with normal AER or microalbuminuria. In addition, eGFR and LDL cholesterol remained in the final model only in individuals with baseline macroalbuminuria (Table 12).

Table 12. Determinants of urinary ADP according to DN stage at baseline of Study II.

Predictor variables	β	SE	p
Normal AER			
Age of onset (years)	-0.013	0.004	<0.001
BMI (kg/m^2)	-0.020	0.011	0.07
Urinary KIM-1 (ng/mmol)	0.005	0.001	<0.0001
AER (mg/24h)	0.013	0.003	<0.0001
HbA_{1C} (%)	0.147	0.025	<0.0001
Urinary L-FABP (μg/μmol)	0.137	0.066	0.04
Sex (M/F)	0.366	0.078	<0.0001
Serum ADP (mg/l)	0.033	0.007	<0.0001
Microalbuminuria			
BMI (kg/m^2)	-0.039	0.021	0.07
Urinary KIM-1 (ng/mmol)	0.005	0.002	0.04
AER (mg/24h)	0.002	0.001	0.03
HbA_{1C} (%)	0.159	0.048	0.001
Urinary L-FABP (μg/μmol)	2.134	0.245	<0.0001
Gender (M/F)	0.481	0.149	0.002
Macroalbuminuria			
Age of onset (years)	-0.019	0.008	0.02
BMI (kg/m^2)	-0.048	0.020	0.02
Urinary KIM-1 (ng/mmol)	0.002	0.001	0.06
eGFR (ml/min/1.73m^2)	-0.018	0.003	<0.0001
AER (mg/24h)	0.001	0.0001	<0.0001
HbA_{1C} (%)	0.103	0.051	<0.05
Urinary L-FABP (μg/μmol)	0.079	0.026	0.003
LDL (mmol/l)	-0.196	0.090	0.03
Serum ADP (mg/l)	0.015	0.007	0.03

β - the degree of change in urinary ADP for every 1 unit of change in the predictor variable, SE – standard error, p – statistical significance, M / F – male / female

5.2.2. Prediction of progression of DN by urinary ADP

In simple Cox analysis as well as in gender adjusted analysis, urinary ADP predicted progression to a higher stage regardless of the DN category at baseline.

The basic Cox proportional hazard models for progression (basic models of progression – BM) at every stage were built according to the methodology described in the Material and methods section. The BM for progression to microalbuminuria comprised the following variables: total cholesterol, history of smoking, HbA_{1C} and WHR. The BM for progression to macroalbuminuria included HbA_{1C}, WHR and triglycerides, while the BM for progression to ESRD had just two components - triglycerides and eGFR (data not shown).

When adjusted with the BM models for each stage, urinary ADP predicted progression to a higher stage with or without adjustments for gender. When further adjustment with AER was applied, however, urinary ADP independently predicted progression only to ESRD. It is noteworthy also that urinary ADP's ability to predict ESRD was independent of gender, urinary L-FABP, urinary KIM-1 or serum ADP (Table 13).

Table 13. Prediction of DN progression by urinary ADP

Variable	Adjustment	Unadjusted or adjusted for sex		Adjusted for BM		Adjusted for BM and AER		Adjusted for BM and Urinary KIM-1		Adjusted for BM and Urinary L-FABP		Adjusted for BM and Serum ADP	
		HR	p	HR	p	HR	p	HR	p	HR	p	HR	p
Normal AER													
Urinary ADP (µg/g)	None	1.32	< 0.001	1.22	0.02	0.98	0.80	0.96	0.76	0.98	0.90	1.22	0.07
	Sex	1.39	< 0.001	1.25	0.01	0.99	0.91	0.97	0.81	1.01	0.94	1.26	0.04
Microalbuminuria													
Urinary ADP (µg/g)	None	1.38	0.007	1.34	0.03	1.01	0.95	1.31	0.07	1.02	0.92	1.30	0.14
	Sex	1.46	0.001	1.35	0.02	1.02	0.89	1.31	0.06	1.04	0.83	1.29	0.15
Macroalbuminuria													
Urinary ADP (µg/g)	None	2.03	< 0.001	1.51	< 0.001	1.30	0.03	1.47	< 0.001	1.36	0.002	1.39	0.001
	Sex	2.05	< 0.001	1.52	< 0.001	1.37	0.01	1.29	0.001	1.39	0.001	1.36	0.001

The results were similar when the competing risk analysis considering death as a competing event for progression at any stage was performed.

5.2.3. Urinary ADP's diagnostic performance and added clinical benefit for prediction of progression to ESRD

The average diagnostic performance over time for urinary ADP and AER was assessed also by AUC of each biomarker or model only for prediction of progression to ESRD. In individuals with baseline macroalbuminuria, urinary ADP identified on average another 5.7% of progressors to ESRD compared to AER (p = 0.04). In addition, urinary ADP added to AER identified on average another 5.6% of progressors to ESRD (p = 0.02), compared with AER alone. Finally, when we compared the average AUCs of urinary ADP with eGFR there was no difference (p = 0.79), although when urinary ADP was added to eGFR there was again an increase in AUC (p = 0.03), meaning an average extra 2.9% of progressors were identified by using urinary ADP together with eGFR, compared with eGFR alone (Table 14).

We assessed only the clinical diagnostic benefit of urinary ADP for prediction of progression to ESRD using time dependent ROC curve analysis at quintiles of follow-up time, since urinary ADP was an independent predictor only for progression to ESRD. The diagnostic ability of urinary ADP increased slightly over time, while diagnostic capability of both AER and eGFR presented a constantly declining AUC in individuals with macroalbuminuria at baseline. Thus, the AUC of urinary ADP was significantly larger than the AUC of AER only after 5.5 years of follow-up. However, the AUC of urinary ADP was no better than the AUC of eGFR at any point. Finally, when urinary ADP was added to basic models for progression to ESRD with or without AER, the models were similar at all time-points. The constant decline in predictive capability for AER and eGFR could not entirely be prevented by addition of urinary ADP to the models (Table 15 and Figure 13).

Table 14. Average diagnostic performance over time using ROC curves analysis for prediction of DN progression by urinary ADP, AER and eGFR.

ROC	AUC	95%CI
AER	0.786	0.736 - 0.829
Urinary ADP	0.842	0.798 - 0.880
eGFR	0.853	0.809 - 0.890
AER+ADP	0.842	0.797 - 0.880
eGFR+ADP	0.882	0.841 - 0.915
BM	0.853	0.809 - 0.890
BM+AER	0.880	0.840 - 0.914
BM+ADP	0.884	0.843 - 0.917
BM+AER+ADP	0.887	0.847 - 0.919

ADP+AER – Cox model formed by urinary ADP and AER used together; ADP+eGFR – Cox model formed by urinary ADP and eGFR used together

Table 15. ROC curves analysis at quintiles of follow-up time for the main progression models in Study **II.**

Progression from macroalbuminuria to ESRD										
Time points (years)	3.54		4.60		6.16		6.97		7.38	
Cases / controls	36/256		47/224		57/160		61/96		63/64	
ROC	AUC	SE	AUC	SE	AUC	SE	AUC	SE	AUC	SE
AER	81.43	4.13	79.55	4.11	75.12	4.26	75.52	4.20	75.55	4.42
eGFR	93.02	2.60	92.21	2.51	90.83	2.32	85.86	3.61	83.15	4.51
ADP	84.48	4.17	84.00	3.42	82.51	3.21	85.19	3.15	85.52	3.50
ADP+AER	84.77	4.18	84.01	3.52	82.22	3.30	84.40	3.24	84.79	3.57
ADP+eGFR	92.46	2.38	92.48	2.12	91.52	1.99	88.75	2.89	86.11	3.87
BM	92.76	4.42	92.36	2.43	90.84	2.27	85.46	3.76	82.70	4.69
BM+AER	93.43	2.20	93.01	2.08	91.66	1.93	87.46	3.24	84.84	4.22
BM+ADP	92.46	2.45	92.55	2.13	91.50	2.02	88.51	3.00	85.84	3.93
BM+AER+ADP	93.01	2.29	93.01	2.04	91.96	1.90	88.49	3.01	85.84	3.95

ADP+AER – Cox regression model with urinary ADP and AER; BM+ADP – Cox regression model of urinary ADP and the BM; BM+AER – Cox regression model of AER adjusted with the BM; BM+AER+ADP – Cox regression model of urinary ADP adjusted with AER and BM.

Since urinary ADP was not an independent predictor of progression to micro- and macroalbuminuria, for these baseline stages, NRI and IDI were not calculated. When NRI or IDI were calculated, in individuals with baseline macroalbuminuria, urinary ADP used on top of AER added a significant reclassification benefit (added clinical benefit) for prediction of progression to ESRD. Urinary ADP added to AER

correctly reclassified 54.9% of individuals at 5 years when NRI_{S-5} was calculated and 9.2% of individuals when IDI_{S-5} was estimated. However, no added clinical benefit was seen by adding urinary ADP to BM plus AER. Furthermore, urinary ADP improved the prediction of progression to ESRD, compared with eGFR alone in logistic models, but not at 5 years. Of note is the fact that neither NRI_{S-10} nor IDI_{S-10} could be estimated, since at 10 years' follow up there was no survival of individuals with baseline macroalbuminuria (Table 16).

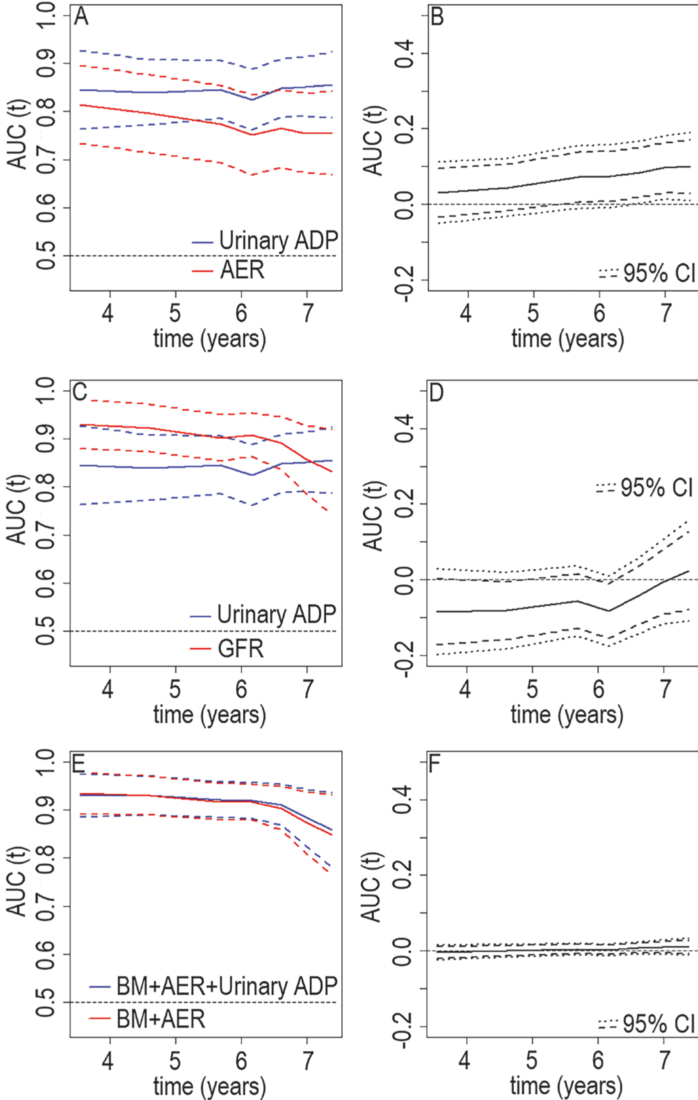

Figure 13. Variation over time of predictive ability (AUC) for identification of progressors to ESRD (A, C, E). Variation of the differences between the AUC of urinary ADP and AER (B) or GFR (D). Variation of the difference between the AUC of BM+AER and BM+AER+ADP (F). BM+AER – basic models for progression to ESRD plus AER, BM+AER+ADP – basic models for progression to ESRD plus AER and ADP.

Table 16. Urinary ADP added reclassification benefit analyzed using NRI and IDI.

Variables	cNRI	NRI_{S-5}	NRI_{S-10}	IDI	IDI_{S-5}	IDI_{S-10}
Progression to ESRD						
ADP+AER *vs* AER	0.794*	0.549*	NA	0.115***	0.092*	NA
ADP+eGFR *vs* eGFR	0.637***	0.188	NA	0.087***	0.039	NA
ADP+BM *vs* BM	0.674***	0.299	NA	0.084***	0.046	NA
BM+AER+ADP *vs* BM+AER	0.420	0.079	NA	0.015	-0.007	NA

ADP+AER – Cox regression model with Urinary ADP and AER; ADP+AER – Cox regression model with urinary ADP and eGFR; BM+ADP – Cox regression model of urinary ADP and the BM; BM+AER – Cox regression model of AER adjusted with the BM; BM+AER+ADP – Cox regression model of urinary ADP adjusted with AER and BM. * - significant p-value ($p < 0.05$), ** - significant p-value ($p < 0.01$), *** - significant p-value ($p < 0.001$).

5.2.4. Urinary ADP and causality for DN progression

Because no SNP was sufficiently strongly ($p < 5 \times 10^{-8}$) associated with the modifiable exposure (urinary ADP levels), the first assumption of a Mendelian randomization study was not fulfilled. Thus, no further analysis could be performed to test causality using the Mendelian randomization approach (Table 17).

Table 17. Top SNPs associated with urinary ADP values.

CHR	SNP	BP	A1	A2	MAF	β	95% CI	P
1	rs1052607	46272113	G	A	0.054	0.20	0.11 – 0.28	4.77×10^{-6}
2	rs4140872	169734842	G	A	0.223	0.10	0.06 – 0.15	8.44×10^{-6}
8	rs17624806	16963457	A	G	0.112	0.12	0.07 – 0.17	4.12×10^{-6}

Models were adjusted for diabetes, gender, duration and two first PCs.
A significant association is considered if $p < 5 \times 10^{-8}$.

5.3. Study III – Urinary KIM-1 and DN progression

In Study III, all eligible individuals with full baseline data and available measurement for urinary KIM-1 at the enrolment visit were included. These 1572 individuals were divided into three groups: 953 individuals with normal AER, 269 with microalbuminuria and 350 individuals with macroalbuminuria.

At baseline, the following variables: diabetes duration, BMI, WHR, SBP and DBP, HbA_{1C}, total cholesterol and AER registered higher values with more advanced stage of DN. The complete clinical characteristics at the enrollment visit are presented in Table 18. Urinary KIM-1 values increased also with the severity of DN at baseline (Table 18 and Figure 14A).

After a median follow-up time of 6.0 years (IQR 5.7 – 6.4), 174 individuals progressed to the next stage. No difference in KIM-1 levels was observed between progressors to microalbuminuria and non-progressors. Urinary KIM-1 levels were higher, however, for progressors from microalbuminuria to macroalbuminuria and from macroalbuminuria to ESRD (Figure 14B).

Table 18. Clinical data at baseline visit for individuals enrolled in Study III.

Variable		Normoalbuminuria	Microalbuminuria	Macroalbuminuria
Number of individuals (M/F)		953 (407/546)	269 (163/106)	350 (190/160)
Age (years)		40.1 ± 12.1	39.2 ± 12.7	41.1 ± 10.5
Diabetes duration (years)		24.2 ± 9.9	26.4 ± 10.7	29.1 ± 8.0
BMI (kg/m^2)		25.2 ± 3.4	25.6 ± 3.5	26.0 ± 3.8
WHR	Men	0.90 ± 0.07	0.92 ± 0.07	0.94 ± 0.07
	Women	0.80 ± 0.06	0.83 ± 0.07	0.84 ± 0.07
History of smoking (%)		39.9	52.8	60.4
SBP (mmHg)		131 ± 16	137 ± 17	144 ± 20
DBP (mmHg)		78 ± 9	81 ± 10	83 ± 10
HbA_{1C} (%)		8.2 ± 1.2	8.8 ± 1.5	9.1 ± 1.6
HDL cholesterol (mmol/l)		1.37 ± 0.37	1.29 ± 0.37	1.21 ± 0.37
LDL cholesterol (mmol/l)		3.03 ± 0.79	3.11 ± 0.79	3.35 ± 0.89
Triglycerides (mmol/l)		0.92 (0.70 - 1.24)	1.08 (0.82 - 1.61)	1.36 (1.01 - 2.05)
AER (mg/24h)		7 (5 - 11)	59 (29 - 110)	453 (168 - 1210)
eGFR (ml/min/1.73 m^2)		88 ± 28	88 ± 38	50 ± 30
Urinary KIM-1 (ηg/mmol)		27.8 (13.6 - 50.3)	33.1 (16.6 - 63.9)	49.5 (26.9 - 92.4)

Normally distributed variables are presented as means ± SD, non-normally distributed variables are presented as median (interquartile range). M / F – male / female.

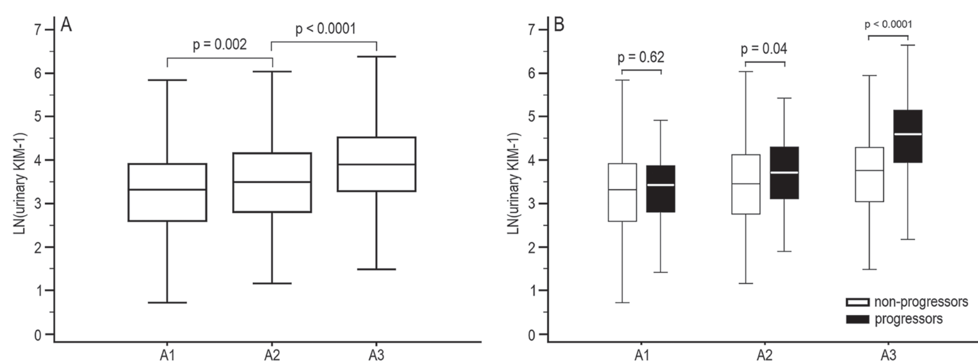

Figure 14. Urinary KIM-1 levels across study groups at baseline. A: Urinary KIM-1 levels according to baseline DN stages. B: Urinary KIM-1 levels at baseline according to DN stage and progression status.

5.3.1. Baseline determinants of KIM-1 levels at each stage

For each baseline stage of DN, all clinical and biochemical variables present in the database were tested for association with baseline urinary KIM-1 concentration (please see the Material and methods section for tested variables).

In individuals with baseline normal AER, the most important variables associated with urinary KIM-1 were: age at enrollment, current smoking status, BMI, CRP, AER and urinary ADP. Of these variables, current smoking, urinary ADP and age at study baseline explained most of the urinary KIM-1 variation (57%). The remaining variables in the model explained another 1% of the urinary KIM-1 variation. In

individuals with baseline microalbuminuria, gender, history of smoking and urinary ADP explained 89% of urinary KIM-1 variation. In addition, HbA1C explained another 9% of its variability. Finally, in individuals with baseline macroalbuminuria the most important determinants of urinary KIM-1 were again current smoking status, AER and urinary ADP. In addition, serum ADP, serum triglycerides, HbA1C and eGFR were also among the most important determinants of urinary KIM-1 levels in individuals with baseline macroalbuminuria (Table 19).

Table 19. Determinants of urinary KIM-1 according to baseline stage of DN in Study III.

Variables	β	SE	p
Normal AER			
Age (years)	0.112	0.003	0.0001
Current Smoking (Y/N)	0.310	0.080	0.0001
BMI (kg/m^2)	-0.021	0.010	0.048
CRP (mg/l)	0.066	0.036	0.06
AER (mg/24h)	0.083	0.046	0.07
Urinary ADP (ηg/mmol)	0.257	0.030	0.0001
Microalbuminuria			
Sex (M/F)	0.323	0.116	0.006
History of Smoking (Y/N)	0.351	0.113	0.002
HbA_{1C} (%)	0.090	0.041	0.03
Urinary ADP (ηg/mmol)	0.220	0.047	0.0001
Macroalbuminuria			
Current Smoking (Y/N)	0.211	0.116	0.07
eGFR ($ml/min/1.73m^2$)	0.006	0.002	0.01
HbA_{1C} (%)	0.070	0.037	0.06
Triglycerides (mmol/l)	0.557	0.135	0.0001
Serum ADP (mg/l)	0.420	0.124	0.001
AER (mg/24h)	0.159	0.056	0.005
Urinary ADP (ηg/mmol)	0.111	0.047	0.02

The common determinants of urinary KIM-1 levels across all DN stages were smoking and urinary ADP. Other common determinants retained in two out of three stages of DN at baseline were HbA_{1C} and AER (Table 19).

5.3.2. Prediction of DN progression by urinary KIM-1

In simple Cox regression, urinary KIM-1 was not predictive for progression to microalbuminuria ($p = 0.86$). In unadjusted analysis, one logarithmic unit increase in urinary KIM-1 was associated with a 4.14 times higher risk of progression from microalbuminuria to macroalbuminuria, as well as a 2.08-fold increase in the risk of progression from macroalbuminuria to ESRD.

After adjusting for basic models of progression, urinary KIM-1 still predicted progression to ESRD ($P < 0.0001$). However, after further adjustments with AER, urinary KIM-1 was no longer an independent predictor of progression to ESRD ($p = 0.17$) (Table 20).

Table 20. Prediction of DN progression by urinary KIM-1

Variable	Unadjusted			Adjusted with BM			Adjusted with BM and AER		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Normal AER									
Urinary KIM-1 (ηg/mmol)	1.06	0.54 - 2.08	0.86	0.98	0.49 - 1.97	0.96	0.80	0.39 - 1.64	0.54
Microalbuminuria									
Urinary KIM-1 (ηg/mmol)	4.14	2.75 - 6.23	< 0.0001	1.61	0.69 - 3.74	0.27	1.07	0.43 - 2.64	0.89
Macroalbuminuria									
Urinary KIM-1 (ηg/mmol)	2.08	1.66 - 2.62	< 0.0001	1.78	1.41 - 2.56	< 0.0001	1.20	0.92 - 1.57	0.17

Variables included in the basic model of progression to microalbuminuria after backward selection of covariates were HbA1C, serum triglycerides and WHR. The basic model for progression to macroalbuminuria included HbA1C, serum triglycerides and WHR. Finally, the basic progression model to ESRD comprised serum triglycerides and systolic blood pressure.

5.3.3. Urinary KIM-1's diagnostic performance for prediction of progression and added clinical benefit

Urinary KIM-1 predicted progression only to ESRD, and even then it was not independent of AER. We therefore assessed urinary KIM-1's diagnostic performance and added benefit only for prediction of progression to ESRD.

Table 21. Average diagnostic performance over time using ROC curves analysis for prediction of DN progression by urinary KIM-1, AER, eGFR and other progression models.

ROC	AUC	95%CI
AER	0.797	0.751 – 0.838
eGFR	0.861	0.821 – 0.896
KIM-1	0.735	0.686 – 0.781
AER+KIM-1	0.797	0.751 – 0.838
eGFR+KIM-1	0.876	0.825 – 0.899
BM	0.699	0.648 – 0.747
BM+AER	0.809	0.764 – 0.849
BM+eGFR	0.861	0.820 – 0.896
BM+KIM-1	0.772	0.724 – 0.815
BM+AER+KIM-1	0.818	0.773 – 0.857
BM+eGFR+KIM-1	0.879	0.839 – 0.911

AER+KIM-1 – model comprising urinary KIM-1on top of AER; eGFR+KIM-1 - model comprising urinary KIM-1on top of eGFR; BM – basic progression model to ESRD comprised serum triglycerides and systolic blood pressure; BM+AER – basic models for progression to ESRD plus AER; BM+eGFR – basic models for progression to ESRD plus eGFR; BM+AER+eGFR – basic models for progression to ESRD plus AER and eGFR; BM+AER+eGFR+KIM1 – basic models for progression to ESRD plus AER, eGFR and urinary KIM-1.

The diagnostic performance of urinary KIM-1 for prediction of progression to ESRD over time was assessed using time dependent ROC curve analysis. As expected, since it was not independent of AER, KIM-1's diagnostic ability was not superior compared to AER or eGFR at any time point or as an average in the time dependent ROC curve analysis (Table 21, Table 22 and Figure 15).

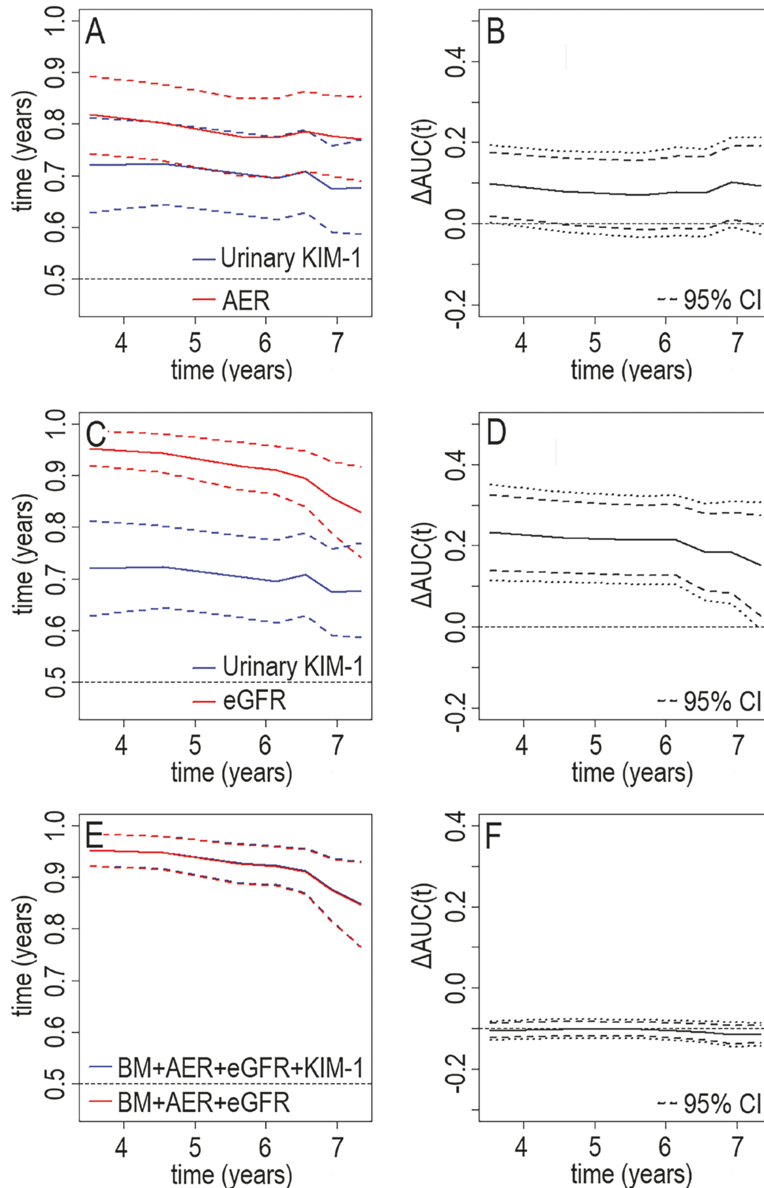

Figure 15. Variation over time of diagnostic performance for prediction (AUC) of progressors to ESRD (A, C, E), as well as variation of the difference between AUCs (B, D, F) for urinary KIM-1 and AER (A and B), urinary KIM-1 and eGFR (B and C) as well as for BM+AER+eGFR and BM+AER+eGFR+KIM1 (E and F). BM – basic progression model to ESRD comprised serum triglycerides and systolic blood pressure; BM+AER+eGFR – basic models for progression to ESRD plus AER and eGFR; BM+AER+eGFR+KIM1 – basic models for progression to ESRD plus AER, eGFR and urinary KIM-1.Δ

In reclassification analysis, undertaken using NRI or IDI generalized for survival data, there was no reclassification benefit at 5 or 10 years' follow up with regard to prediction of progression to ESRD (Table 23).

Table 22. ROC curves analysis at quintiles of follow-up time for the main models of progression to ESRD in Study III.

Time points (years)	3.53		4.55		6.15		6.92		7.34	
Individuals (cases/controls)	40/274		52/240		63/171		66/103		68/69	
ROC	AUC	SE	AUC	SE	AUC	SE	AUC	SE	AUC	SE
AER	81.84	3.84	80.27	3.78	77.37	3.90	77.67	3.92	77.07	4.19
eGFR	95.31	1.72	94.38	1.89	91.01	2.38	85.79	3.56	82.86	4.48
KIM1	72.05	4.69	72.34	4.06	69.54	4.09	67.48	4.24	67.77	4.67
KIM1+AER	81.94	3.93	80.68	3.71	77.81	3.81	77.84	3.84	77.42	4.14
KIM1+eGFR	94.66	1.69	94.32	1.71	91.21	2.28	86.06	3.54	83.10	4.50
BM	72.68	4.53	73.83	4.01	70.44	3.95	67.38	4.46	69.40	4.67
BM+AER	83.09	3.90	82.46	3.61	79.47	3.64	79.06	3.84	78.73	4.09
BM+eGFR	95.04	1.67	94.53	1.82	91.02	2.31	85.50	3.66	82.85	4.57
BM+KIM1	77.50	4.39	78.40	3.76	75.54	3.85	72.13	4.24	73.31	4.45
BM+AER+KIM1	83.34	3.95	82.74	3.61	79.74	3.64	79.12	3.88	79.17	4.12
BM+AER+eGFR+KIM1	94.72	1.59	94.66	1.59	91.52	2.19	85.98	3.63	83.18	4.54

AER+KIM-1 – model comprising urinary KIM-1on top of AER; eGFR+KIM-1 - model comprising urinary KIM-1on top of eGFR; BM – basic progression model to ESRD comprised serum triglycerides and systolic blood pressure; BM+AER – BM for progression to ESRD plus AER; BM+eGFR – BM for progression to ESRD plus eGFR; BM+AER+eGFR – BM for progression to ESRD plus AER and eGFR; BM+AER+eGFR+KIM1 – BM for progression to ESRD plus AER, eGFR and urinary KIM-1.

Table 23. KIM-1 added reclassification benefit for progression to ESRD analyzed using NRI and IDI.

Variables	cNRI	NRI_{S-5}	NRI_{S-10}	IDI	IDI_{S-5}	IDI_{S-10}
KIM1+AER *vs* AER	0.264	-0.010	NA	0.024	0.003	NA
KIM1+eGFR *vs* eGFR	0.627***	-0.063	NA	0.079***	0.014	NA
KIM1+BM *vs* BM	0.544***	0.413*	NA	0.099***	0.051	NA
KIM1+AER+BM *vs* AER+BM	0.243	-0.174	NA	0.023	0.001	NA
KIM1+eGFR+BM *vs* eGFR+BM	0.465**	0.098	NA	0.063**	0.008	NA
KIM1+eGFR+AER+BM *vs* KIM1+eGFR+AER	0.327	-0.273	NA	0.020	-0.001	NA

BM – basic progression model (Cox model formed by serum triglycerides and systolic blood pressure); KIM-1+AER – Cox model formed by urinary KIM-1 and AER; KIM-1+eGFR – Cox model formed by urinary KIM-1 and eGFR; AER+BM – Cox model formed by AER and BM; eGFR+BM – Cox model formed by eGFR and BM; KIM-1+BM – Cox model formed by urinary KIM-1 and BM; KIM1+AER+BM – Cox model formed by urinary KIM-1, AER and BM; KIM1+eGFR+BM – Cox model formed by urinary KIM-1, AER and BM; * - significant p-value ($p < 0.05$), ** - significant p-value ($p < 0.01$), *** - significant p-value ($p < 0.001$).

5.3.4. Urinary KIM-1 and causality for DN progression

5.3.4.1. Identification of the instrumental variable and the first assumption – GWAS on KIM-1

The GWAS on ln (KIM-1) identified 49 SNPs with a *p-value* < 5×10^{-8} (the genome-wide significance). All SNPs were on chromosome 5q33.3 in the region that includes the *KIM1* gene (*HAVCR1*: hepatitis A virus cellular receptor 1) (Figure 16 and Table 24). The strongest association was observed for rs2036402 with $P = 6.5\times10^{-38}$

(ß = -0.51, i.e., each copy of the minor G allele decreases ln(KIM-1) by 0.51; 95% CI -0.47 – -0.54) (Figure 16). After conditional analysis on rs2036402, no other SNP reached genome-wide significance, suggesting that rs2036402 explains most of the association seen on the locus (Table 24 and Table 25).

Table 24. Top SNPs associated with urinary KIM-1 levels. Models were adjusted for diabetes, gender, duration and two first PCs.

CHR	SNP	BP	A1	A2	MAF	B	95% CI	P	p adjusted for rs2036402
5	rs2036402	156396820	C	T	0.24	-0,51	-0,47 – -0,54	6.48×10^{-38}	NA
5	rs6889164	156399356	C	T	0.24	-0,51	-0,47 – -0,54	6.83×10^{-38}	NA
5	rs13173581	156402386	T	C	0.24	-0,5	-0,47 – -0,54	7.71×10^{-38}	NA
5	rs1039438	156409348	A	G	0.24	-0,5	-0,47 – -0,54	7.81×10^{-38}	NA
5	rs12522248	156412004	C	T	0.26	-0,49	-0,46 – -0,53	4.45×10^{-37}	0.18
5	rs11740496	156367980	G	A	0.24	-0,5	-0,46 – -0,54	1.29×10^{-36}	0.73
5	rs11740499	156364319	A	G	0.24	-0,5	-0,46 – -0,54	1.54×10^{-36}	0.71
5	rs12515585	156362728	G	A	0.24	-0,5	-0,46 – -0,54	1.76×10^{-36}	0.68
5	rs7719994	156362654	C	T	0.24	-0,5	-0,46 – -0,54	1.79×10^{-36}	0.68
5	rs13169621	156359552	A	G	0.23	-0,51	-0,48 – -0,56	2.46×10^{-36}	0.93
5	rs13181803	156361755	G	C	0.24	-0,5	-0,46 – -0,54	4.05×10^{-36}	0.50
5	rs13169465	156359494	A	G	0.24	-0,5	-0,46 – -0,54	4.40×10^{-36}	0.49
5	rs13169155	156359241	A	G	0.24	-0,5	-0,46 – -0,54	4.55×10^{-36}	0.48
5	rs6863148	156339221	A	G	0.24	-0,5	-0,46 – -0,54	8.76×10^{-36}	0.43
5	rs7700944	156298759	A	G	0.21	-0,51	-0,47 – -0,55	5.11×10^{-32}	0.89
5	rs6555760	156297944	A	T	0.21	-0,51	-0,47 – -0,55	6.36×10^{-32}	0.89
5	rs2862058	156287953	G	A	0.21	-0,51	-0,47 – -0,55	7.24×10^{-32}	0.89
5	rs1345617	156284081	C	G	0.21	-0,51	-0,47 – -0,55	8.47×10^{-32}	0.89
5	rs1345618	156283925	C	T	0.21	-0,5	-0,46 – -0,55	8.97×10^{-32}	0.89
5	rs2279804	156411782	T	C	0.42	-0,4	-0,37 – -0,43	3.04×10^{-29}	0.0001
5	rs953568	156410210	A	T	0.42	-0,4	-0,37 – -0,43	3.61×10^{-29}	0.0001
5	rs953569	156409978	G	T	0.41	-0,4	-0,37 – -0,43	4.39×10^{-29}	0.0006
5	rs6555820	156404727	A	C	0.41	-0,4	-0,37 – -0,42	5.47×10^{-29}	0.0007
5	rs868529	156363714	T	A	0.40	-0,39	-0,36 – -0,42	8.52×10^{-28}	0.002
5	rs1393206	156362231	A	T	0.20	-0,47	-0,43 – -0,51	6.05×10^{-26}	0.08
5	rs7732745	156279792	T	C	0.18	-0,42	-0,38 – -0,46	6.72×10^{-21}	0.12
5	rs10070224	156278313	C	T	0.16	-0,47	-0,42 – -0,52	7.45×10^{-21}	0.69
5	rs12187482	156269565	G	A	0.16	-0,47	-0,42 – -0,52	7.48×10^{-21}	0.69
5	rs7720464	156268109	G	A	0.15	-0,49	-0,44 – -0,54	2.05×10^{-20}	0.62
5	rs4704821	156267542	A	C	0.15	-0,49	-0,44 – -0,55	8.16×10^{-20}	0.58
5	rs2116787	156432559	G	A	0.15	-0,43	-0,39 – -0,47	1.97×10^{-18}	0.86
5	rs1501909	156398757	T	G	0.45	0,3	0,28 – 0,32	6.39×10^{-17}	0.02
5	rs17054137	156405508	A	G	0.45	0,3	0,28 – 0,32	6.52×10^{-17}	0.02

β on ln(KIM-1) was estimated per one copy of A1. p – statistical significance of the association between SNPs and ln(KIM-1) was calculated for models adjusted with diabetes duration and two first PCs; p adjusted for rs2036402 – statistical significance of the association between SNPs and ln(KIM-1) was estimated after addition to the previous model of rs2036402, as an independent covariate. P values considered statistically significant were less than 5×10^{-8}.

Finally, the IV was rs2036402, a SNP in the *HAVCR1* gene, with the strongest independent association with the urinary KIM-1 levels. The imputed rs2036402

allele dosage data was converted to the most likely genotypes, accepting genotype calls with > 0.9 genotype likelihood as estimated by the MACH imputation software (Figure 16 and Table 24 and Table 25).

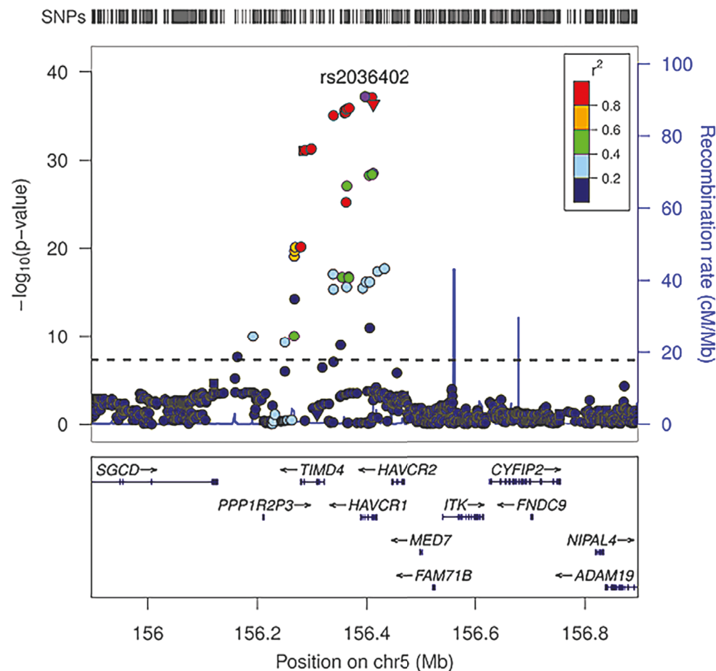

Figure 16. The association plot of *HAVCR1* region with all SNPs associated with urinary KIM1 levels. The threshold for genome-wide significance ($p < 5 \times 10^{-8}$) is marked by the dashed horizontal line. For this association analysis, adjustments for the two PCs as well as for gender and diabetes duration were used. This plot visualization used LocusZoom software1 ([http://csg.sph.umich.edu/locuszoom/) (449).

Table 25. Association of rs2036402 (the top SNP in *HAVCR1* gene that encodes KIM-1) with urinary KIM-1 levels after different adjustments.

Adjustment covariates for rs2036402	β	95% CI	p
Unadjusted	-0.51	-0.58 – -0.43	3.54×10^{-38}
Duration	-0.51	-0.58 – -0.43	7.08×10^{-38}
Duration and PCs	-0,51	-0,47 – -0,54	6.48×10^{-38}
Duration and ln(AER)	-0.49	-0.57 – -0.43	5.69×10^{-42}
Duration, PCs and ln(AER)	-0,49	-0,45 – -0,52	6.99×10^{-40}

β – effect on ln(KIM-1) per one copy of rs2036402 minor C allele; 95%CI – 95% confidence intervals for β; p – statistical significance for association between the top KIM-1 genetic variant (rs2036402) and urinary KIM-1 levels. Considered significant if $p < 5 \times 10^{-8}$; PCs – the first two principal components;

5.3.3.2. The second assumption fulfillment

Genetic variants are assumed to be randomly distributed before birth. Consequently, at birth all subjects are randomly assigned to different levels of urinary KIM-1. Thus, it is also assumed that subjects' allocation to different levels of urinary KIM-1 is independent of any confounders that appear a long time after birth (e.g., HbA_{1C}).

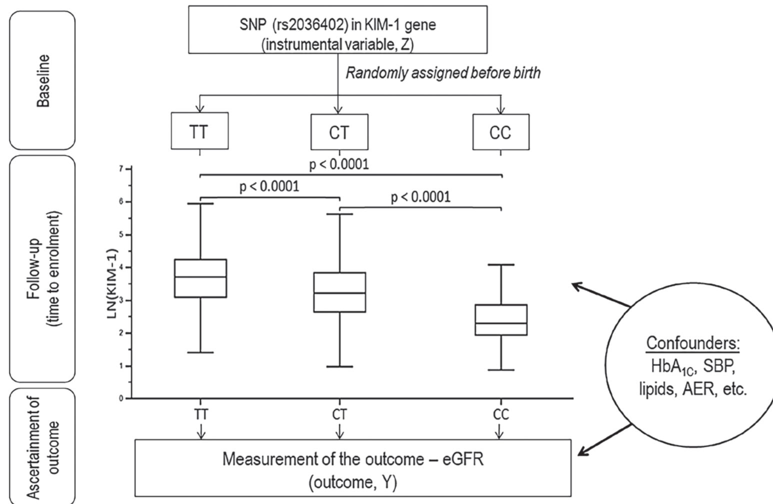

Figure 17. Urinary KIM-1 levels according to genotypes and the Mendelian randomization second assumption fulfilment. KIM-1 levels were significantly different among different genotypes ($p < 0.0001$). Consequently, at birth all subjects are randomly assigned to different levels of urinary KIM-1. It is assumed that if KIM-1 is causal for the loss of kidney function and it acts for a long enough period (time from birth to study baseline), then those individuals with high levels of urinary KIM-1 should have lower eGFR values at the first study visit.

Since the study subjects are randomly allocated to different levels of urinary KIM-1 long before birth, the second assumption of the Mendelian randomization analysis is considered a priori true (Figure 17).

5.3.3.3. The third assumption fulfillment

To test if the association between the eGFR (outcome, Y) and *KIM1* SNP (IV, Z) is mediated only through urinary KIM-1 levels, two conditions must be fulfilled: a) the IV (rs2036402) is not associated with any other variable in our database; b) the IV (rs2036402) is either not associated with eGFR, or if associated this association should disappear when adjusted with urinary KIM-1 levels.

For the first condition, rs2036402 was tested for association with all the variables present in the database by simple linear regressions or χ^2 test. No variable was associated with rs2036402, except for urinary KIM-1 levels ($p < 0.0001$). This result validated the first condition from the last assumption of the MR approach (Table 26).

To check the validity of the second condition of the last assumption that the IV (rs2036402) acts on the outcome (eGFR) only through the exposure (urinary KIM-1) without pleiotropic effects we performed a regression of the IV on eGFR. This regression showed that rs2036402 was associated with GFR in unadjusted analysis ($p = 0.02$). When this association was adjusted with urinary KIM-1 levels, the association completely vanished ($p = 0.48$), suggesting that the rs2036402 acts on eGFR through urinary KIM-1. Association between rs2036402 and eGFR was also adjusted for duration, PCs and AER, but the effect did not vanish as in the case of

urinary KIM-1. These results confirmed a weak association between rs2036402 and eGFR, but the SNP acted on eGFR only through urinary KIM-1 levels, as initially assumed. Thus, the last assumption of the MR study, that the IV (rs2036402) acts through the exposure (KIM-1) without pleiotropic effects, was fulfilled.

Table 26. Variables associated with rs2036402 present in the database.

Variable	Association with rs2036402		
	β	SE	p
Age (years)	-0.009	0.005	0.06
Age of onset (years)	-0.004	0.006	0.46
Duration (years)	-0.009	0.006	0.12
BMI (kg/m2)	-0.016	0.015	0.29
History of smoking (%)	-	-	0.68
SBP (mmHg)	-0.003	0.003	0.32
DBP (mmHg)	0.003	0.006	0.62
HbA_{1C} (%)	0.005	0.038	0.90
Total cholesterol (mmol/l)	0.004	0.057	0.94
HDL cholesterol (mmol/l)	0.066	0.143	0.64
LDL cholesterol (mmol/l)	-0.002	0.066	0.97
ln(Triglycerides)	-0.190	0.108	0.08
ln(AER)	0.004	0.027	0.88
ln(KIM-1)	-0.460	0.059	<0.0001
Sex (M/F)	-	-	0.81
WHR	-0.942	0.642	0.14

Table 27. Association between KIM-1 genetic variant and eGFR, presented with different adjustments.

Adjustment covariates for rs2036402	B	95% CI	*P*
Unadjusted	3.25	0.49 – 6.00	0.02
Ln(KIM-1)	1.05	-1.83 – 3.93	0.48
Duration	2.55	-0.07 – 5.17	0.07
Duration and PCs	2,68	0,20 – 36,86	0.05
Duration and ln(AER)	2.03	-0.13 – 4,74	0.06
Duration, PCs and ln(AER)	2,03	0,18 – 23,49	0.11
Duration, PCs and ln(KIM-1)	0,69	0,04 – 10,79	0.62

The effect β on GFR was estimated per one copy of rs2036402 minor C allele.
PCs represented the first 2 PCs.

5.3.3.4. Causal link between KIM-1 and eGFR

The conventional (observational) estimate of the relationship between KIM-1 and eGFR was obtained using a linear regression. In the linear regression one logarithmic unit increase in urinary KIM-1 was associated with a 4.52 ml/min lower eGFR. This inverse association remained significant after different adjustments such as diabetes duration or HbA_{1C}, but disappeared after adjusting for AER (p = 0.70) (Table 28).

Table 28. Comparison of estimates from instrumental analysis and observed association between KIM-1 and eGFR.

Instrumental variable (IV)	F	IV estimate			Observational estimate			E (p)
		B	95% CI	p	B	95% CI	p	
Unadjusted					-4.522	-6.238 – -2.807	< 0.001	
rs2036402	93.66	-6.786	-12.027 – -1.546	0.01				0.37
Duration					-4.066	-5.699 – -2.434	< 0.001	
rs2036402	63.05	-5.654	-10.647 – -0.661	0.03				0.51
Duration+HbA1c					-3.827	-5.493 – -2.161	< 0.001	
rs2036402	64.67	-5.667	-10.681 – -0.655	0.03				0.45
Duration+AER					0.325	-1.305 – 1.956	0.70	
rs2036402	113.93	-5.044	-9.865 – -0.224	0.04				0.02
Duration+HbA1c+AER					0.114	-1.525 – 1.755	0.89	
rs2036402	103.23	-5.106	-9.920 – -0.292	0.04				0.02

Instrumental analysis (2SLS) was performed for calculation of the 2-stage estimator for the causal effect of the modifiable exposure (urinary KIM-1) on the outcome (eGFR), using rs2036402 in *KIM-1* gene as the IV. Association between urinary KIM-1 levels and eGFR was evaluated with a multiple linear regression. The differences between the IV estimators and the conventional regression based estimators (endogeneity) were tested by a Durbin-Wu test, to see if the differences were statistically significant. The covariates used for adjustments were the following: Duration – diabetes duration; Duration+HbA1C – diabetes duration and HbA_{1C}; Duration+HbA_{1C}+AER – diabetes duration, HbA_{1C} and AER; Duration – duration of T1DM; F *(first-stage regression)* – F-statistics from the first-stage regression of the 2SLS; The causal effect (β) of urinary KIM-1 on eGFR was estimated per one standard deviation of ln(KIM-1). Statistical significance (p) was considered significant at values of less than 0.05.

The causal estimate (or the IV estimate) of the effect of KIM-1 on eGFR was obtained using a 2SLS. The 2SLS showed that one logarithmic unit increase in urinary KIM-1's levels was associated with a 6.79 ml/min (95% CI -12.03 – -1.55) lower eGFR in the unadjusted analysis. Urinary KIM-1 increase remained independently associated with a lower eGFR even after adjustment with diabetes duration, HbA_{1C}, AER or all of them (*p* = 0.038). In comparison with the linear regression, the IV estimated effect sizes of urinary KIM-1 on eGFR were consistent with and without adjustments, ranging from -6,79 ml/min in unadjusted analysis to -5.11 ml/min in full adjusted analysis (Table 28).

Finally, the F-statistics from the first regression of the 2SLS, evaluating the strength of the IV, were far higher than 10, confirming that the balance between our sample size and the strength of the IV was sufficient for this type of study (Table 28).

5.3.3.5. Endogeneity testing

The endogeneity was non-significant in the unadjusted analysis (p = 0.37) and after adjustment for diabetes duration (p = 0.51) or HbA_{1C} (p = 0.45). This result could be interpreted as no difference between the observed estimator (from linear regression) and the IV estimator. However, when the 2SLS model was adjusted for AER, significant endogeneity was found (p < 0.05), indicating a significant difference between the two estimators (Table 28).

5.4. Study IV – Urinary biomarkers and prediction of macrovascular complications and mortality

Study IV comprised individuals with T1DM without ESRD or a previous cardiovascular event.

Table 29. Clinical characteristics at baseline for individuals enrolled.

Variable		Value
Sex (M/F)		1174 / 1155
Age (years)		37.17 ± 11.8
Diabetes duration (years)		21.2 ± 11.4
BMI (kg/m^2)		25.1 ± 3.6
WHR	Male	0.90 ± 0.07
	Female	0.81 ± 0.06
History of smoking (%)		49.9
Current smoking (%)		25.6
SBP (mmHg)		132 ± 17
DBP (mmHg)		79 ± 10
HbA_{1C} (%)		8.4 ± 1.5
Total cholesterol (mmol/l)		4.90 ± 0.92
LDL cholesterol (mmol/l)		3.03 ± 0.82
HDL cholesterol (mmol/l)		1.34 ± 0.38
Triglycerides (mmol/l)		0.99 (0.75 – 1.40)
AER (mg/24 h)		10 (6 – 36)
eGFR (ml/min/1.73 m2)		84 (68 – 103)
CRP (mg/l)		4.30 (1.19 – 3.89)
Proliferative retinopathy (%)		26.2
Antihypertensive medication (%)		36.6
Diabetic nephropathy (%)		28.1
Urinary L-FABP (µg/µmol)		0.05 (0.01 – 0.13)
Urinary ADP (µg/g)		0.40 (0.03 – 0.22)
Urinary KIM-1 (ŋg/mmol)		29.8 (13.6 – 53.8)

Categorical data are presented as numbers or percentages (%). Continuous data are presented as means ± SD if they are normally distributed or as median (inter- quartile range) for non-normally distributed variables. M / F – male / female, CAD – coronaryartery disease, PVD - peripheral vascular disease, CVD – cardiovascular disease.

The median age of the 2329 individuals was 37 years, and the duration of diabetes at baseline was 16 years. Of these individuals, a large number were male (1174 vs 1155), 25.6% were currently smoking, while 49.9% of them had a history of smoking. The overall mean BMI was 25 kg/m^2. The clinical characteristics of this population are presented in Table 28.

The median follow-up time in study IV was 14.1 years (IQR 12.5 – 16.0). During follow-up 240 (10.1%) individuals presented CAD, 116 (5%) presented PVD, 133 (5.8%) suffered a stroke, 15.1% had CVD, while 269 (11.3%) of them died.

5.4.1. Prediction of CVD, CAD, PVD, stroke and mortality by the tested urinary biomarkers

5.4.1.1. CAD, PVD and CVD

All urinary biomarkers had higher levels in those experiencing an incident CAD, PVD or CVD event (Table 30).

In addition, in simple Cox regressions, as well as after adjustment with the model of TRF, all biomarkers predicted incident CVD, CAD or PVD events. After further adjustments with eGFR, all biomarkers predicted CVD and PVD, while only urinary ADP predicted CAD. After further adjustments with AER were performed, urinary ADP and urinary KIM-1 predicted CVD, while urinary KIM-1 was the only biomarker predicting PVD after Bonferroni correction was applied (Table 30).

5.4.1.2. Stroke

Compared with those without incident strokes, individuals who experienced an incident stroke had higher levels for all 3 urinary biomarkers.

Urinary ADP predicted incident strokes independently of the TRF model, as well as when supplementary adjustment for eGFR was applied. However, when further adjustments with AER were applied, urinary ADP was no longer an independent predictor of stroke after Bonferroni correction was applied.

Urinary KIM-1 predicted incident stroke in unadjusted analysis as well as after adjustment with the TRF model.

Urinary L-FABP was the only biomarker that independently predicted stroke after adjustment with TRF as well as after further adjustment with eGFR and AER (Table 31).

5.4.1.3. Mortality

Again, all baseline levels of the 3 biomarkers were higher in individuals who died during follow up, compared with the survivors' levels.

All biomarkers predicted mortality in unadjusted analysis as well as after adjustments with TRF. After further adjustment with eGFR, however, only urinary L-FABP and urinary ADP still predicted mortality. Finally, only urinary L-FABP independently predicted mortality when adjusted with TRF, eGFR and AER (Table 31).

Table 30. Comparison between each biomarker's levels for each outcome.

	Coronary artery disease		
Variables	No CAD	CAD	p
Urinary L-FABP (µg/µmol)	0.051 (0.100 – 0.125)	0.123 (0.032 – 0.542)	< 0.0001
Urinary ADP (µg/g)	0.081 (0.032 - 0.200)	0.149 (0.045 - 0.576)	< 0.0001
Urinary KIM-1 (ηg/mmol)	27.727 (13.043 - 51.521)	40.870 (18.679 - 66.222)	< 0.0001
	Peripheral vascular disease		
Variables	No PVD	PVD	p
Urinary L-FABP (µg/µmol)	0.050 (0.010 – 0.054)	0.243 (0.069 – 1.349)	< 0.0001
Urinary ADP (µg/g)	0.081 (0.032 - 0.203)	0.256 (0.082 - 1.213)	< 0.0001
Urinary KIM-1 (ηg/mmol)	27.713 (13.044 - 51.429)	49.787 (26.810 - 79.335)	< 0.0001
	Stroke		
Variables	No Stroke	Stroke	p
Urinary L-FABP (µg/µmol)	0.051 (0.010 - 0.128)	0.1681 (0.044 - 0.813)	< 0.0001
Urinary ADP (µg/g)	0.081 (0.032 - 0.208)	0.222 (0.067 - 0.889)	< 0.0001
Urinary KIM-1 (ηg/mmol)	28.148 (13.103 - 51.521)	45.652 (20.916 - 73.257)	< 0.0001
	Cardiovascular disease		
Variables	No CVD	CVD	p
Urinary L-FABP (µg/µmol)	0.046 (0.008 - 0.112)	0.129 (0.037 - 0.669)	< 0.0001
Urinary ADP (µg/g)	0.074 (0.031 - 0.186)	0.1719 (0.058 - 0.831)	< 0.0001
Urinary KIM-1 (ηg/mmol)	26.633 (12.446 - 49.161)	43.333 (20.000 - 72.813)	< 0.0001
	Mortality		
Variables	Survivors	Dead	p
Urinary L-FABP (µg/µmol)	0.048 (0.009 - 0.119)	0.175 (0.051 - 0.987)	< 0.0001
Urinary ADP (µg/g)	0.078 (0.032 - 0.194)	0.201 (0.062 - 1.017)	< 0.0001
Urinary KIM-1 (ηg/mmol)	27.273 (12.873 - 50.870)	43.431 (20.548 - 74.018)	< 0.0001

Biomarkers levels are presented as median (interquartile range). Comparison between groups was performed using Mann-Whitney test for independent samples. Statistically significant difference was considered if $p < 0.01$ (according to Bonferroni correction for 5 outcomes).

5.4.2. L-FABP's diagnostic performance and added clinical benefit for prediction of stroke and mortality

Urinary L-FABP was an independent predictor for stroke and mortality. For these two outcomes, we performed further analysis on urinary L-FABP's diagnostic performance and added clinical benefit.

The average predictive performance (AUC) for stroke of urinary L-FABP was similar to that of eGFR ($p = 0.72$) or AER ($p = 0.56$), and superior to other well-known cardiovascular predictors (Table 32) (450). Notably, adding urinary L-FABP to the TRF model improved the diagnostic performance for stroke ($p = 0.04$). The added diagnostic benefit (increment in AUC) of urinary L-FABP used on top of the TRF was similar to that of AER. Finally, used on top of the TRF models already containing AER or eGFR, urinary L-FABP no longer improved the prediction of stroke (Table 32).

Table 31. Prediction of incident incident outcomes using Cox proportional hazard models with the individuals' clinical data from the baseline visit.

Outcome	Variable tested	Unadjusted (univariate)			Adjusted for TRF			Further adjusted for eGFR			Further adjusted for AER		
		HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Coronary artery Disease	AER (mg/24h)	1.41	1.33 - 1.49	< 0.0001	1.28	1.15 - 1.41	< 0.0001	1.24	1.11 - 1.40	0.0002	NA	NA	NA
	Urinary L-FABP (µg/µmol)	1.45	1.35 - 1.55	< 0.0001	1.21	1.11 - 1.32	< 0.0001	1.12	1.02 - 1.23	0.02	1.00	0.89 - 1.12	0.97
	Urinary ADP (µg/g)	1.38	1.29 - 1.48	< 0.0001	1.19	1.09 - 1.30	< 0.0001	1.14	1.04 - 1.25	0.006	1.09	0.97 - 1.22	0.15
	Urinary KIM-1 (ηg/mmol)	1.40	1.25 - 1.58	< 0.0001	1.17	0.96 - 1.26	< 0.0001	1.06	0.93 - 1.22	0.37	0.76	0.88 - 1.19	0.76
Peripheral vascular disease	AER (mg/24h)	1.66	1.53 - 1.80	< 0.0001	1.43	1.28 - 1.59	< 0.0001	1.36	1.20 - 1.54	< 0.0001	NA	NA	NA
	Urinary L-FABP (µg/µmol)	1.61	1.48 - 1.76	< 0.0001	1.30	1.16 - 1.45	< 0.0001	1.19	1.05 - 1.35	0.006	1.10	0.94 - 1.29	0.25
	Urinary ADP (µg/g)	1.57	1.43 - 1.72	< 0.0001	1.31	1.16 - 1.46	< 0.0001	1.20	1.05 - 1.36	0.007	1.15	0.97 - 1.36	0.10
	Urinary KIM-1 (ηg/mmol)	1.79	1.53 - 2.01	< 0.0001	1.51	1.24 - 1.84	< 0.0001	1.43	1.17 - 1.73	0.0004	1.38	1.11 – 1.73	0.004
Stroke	AER (mg/24h)	1.45	1.34 - 1.56	< 0.0001	1.27	1.15 - 1.42	< 0.0001	1.24	1.11 - 1.40	0.0002	NA	NA	NA
	Urinary L-FABP (µg/µmol)	1.55	1.42 - 1.68	< 0.0001	1.33	1.20 - 1.49	< 0.0001	1.28	1.14 - 1.44	< 0.0001	1.24	1.06 - 1.44	0.009
	Urinary ADP (µg/g)	1.52	1.39 - 1.67	< 0.0001	1.30	1.17 - 1.45	< 0.0001	1.25	1.11 - 1.41	0.0002	1.19	1.01 - 1.39	0.03
	Urinary KIM-1 (ηg/mmol)	1.55	1.33 - 1.81	< 0.0001	1.24	1.04 - 1.49	0.02	1.21	1.01 - 1.45	0.04	1.13	0.92 - 1.39	0.22
Cardiovascular Disease	AER (mg/24h)	1.49	1.42 - 1.56	< 0.0001	1.27	1.19 - 1.36	< 0.0001	1.23	1.14 - 1.32	< 0.0001	NA	NA	NA
	Urinary L-FABP (µg/µmol)	1.53	1.45 - 1.62	< 0.0001	1.30	1.21 - 1.39	< 0.0001	1.23	1.14 - 1.32	< 0.0001	1.11	1.00 - 1.23	0.05
	Urinary ADP (µg/g)	1.48	1.40 - 1.57	< 0.0001	1.27	1.18 - 1.36	< 0.0001	1.22	1.12 - 1.31	< 0.0001	1.16	1.05 - 1.29	0.003
	Urinary KIM-1 (ηg/mmol)	1.56	1.42 - 1.72	< 0.0001	1.27	1.14 - 1.42	< 0.0001	1.24	1.11 - 1.38	0.0002	1.19	1.05 - 1.35	0.007
Mortality	AER (mg/24h)	1.50	1.42 - 1.58	< 0.0001	1.32	1.23 - 1.42	< 0.0001	1.28	1.18 - 1.39	< 0.0001	NA	NA	NA
	Urinary L-FABP (µg/µmol)	1.56	1.47 - 1.66	< 0.0001	1.34	1.24 - 1.45	< 0.0001	1.29	1.18 - 1.39	< 0.0001	1.22	1.09 - 1.36	0.0003
	Urinary ADP (µg/g)	1.50	1.41 - 1.60	< 0.0001	1.31	1.21 - 1.41	< 0.0001	1.24	1.14 - 1.35	< 0.0001	1.14	1.02 - 1.27	0.02
	Urinary KIM-1 (ηg/mmol)	1.55	1.39 - 1.74	< 0.0001	1.23	1.08 - 1.40	< 0.0001	1.18	1.04 - 1.34	0.01	1.10	0.95 -1.27	0.21

The TRF models for prediction of every outcome comprised the following variables: age, sex, diabetes duration, HDL-C, triacylglycerol, HbA_{1C}, history of smoking, SBP, BMI and proliferative retinopathy. On top of this traditional risk factor, further adjustments for eGFR and AER were performed. The full description of statistical analysis was described in the Methods section. TRF – traditional risk factors model

Table 32. Average diagnostic performance using ROC curves analysis for prediction of stroke and mortality by urinary L-FABP in comparison with well-known risk factors.

Variable	AUC	95%CI	L-FABP	
			Diff.	p
Stroke				
HDL cholesterol	0.520	0.499 - 0.541	0.188	< 0.0001
LDL cholesterol	0.584	0.563 - 0.604	0.124	0.01
HbA_{1C}	0.596	0.575 - 0.616	0.112	0.008
SBP	0.686	0.667 - 0.706	0.022	0.68
eGFR	0.710	0.689 - 0.730	-0.002	0.72
AER	0.709	0.688 - 0.729	-0.001	0.56
Urinary L-FABP	0.708	0.687 - 0.728	-	-
Mortality				
HDL cholesterol	0.556	0.535 - 0.576	0.168	< 0.0001
LDL cholesterol	0.576	0.555 - 0.596	0.148	< 0.0001
HbA_{1C}	0.587	0.566 - 0.607	0.137	< 0.0001
SBP	0.689	0.670 - 0.708	0.035	0.13
eGFR	0.743	0.724 - 0.760	-0.019	0.37
AER	0.727	0.709 - 0.746	-0.003	0.82
Urinary L-FABP	0.724	0.705 - 0.742	-	-

Table 33. Average diagnostic performance using ROC curves analysis for the main comparisons between models used in the study.

Variable	AUC	95%CI
Stroke		
TRF	0.805	0.786 - 0.819
TRF+eGFR	0.810	0.788 - 0.821
TRF+AER	0.824	0.799 - 0.831
TRF+L-FABP	0.822	0.804 - 0.839
TRF+eGFR+AER	0.824	0.806 - 0.841
TRF+eGFR+L-FABP	0.822	0.804 - 0.839
TRF+AER+L-FABP	0.826	0.809 - 0.843
TRF+eGFR+AER+L-FABP	0.826	0.809 - 0.843
Mortality		
TRF	0.833	0.815 - 0.849
TRF+eGFR	0.840	0.822 - 0.856
TRF+AER	0.848	0.831 - 0.864
TRF+L-FABP	0.849	0.832 - 0.865
TRF+eGFR+AER	0.849	0.832 - 0.864
TRF+eGFR+L-FABP	0.850	0.834 - 0.866
TRF+AER+L-FABP	0.851	0.835 - 0.867
TRF+eGFR+AER+L-FABP	0.852	0.835 - 0.867

TRF – traditional risk factors model; TRF+GFR – Cox model formed by TRF and GFR; TRF+AER – Cox model of TRF and AER; TRF+L-FABP – Cox model of TRF and urinary L-FABP; TRF+GFR+AER – Cox model of TRF together with GFR and AER; TRF+GFR+L-FABP – Cox model of TRF together with GFR and urinary L-FABP; TRF+AER+L-FABP – Cox model of TRF together with AER and urinary L-FABP; TRF+GFR+AER+L-FABP – Cox model of TRF together with GFR, AER and urinary L-FABP.

Table 34. ROC curves analysis at quintiles of follow-up time for progression models.

Time points (years)	12.1		13.4		14.5		16.0		16.2	
Cases / controls	102/2125		125/1858		138/1328		147/797		150/531	
ROC	AUC	SE	AUC	SE	AUC	SE	AUC	SE	AUC	SE
Stroke										
eGFR	71.03	3.42	69.87	3.21	71.46	3.03	72.51	2.96	72.28	3.02
AER	70.39	3.15	68.97	2.93	68.05	2.98	65.27	2.99	64.57	2.99
Urinary L-FABP	68.10	3.06	67.38	2.75	65.83	2.79	62.88	2.95	61.99	2.90
TRF	84.61	1.85	83.21	1.95	84.72	1.76	85.02	1.84	84.27	1.99
TRF+eGFR	84.58	2.05	83.01	2.04	84.66	1.84	85.32	1.82	84.80	1.94
TRF+AER	86.14	1.73	84.38	1.84	85.53	1.71	86.09	1.73	85.19	1.89
TRF+L-FABP	86.41	1.71	83.39	2.10	85.29	1.90	85.77	1.87	85.07	1.99
TRF+eGFR+AER	86.04	1.77	84.29	1.85	85.57	1.70	86.22	1.71	85.38	1.87
TRF+eGFR+L-FABP	86.06	1.83	83.12	2.12	85.16	1.90	85.79	1.86	85.18	1.97
TRF+AER+L-FABP	86.62	1.71	83.94	2.00	85.51	1.84	86.19	1.81	85.39	1.94
TRF+eGFR+AER+L-FABP	86.55	1.72	83.86	2.01	85.52	1.83	86.21	1.80	85.48	1.93
Mortality										
eGFR	69.97	2.35	29.47	2.26	26.69	2.05	26.99	2.09	27.26	2.19
AER	71.56	2.23	71.16	2.19	69.83	2.18	68.56	2.25	67.46	2.36
Urinary L-FABP	68.81	2.31	68.74	2.22	67.92	2.18	66.39	2.82	65.20	2.39
TRF	81.68	1.71	82.35	1.62	83.56	1.51	83.59	1.54	84.26	1.63
TRF+eGFR	81.64	1.79	82.31	1.70	83.88	1.55	83.87	1.56	84.51	1.63
TRF+AER	82.78	1.72	83.57	1.63	84.49	1.52	84.48	1.53	84.73	1.63
TRF+L-FABP	83.06	1.65	83.78	1.56	84.77	1.45	84.60	1.49	84.77	1.60
TRF+eGFR+AER	82.60	1.75	83.40	1.65	84.52	1.52	84.53	1.54	84.82	1.62
TRF+eGFR+L-FABP	82.88	1.69	82.60	1.59	84.83	1.46	84.61	1.50	84.80	1.60
TRF+AER+L-FABP	83.06	1.69	83.87	1.59	84.84	1.48	84.75	1.51	84.90	1.61
TRF+eGFR+AER+L-FABP	82.97	1.70	83.81	1.60	84.88	1.48	84.78	1.51	84.94	1.61

TRF – traditional risk factors model; The abreviation of the main models used in the study are presented in the legend of Table 33.

Urinary L-FABP's average AUC for prediction of mortality when compared with eGFR ($p = 0.37$) or with AER ($P = 0.82$) was similar (Table 32). Addition of urinary L-FABP to the model comprising the TRF added a significant clinical benefit for prediction ($p = 0.001$) and the increment was like the one of AER ($p = 0.76$) (Table 33). Notably, the average diagnostic performance of urinary L-FABP for prediction of mortality was much better when compared with other well-known cardiovascular risk factors (Table 32) (450).

Urinary L-FABP's clinical diagnostic performance variation for prediction of stroke and mortality was also assessed using time dependent ROC curve analysis at quintiles of follow-up time (Table 34 and Figure 18). For both outcomes of study IV and at any time point urinary L-FABP was no better than AER or eGFR, while TRF was at all time points superior to urinary L-FABP, AER or eGFR . Urinary L-FABP used on top of TRF, eGFR and AER provided no added diagnostic benefit for prediction of stroke or mortality.

When NRI and IDI for logistic model were calculated, there was no added reclassification benefit on top of AER or eGFR for any of the two outcomes in study IV (Table 35).

When NRI and IDI generalized for survival data were calculated, the largest reclassification benefit for stroke was observed on top of TRF at 5 years ($NRI_{3\text{-}S5}$ = 14.6%). However, there was no reclassification benefit of adding L-FABP on top of AER and/or eGFR (Table 35).

Table 35. Urinary L-FABP added predictive benefit.

Models	$NRI_{3\text{-}lm}$	SE	p	$NRI_{3\text{-}S5}$	$NRI_{3\text{-}S10}$	IDI_{lm}	SE	p	IDI_{S5}	IDI_{S10}
Stroke										
TRF+L-FABP *vs* TRF	0.179	0.050	< 0.001	0.146	0.073	0.013	0.004	0.001	0.026	0.041
TRF+ eGFR *vs* TRF	0.010	0.040	0.01	0.040	0.061	0.008	0.002	< 0.001	0.006	0.005
TRF+AER *vs* TRF	0.062	0.028	0.03	0.044	-0.132	0.008	0.001	< 0.001	0.011	0.022
TRF+L-FABP+eGFR *vs* TRF+eGFR	0.076	0.033	0.04	-0.156	-0.027	0.007	0.003	0.02	0.020	0.035
TRF+L-FABP+AER *vs* TRF+AER	0.073	0.032	0.02	-0.057	-0.100	0.005	0.002	0.01	0.011	0.022
TRF+eGFR+AER+L-FABP *vs* TRF+eGFR+AER	0.045	0.027	0.10	-0.053	-0.101	0.005	0.002	0.01	0.010	0.022
Mortality										
TRF+L-FABP *vs* TRF	0.074	0.027	0.005	0.203	0.078	0.043	0.007	< 0.001	0.036	0.041
TRF+eGFR *vs* TRF	0.086	0.040	0.03	-0.026	0.056	0.007	0.002	< 0.001	0.003	0.005
TRF+AER *vs* TRF	0.014	0.020	0.47	-0.064	-0.057	0.006	0.002	< 0.001	0.004	0.022
TRF+L-FABP+eGFR *vs* TRF+eGFR	0.064	0.023	0.006	-0.226	-0.051	0.028	0.005	< 0.001	0.031	0.035
TRF+L-FABP+AER *vs* TRF+AER	0.026	0.020	0.20	-0.218	-0.099	0.010	0.003	0.001	0.024	0.022
TRF+eGFR+AER+L-FABP *vs* TRF+eGFR+AER	0.027	0.019	0.16	-0.239	-0.089	0.009	0.003	0.004	0.023	0.021

$NRI_{3\text{-}lm}$ - net reclassification index with 3 thresholds for logistic model; $NRI_{3\text{-}S5}$ - net reclassification index with 3 thresholds generalized for survival data (calculated for 5 years survival); $NRI_{3\text{-}S10}$ - net reclassification index with 3 thresholds generalized for survival data (calculated for 10 years survival); IDI – integrated discrimination improvement; IDI_{S5} - integrated discrimination improvement generalized for survival data (calculated for 5 years survival); NRI_{S10} - integrated discrimination improvement generalized for survival data (calculated for 10 years survival); 95% CI – 95% confidence interval; *p* –statistical significance; TRF – traditional risk factors model; TRF+eGFR – Cox model formed by TRF and eGFR used together; TRF+AER – Cox model formed by TRF and AER used together; TRF+L-FABP – Cox model formed by TRF and urinary L-FABP used together; TRF+eGFR+AER – Cox model formed by TRF together with eGFR and AER; TRF+eGFR+L-FABP – Cox model formed by TRF together with eGFR and urinary L-FABP; TRF+AER+L-FABP – Cox model formed by TRF together with AER and urinary L-FABP; TRF+eGFR+AER+L-FABP – Cox model formed by TRF together with eGFR, AER.

5.4.3. L-FABP and causality for stroke or mortality

Causality could not be assessed because the first assumption of the Mendelian randomization analysis was not fulfilled. For further details please see the Study I.

6. DISCUSSION

6.1. Methodological evaluation – strengths and weaknesses of the studies

6.1.1. Outcome measures used in the studies

6.1.1.1. DN progression based on AER – early stages of DN

In studies I, II and III the individuals with T1DM were classified as "incipient" DN (microalbuminuria), clinical overt DN (macroalbuminuria) and ESRD, while passage to a higher stage was considered as progression of DN. The AER-based progression of DN, however, has some limitations, both at the early and the late stages of the disease. At the early stages DN progression is based on AER. The AER increase over time usually mirrors the DN progression towards ESRD for the majority of affected individuals (88). Some, however, can progress to ESRD without having any increase at all in AER (94, 451, 452).

An increase in AER is mainly regarded as a reflection of progression of glomerular injury (453). Consequently, any attempt to predict such progression by novel tubular biomarkers has 3 practical problems. First, the prediction of glomerular injury by using biomarkers of tubular injury implies a relationship between the two apparently independent processes. Second, when the outcome is progression of DN, based on AER change to a higher category, it is difficult for any other biomarker to outperform the gold standard outcome, the AER. Third, by using the AER-based progression of DN as an outcome we are practically testing our biomarkers for prediction of only one phenotype of DN. This is problematic, since it is well-known that DN has two phenotypes – one phenotype based on AER and the other based on eGFR decline – and they do not fully overlap (96, 108).

Considering these aspects, prediction of AER-based progression of DN using novel biomarkers should be of even higher clinical value, if the prediction would be independent of AER. In addition, a biomarker that does not predict AER-based progression of DN independently of AER may have a glomerular component or alternatively may reflect a tubular component of AER. Finally, a biomarker that does not predict AER-based progression of DN may still predict progression of DN based on the slope of the renal function decline.

One option to overcome these limitations would be to also test the other phenotype of DN, which is based on the decline of eGFR. This phenotype may better reflect the ultimate outcome of DN than AER does. Unfortunately, the slopes of renal decline were not yet available at the time of this analysis. However, even if this second phenotype of DN progression was not tested, the results are still relevant, considering that the AER-based progression of DN is widely used in clinical practice.

6.1.1.2. DN progression based on ESRD outcome – the late stage of DN

In studies I, II and III, progression to ESRD was considered when the individuals with T1D started dialysis or received a kidney transplant (430, 431, 454). The start of dialysis is widely used in clinical research and considered to be a hard endpoint. However, the timing of dialysis initiation is variable, being a complex interplay between at least 3 interrelated and dynamic processes (physician practices and local guidelines, acute medical episodes or procedures and patient–physician dynamics) (455, 456). Similarly, kidney transplantation is also a hard endpoint, but again the timing of transplantation may vary, being influenced by age, access to transplantation, type of transplant procedure and organ availability (457, 458). Thus, even if both are relevant outcomes, the time to the event may vary in both cases. Therefore, the reflection of the clinical or biochemical status of kidney function may not be accurate when considering these two outcomes, because of the variable time-to-event information (459).

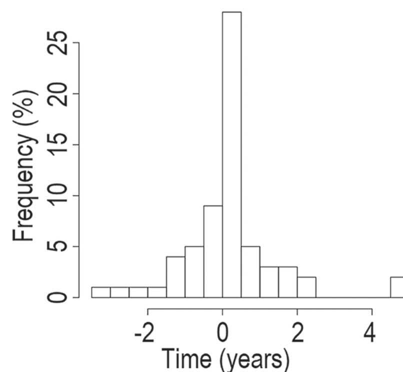

Figure 18. Histogram of the time differences in years between the time of ESRD and the time of the first eGFR value < 10 ml/min/1.73m^2 in individuals with macroalbuminuria at baseline from the FinnDiane Study.

These notions should be kept in mind when interpreting the results regarding the prediction of progression at the late stages of DN. On the other hand, progression at these late stages of DN should be tested using other potential outcomes that better reflect the kidney function. The need for other outcomes is confirmed by partial *posthoc* analysis of the FinnDiane participants with macroalbuminuria at baseline. The analysis of the macroalbuminuric individuals with regard to the time between the occurrence of the first eGFR value lower than 10 ml/min/1.73m^2 and the time of ESRD outcome showed important differences (Figure 18). Approximately 15% of the subjects reaching ESRD had an eGFR below 10 ml/min/1.73m^2 at least 6 months prior to the ESRD, while 5% had an eGFR below 10 ml/min/1.73m^2 at least one year before ESRD. In contrast, in 10 to 15% of the subjects the decision to start dialysis more than 6 months earlier was probably not because of an eGFR below 10 ml/min/1.73m^2. Only in about 35% of the macroalbuminuric individuals who reached ESRD was the dialysis started ± 6 months from the first eGFR value being below 10

ml/min/1.73m^2. Other outcomes such as the decline in renal function also predict progression to renal insufficiency in individuals with DN (93).

Despite its limitations, however, the definition of the ESRD outcome as used in studies I, II and III was still considered a hard clinical outcome as widely accepted in observational and clinical trials. Furthermore, at the time of publication of these papers the data on the slopes of renal function decline in the FinnDiane study were not available.

6.1.1.3. CVD and mortality outcomes

The cardiovascular and mortality outcomes were obtained by linking the FinnDiane database with two national registries – the Finnish HDR and the Finnish CDR. The Finnish HDR has a high level of accuracy and completeness of the collected cardiovascular data (460). The Finnish CDR also reflects the mortality statistics accurately (461). It is worth noting that linking of the FinnDiane database with these two registries had previously been done and validated (462).

6.1.2. FinnDiane data collection and follow-up

The FinnDiane Study represents a high-quality sample collection and is at the moment probably one of the largest collections of data on T1DM with and without DN in the world. The number of individuals carefully characterized exceeds 8000, and blood, serum, plasma, urine and DNA samples are available for research purposes.

The collection system for the FinnDiane database is provided by the BC Platform, a world leader in providing powerful data management and analysis solutions for biomedical research and healthcare information technologies (http://www.bcplatforms.com/).

Follow up of the participants is done by careful review of the medical records or by using extensive data from the Finnish registries (comorbidities, cardiovascular and renal events, causes of death, use of medications).

All the characteristics above (large number of participants, long follow-up and high-quality phenotypic characterization) are considered important strengths of this study.

6.1.3. Samples, storage and biomarker measurement and biovariability

One potential limitation may be the fact that the samples were collected from 1997 onwards, while the actual biomarkers were not measured until 2009. During this time, however, all samples were stored at stable -20°C conditions. The quality and stability of the storage was verified by comparing the baseline urinary creatinine concentration with the urinary creatinine concentration at the time the biomarkers were measured, with a 0.99 correlation coefficient.

To our knowledge there are no studies regarding the effect of storage on urinary ADP variability. However, storage at -30°C for 33 months or three cycles of freezing/

defreezing had no discernible effect on serum adiponectin levels (463). It is worth mentioning that in the present study it was not possible to measure the various urinary ADP isoforms, but previous studies have shown that the high molecular weight (HMW) ADP is the main Urinary ADP isoform in individuals with diabetes (424, 464).

Regarding the urinary L-FABP and KIM-1 stability, we found only one study with different processing conditions at sample collection or during storage. This study showed that both urinary biomarkers have high short-term stability, independent of storage or processing protocols (465).

However, all three urinary biomarkers are peptides, which can be compared for example to C-peptide. C-peptide was much more stable in urine than in the serum after 72h at room temperature or after storage, due to the absence of proteases in urine (466).

Another potential limitation could be that the biomarker values are based on a single measurement from a 24h urine sample collected at baseline. However, there have been no studies so far regarding the biovariability of the three studied urinary biomarkers. In addition, regarding their serum concentrations the available studies suggest minimal short- and long-term variability (467, 468). To decrease the potential variability of the studied biomarkers, all measured values were normalized with urinary creatinine (even if the samples were measured from 24h urine collections).

Since there are studies showing that the concentrations of these biomarkers can be influenced by the presence of leukocyte esterase, nitrite, hematuria or proteinuria in the tested urine sample, a concomitant urine sample for urinalysis was also collected and tested (468). If in this second urine sample there was any sign of infection or acute abnormality, additional tests were performed, and sampling was repeated after proper treatment.

Finally, since no critical variability of the tested biomarkers has been described and measures were taken to ensure similar sampling conditions, additional measurements would only strengthen the results.

6.1.4. Generalizability (external validity), internal validity (study power)

The FinnDiane Study is a large multicenter cohort from more than 80 centers comprising almost 25% of the individuals with T1DM in Finland. The large number of individuals enrolled, together with the nationwide spread of the study, which mirrors the distribution of the Finnish population, makes this study representative of the Finnish population with T1DM.

Given the unique genetic characteristics of the Finnish population, however, the results from this study may need to be replicated in other populations before they can be considered globally generalizable.

We conducted these studies without determining the sample size and study power necessary for the study, but we were indeed using all available samples for the biomarker measurements, as well as the complete follow-up data available at

the time of the studies. However, in order to provide a correct interpretation of the results and to avoid any pitfalls regarding negative results, we tested the power of each study in a post-hoc analysis (469). Since our studies could not always reject the null hypothesis with a power of 0.8, the negative results from these studies should be interpreted with caution. Thus, even stronger emphasis needs to be placed on the positive results and the predictive ability of the studied biomarkers.

6.1.5. Developments in statistical methods for evaluation of biomarkers

In recent decades the statistical methods for the analysis of biomarkers have evolved from binary *cross-sectional outcomes* to time to event analyses. In addition, given the multitude of biomarkers, additional indexes have been developed and statistical tests have gone from discriminant analysis to regressions and then to survival and competing risk analyses (435, 470, 471).

Over the last 10 years, time-to-event analysis has come to be widely used for the evaluation of biomarker *prediction ability*, although other statistical tests also have become necessary. Initially, the ROC analysis and C-statistics were necessary for *discrimination* between individuals with and without events for a certain biomarker (472). The ROC curve analysis was adapted for survival data as an average and at different time-points (473, 474). Then, in another step in the evolution, due to the multitude of biomarkers the *reclassification* analysis was required to evaluate the added reclassification benefit of a new biomarker compared with the current gold standard. Thus, new indexes have been published for logistic models such as the NRI and IDI (438, 475). Furthermore, adaptations of these indexes have also been developed (continuous NRI, IDI generalized for survival data, NRI generalized for survival data, etc.) to overcome the limitations of the initial indexes in relation to the time-to-event analysis (476). Despite all these adaptations and generalizations, the debate is still ongoing, as to whether they are appropriate for the assessment of a biomarker's added benefits (477).

The developments in the statistical methods used in studies I to IV took place during the 5-year period when our studies were conducted. Therefore, the latest adaptations of these statistical methods were used in study IV. Additional calculations of all the indexes according to the procedures in study IV have also been done in this thesis. It should, however, be pointed out that even if the additional indexes for the prediction and reclassification were calculated in order to provide a uniform presentation of the data in this thesis, the results did not change to any significant extent, thereby underlining their robustness.

The latest development in biomarker assessment beyond the added reclassification benefit is the *evaluation of causality*. Causality can be evaluated either by a randomized controlled trial (intervention with a drug that influences the biomarker level) or by a MR approach, which was done in studies I–IV.

The MR can be considered a "natural" form of a randomized controlled trial: the subjects are randomly allocated before birth to different "treatment groups" (i.e., different concentrations of a biomarker) according to their genotype and followed

up from birth until the moment of the outcome measurement (i.e., baseline visit in these studies). If the biomarker is causal, then the individuals with low levels of the biomarker should have a different outcome compared with those with high levels of the biomarker.

The basic assumption of the MR analysis, that a genetic variant(s) (instrument, IV) modifies the exposure to a certain risk factor (modifiable exposure = biomarker of interest) by changing its levels, unrelated to any confounding factors, has largely been accepted (478).

However, the "lifetime effect" interpretation commonly seen in MR studies has been challenged by some researchers (479, 480). They claim that a lifetime effect is not statistically justified in the case of time-varying exposures or nonlinear relationships between the IV and the exposure. To exclude a non-linear relationship between our instrument and urinary KIM-1 we performed, in addition to verification of the linear regression assumption and after the publication of the manuscript, the Ramsey Regression Equation Specification Error Test (RESET) to see whether polynomial terms are needed. At RESET test, no unlinear relationship was seen between rs2036402 and urinary KIM-1 ($p = 0.19$) or between urinary KIM-1 and eGFR ($p = 0.09$). Consequently, in our study there is no time-varying exposure and no need to specify a period average causal effect.

In addition, reverse causation as another potential bias in the MR studies was also suggested (481). However, for many reasons it may not be possible in a MR study for the variant-outcome association to suffer from reverse causation (random allocation of genetic variants during meiosis, time does not flow backwards, etc.). However, the reverse causation between the outcome variable (eGFR) at an earlier timepoint or a proximal precursor of the outcome (AER) and the risk factor (urinary KIM1), which can bias estimates of the effect of the risk factor on the outcome, may not be excluded. To overcome this potential bias we adjusted the final analysis for AER and the causality was still valid. Unfortunately, we had no data regarding eGFR at birth in order to completely exclude this criticism, but there are unlikely to have been important eGFR differences at birth in this study since all patients with kidney disease from other causes except diabetes were excluded from the study. Moreover, the reverse causality is quite improbable in this case, since in the pathophysiological pathway the increase of urinary KIM-1 concentrations appears before eGFR decline.

However, the MR analysis may fall into a series of analytical traps such as using a weak IV, pleiotropy, linkage disequilibrium, etc., mainly because of the large volume of genetic variants and exposures available. It is attractive to compute genetic risk scores to better explain the phenotype and to increase the power of the study, but when these risk scores are not completely understood, the risk of pleiotropy and other inferential complications increases (482). To avoid these problems, the option used here was to test a single genetic variant, to analyze its independence from other SNPs and to verify any potential pleiotropy. In study III we took all these precautions into account to avoid any likely pitfalls.

Although study III probably represents one of the largest studies regarding the DN progression analysis, it may still be considered small for a MR analysis. This is, however, compensated by accurate clinical measurements together with a strong

IV, more than sufficient for our sample size, as proved by the large F-statistic values. Additionally, to increase the precision of our IV and to reduce any probable instrument bias, we adjusted the 2SLS analysis with other measured covariates (diabetes duration, HbA_{1C} and AER), which are not on the causal pathway between the exposure and the outcome, but which could explain the variation of the outcome.

6.1.6. Other aspects of the statistical analysis

One limitation of the studies regarding the urinary L-FABP may be that we had no data on anemia. Anemia may have been present already at the early stages of DN and could increase urinary L-FABP, if it is severe enough (32,33). However, severe anemia at least was not an issue in these studies, since no subject was receiving erythropoietin or any other treatment for anemia, and all participants with ESRD at baseline were excluded.

One limitation in study II on adiponectin could be that there were some gender differences in the urinary ADP concentrations. However, to diminish the influence of gender on the Cox regression analysis, all results are presented adjusted for gender.

Despite all the potential limitations described above, these do not influence the results or their interpretation to any significant extent, while on the other hand the multiple strengths of the studies make them unique, reliable and highly representative of the Finnish population, thus driving the possibility of replication in other populations.

6.2. Clinical implications and interpretation of the results

There are two important findings unveiled by these studies. First, the tubular biomarkers predict the progression of DN at different stages, and KIM-1 is very likely to play a causal role in the loss of kidney function, broadening the glomerulocentric view of DN. The movement from a glomerulocentric view of DN progression to a more global view involving also the tubular function is based on the following results: (1) Urinary L-FABP predicted the AER-based progression of DN at all stages, while urinary adiponectin predicted only the progression to ESRD. (2) Urinary KIM-1 very likely plays a causal role in the loss of kidney function, independently of AER and HbA_{1C}.

Second, the tubular biomarkers may also reflect cardiovascular disease development. This statement is based on the following results: (1) Urinary L-FABP is a predictor of stroke, but not the other cardiovascular outcomes such as CAD, PVD or CVD. (2) Urinary KIM-1 predicted PVD and CVD, while urinary ADP predicted only CVD.

6.2.1. Study I – Urinary L-FABP prediction of DN progression

This study showed that, at any AER-based baseline stage of DN, urinary L-FABP was an independent predictor of progression of DN. This prediction of DN progression

was, however, no better than that of AER at any stage. Moreover, the prediction did not improve when urinary L-FABP was used together with AER.

In previous studies in individuals with T1DM, when analyzed as quartiles, urinary L-FABP was suggested to be a predictor of progression to microalbuminuria (408). Thus, our data confirm that L-FABP predicts the progression of DN at this early stage and may be important for the individuals with normal AER, which is the stage where there is no biomarker or algorithm to estimate the risk of onset of microalbuminuria.

In addition to the prediction of AER-based progression (progression of glomerular damage), urinary L-FABP has previously been shown to be closely associated with structural and functional tubular kidney damage (405, 483). Such a link between tubular injury and the early AER-based progression of DN could be a reason to use a biomarker panel reflecting both structural and functional changes due to DN in the clinic at this early stage.

Interestingly, in the microalbuminuria group, a high urinary L-FABP independently predicted a higher risk of progression to macroalbuminuria [HR = 1.40; 95%CI (1.10 – 1.79)], before the adjustment with AER. After adjustment with AER, however, a high urinary L-FABP predicted a surprisingly lower risk of progression [HR = 0.67; 95% CI 0.47 – 0.95)]. This difference in the risk of progression may have a few potential explanations. One could be the lower statistical power in this group (46 progressors). In the individuals with microalbuminuria compared with the other groups, a stronger correlation between AER and L-FABP (r = 0.49) was seen, which could be another potential explanation. These two alternatives may not, however, explain why urinary L-FABP was as an independent predictor in the first place. Since urinary L-FABP lost its predictive ability after the adjustment for ACE inhibitors or for any antihypertensive medication, a third explanation may be that the effect, seen only in the microalbuminuria group, is an effect of medication. Such an effect is no surprise, as treatment with ACE inhibitors reduces the AER and/or urinary L-FABP levels and thereby influences the progression of DN (405). Last but probably the most attractive explanation may be that L-FABP plays a protective role against the tubular stress induced by FFA overload triggered by elevated AER, as others have suggested (484).

Finally, it is very important to highlight that, in study I, urinary L-FABP predicted progression at all stages of DN. This prediction, in parallel with the worsening of the AER-based stage of DN, was due to the continuous increase in the urinary L-FABP concentrations, an observation also confirmed in individuals with type 2 diabetes (485).

The predictive accuracy in the ROC curve analysis, at all stages and timepoints, was similar to that for AER. An explanation for the similar accuracy could be the AER-based definition of DN progression. Using an AER-based definition of DN makes it very difficult for any other variable to outperform the gold standard (in this case the AER change), when adjusted for that gold standard variable. Despite the adjustment for the outcome, it is important to underline that urinary L-FABP predicted the progression at all stages independently of AER and with similar accuracy.

Another important aspect is that even if the accuracy was similar in the reclassification analysis, urinary L-FABP, on top of the basic models and AER, added

significant reclassification benefit, but only in the individuals with normal AER. This reclassification benefit, seen only in those with normal AER, may have clinical implications, since these individuals have no other signs of kidney damage, while some of them may have the rather rare condition, non-albuminuric DN, observed in only 2% of individuals with T1DM (94). Thus, a future perspective for the use of L-FABP in clinical practice may be real, if replication appears in other populations. Meanwhile, it is noteworthy that L-FABP has already been promulgated by the Ministry of Health, Labour and Welfare in Japan as a new tubular biomarker for use in clinical practice (486).

6.2.2. Study II – Urinary ADP and DN progression

Study II showed that urinary ADP was not a predictor of disease progression independent of AER at the early stages of DN. In contrast, at the late stages such as macroalbuminuria, urinary ADP was a strong and independent predictor of progression to ESRD. In addition, the prediction of progression to ESRD improved in the individuals with macroalbuminuria at baseline, when urinary ADP was used together with AER or eGFR.

Urinary ADP predicted the progression from normal AER to microalbuminuria and from microalbuminuria to macroalbuminuria independently of the basic models of progression, but not independent of AER. An explanation for this could be the interaction between urinary ADP and AER, which might reflect that these two molecules share common pathophysiological mechanisms or common soil triggering their increased concentrations. These pathophysiological similarities are discussed in a later section of the discussion – pathophysiological implications of the results. Another explanation for the lack of independent prediction may be that albuminuria is also an important predictor of progression as well as an outcome at these early DN stages (487). Thus, adjusting for the outcome and the strongest predictor may naturally lead to the lack of independent prediction of AER-based progression of DN.

However, the main observation was that urinary ADP was an independent predictor of progression to ESRD. This finding was due to an increase in urinary ADP in parallel with AER, which was also observed in a small previous study that unfortunately did not explore the association further (427). The present study II went further and showed that urinary ADP predicted the progression to ESRD independently of the basic progression model, AER, eGFR, urinary L-FABP, urinary KIM-1 and serum ADP. This independent prediction is even more interesting, since all variables were also associated with the urinary ADP concentrations, suggesting that urinary ADP captures also other mechanisms of DN progression.

Thus, urinary ADP predicted progression to ESRD, was a better predictor than AER, and added significant clinical benefit to the prediction of progression to ESRD when used together with eGFR or AER. From the clinical point of view, assessment of urinary ADP, on top of AER or eGFR, may help assessment of DN progression at this stage, given the well-known limitations of AER or eGFR (77, 78, 451).

Biomarker comparison regarding IDI and NRI with other studies may be difficult because of the lack of comparable data. Despite these inconveniences, urinary ADP

had IDI and NRI data comparable to another promising biomarker, namely the soluble tumor necrosis factor alpha receptor 1 (sTNFαR1) (311). The added clinical benefit of urinary ADP regarding the progression to ESRD may also be related to the fact that in this group urinary ADP probably captures more mechanisms than any other biomarker.

The fact that urinary ADP is a strong predictor of progression to ESRD is well correlated with the loss of eGFR over time. Given that eGFR loss at the earlier stages of DN represents a new paradigm of disease progression, urinary ADP may also be a promising biomarker for the prediction of eGFR-based progression at the earlier stages of DN, but further studies are needed to confirm this hypothesis (93, 488).

6.2.3. Study III – Urinary KIM-1, DN progression and loss of eGFR

Study III showed that urinary KIM-1 was not an independent predictor of AER-based progression of DN at the early stages in individuals with T1DM and with normal AER or microalbuminuria at baseline. Urinary KIM-1 was also not an independent predictor of DN progression to ESRD, after adjustment for AER. Furthermore, KIM-1 showed no prognostic benefit beyond AER or eGFR. However, study III did demonstrate a strong genetic impact on the urinary KIM-1 concentrations and an independent causal relationship between urinary KIM-1 and lower eGFR at study enrolment.

Study III is one of the largest studies in individuals with T1DM, which assesses the predictive value and the clinical benefit of urinary KIM-1 for the prediction of AER-based progression of DN. In individuals with T1DM, two previous small studies on urinary and plasma KIM-1 showed contradictory results regarding its predictive capability for DN progression (419, 489). In individuals with T2DM, KIM-1 predicted the decline of eGFR in unadjusted analysis, but not the progression to macroalbuminuria, whereas the progression to ESRD was not evaluated (418). Given these previous discordant data, the observation that urinary KIM-1 did not predict either early AER-based progression of DN or late progression to ESRD independently of AER is an important finding. This is surprising, since urinary KIM-1 has been considered to be a sensitive and specific marker of proximal tubular damage, and tubulo-interstitial damage has been proposed to be one of the final pathways leading to ESRD (490).

In study III, the association between urinary KIM-1 and lower eGFR seen in the linear regression analysis was also not independent of AER. The explanation for this could be a strong interaction between urinary KIM-1 and AER. Such a strong correlation was seen in our study and also confirmed by others (490). An alternative explanation may be the strong genetic determination of the urinary KIM-1 concentrations. This was demonstrated in study III, which showed a strong and independent genetic impact on the urinary KIM-1 concentrations.

Based on the genetic determination, the subsequent MR analysis showed a causal relationship between the increased urinary KIM-1 concentrations and low eGFR, with one-unit change of ln(KIM-1) being associated with a -5.0 to -6.8 ml/min/1.73m^2 decrease in eGFR. Importantly, the causality was independent of diabetes duration, glycemic control or AER. Such an independent causal effect of urinary KIM-1 on

the loss of renal function is an interesting finding, since the observational study showed no independent predictive ability of urinary KIM-1 as to DN progression. Given this independent causal relationship, the genetic impact on the urinary KIM-1 concentrations may be the explanation for the inability to predict DN progression in the observational studies, which do not take into consideration the effect of the genes on the urinary KIM-1 concentrations.

The effect of AER, the importance of the genetic background as well as the influence of other confounding factors on the relationship between urinary KIM-1 and eGFR are confirmed by the differences between the observational estimators from the linear regression analysis and the IV estimates. The effect size in the simple linear regression was similar to the IV estimator, but was no longer significant after adjustment for AER, whereas the IV estimator remained robust even after adjustment for AER. Furthermore, in the MR analysis the AER adjusted estimators were different, with constantly smaller observational estimators and increasing endogeneity. There are three possible explanations for the differences between these estimates. The first one could be that a lifetime effect of urinary KIM-1 on eGFR is more evident in the IV analysis, and may be attenuated in a standard regression using a single measurement of urinary KIM-1, AER or eGFR. It is possible that a single measurement of the biomarker and the outcome may not capture the total effect of urinary KIM-1 on the eGFR. The second explanation could be negative confounding, since further adjustments for RAAS-inhibitor treatment or any other antihypertensive medications decreased the endogeneity (data not shown) between the estimators. The third likely explanation may be based on the tubulo-glomerular feedback theory, which may suggest a more complex relationship between eGFR and urinary KIM-1 that is mediated through AER with potential reverse causality (490).

Finally, one important fact should be emphasized – prediction is not the same as causality. While for a biomarker the predictive value can be inferred using a prospective study and Cox regressions or Fine-Gray competing risk analysis, the causality can be confirmed either using a randomized controlled trial or by a MR approach (if all assumptions for this type of study are fulfilled). In study III, Cox regression was used to assess the predictive value of urinary KIM-1, and it was shown that urinary KIM-1 was not independent of AER when the progression of DN was based on AER. On the contrary, the causality for the loss of kidney function (eGFR) was claimed based on the MR study with a 2SLS analysis, where the urinary KIM-1 effect was independent of diabetes duration, HbA_{1C} or AER, all being important confounders in the observational analysis. Unlike the observational studies, the MR analysis is a robust, "free of bias" or "free of confounders" analysis similar to the "gold standard" for drug evaluation in clinical practice – the randomized controlled trials. In an MR analysis the subjects are randomly allocated to their genotype groups. This allocation (randomization) takes place before birth, much earlier than the potential actions of any confounders (e.g., AER, HbA_{1C}, etc.). Then if urinary KIM-1 is causal for the loss of eGFR, years later a difference in the outcome (eGFR) will be observed between the subjects with high, intermediate and low urinary KIM-1 concentrations. Given these characteristics, the MR analysis effect size is a very robust one that should provide a true estimate of the causal effect of urinary KIM-1 on eGFR, even in the presence of confounders.

From the clinical point of view these results are important since they show that, despite the complex relationship between urinary KIM-1, AER and eGFR, the association of urinary KIM-1 with lower eGFR represents a causal relationship. In addition, this study is an excellent example why a causal biomarker may not be useful in clinical practice for the prediction of AER-based DN progression. One solution to this could be to also consider the effect of the genotype to predict the progression. A second option could be to consider another phenotypic characteristic of the progression that is less confounded by AER, such as the eGFR loss over time (93). Studies performed after the publication of our study III confirmed the independent predictive abilities of the urinary or the serum KIM-1 on the loss of eGFR (491-493). Nevertheless, the complex relationship between urinary KIM-1, AER and eGFR reflecting tubular injury, glomerular damage and kidney dysfunction is another strong argument that DN is a multifactorial disease and is more than just glomerular damage.

6.2.4. Study IV – Urinary L-FABP, macrovascular complications and mortality

In study IV, of the five outcomes tested, urinary L-FABP was an independent predictor of incident stroke and mortality. For these two outcomes, urinary L-FABP was a better predictor than well-known cardiovascular risk factors (such as LDL cholesterol, HDL cholesterol, HbA_{1C} or SBP) and was as good a predictor as eGFR or AER. However, urinary L-FABP did not predict the other tested cardiovascular endpoints (CAD, PVD and overall CVD events), when adjusted for AER, while for all outcomes except stroke the AER was a strong predictor independent of L-FABP.

Since only a marginal association between a SNP in the *FABP1* gene and stroke has been reported previously, the urinary L-FABP's independent prediction of stroke is a novel finding (494). In study IV, urinary L-FABP not only predicted stroke independently of known risk factors such as age, sex, BMI, diabetes duration, SBP, triglycerides, HDL-C, glycemic control and proliferative diabetic retinopathy, but the prediction was also independent of eGFR and AER.

Another important finding in study IV was that urinary L-FABP was an independent predictor of all-cause mortality. Its predictive ability was suggested in a previous study by unadjusted analysis, but that study did not explore this relationship further (408). Compared with the earlier study, in study IV, urinary L-FABP predicted mortality independently after multiple adjustments with well-known cardiovascular risk factors as well as after additional adjustments for eGFR and AER.

Since urinary L-FABP predicted only two of the five outcomes, one can argue that these findings are just a statistical artefact. However, this is not the case, considering that we applied the most rigorous statistical analysis. First, adjustments were taken into account in the most extended statistical model, including all well-known risk factors for cardiovascular outcomes. Second, before claiming the results, we applied Bonferroni correction to all tested outcomes, which is the most stringent correction for multiple testing. Given this stringent statistical analysis, and until other studies are performed with a power above 0.80, we have reason to believe that urinary L-FABP predicts at least stroke and mortality if not all cardiovascular outcomes.

One probable reason for the different predictive ability of urinary L-FABP in the case of CAD and PVD compared to stroke may be related to the way these outcomes are defined, as well as their slightly different pathophysiology. However, the definitions of outcomes have been used before and demonstrated to be reliable, and they are based on data from high quality national registries as well as scrutiny of the medical files. These are good reasons to claim that urinary L-FABP is indeed an independent predictor of stroke and all-cause mortality in Finnish individuals with T1DM.

Urinary L-FABP did not add any benefit on top of the full models, but it is important to note that study IV was not able to differentiate between ischemic and hemorrhagic stroke. Still, the clinical value of this finding is emphasized by the fact that urinary L-FABP was a better predictor of stroke than the traditional risk factors and was as good as eGFR or AER in the ROC curve analysis. In addition, recently published data have confirmed that other FABPs are also strong independent predictors of stroke and able to improve the currently used risk stratification protocols for the identification of ischemic stroke (495, 496). Thus, urinary L-FABP may also be a useful tool for etiological risk stratification of stroke, though unfortunately this study was not able to explore this hypothesis.

When NRI was calculated, adding urinary L-FABP to the model of traditional risk factors enabled correct reclassification of 17.9% of the individuals, compared with only 10% and 6% provided by eGFR and AER, respectively. However, when NRI and IDI were evaluated, adding urinary L-FABP on top of AER or eGFR did not improve the long-term prediction of stroke. As in the case of stroke, urinary L-FABP was as good a predictor of all-cause mortality as eGFR or AER. Again, added on top of traditional cardiovascular risk factors, urinary L-FABP correctly reclassified 7.5% of subjects, compared with 8.6% and 1.4% for eGFR and AER, respectively.

6.3. Pathophysiological perspective of the results

6.3.1. Urinary L-FABP

6.3.1.1. Perspective on DN progression

When baseline staging of DN and progression were based on AER, urinary L-FABP predicted the progression at all stages. Urinary L-FABP's predictive ability is a consequence of the continuous increase in the urinary L-FABP concentrations alongside the worsening of kidney disease. This increase has not been completely explained, but may reflect different mechanisms involved at each stage of the DN development.

In individuals with normal AER, hyperglycemia triggers oxidative stress and activation of the intrarenal RAAS (497, 498). Increased oxidative stress and RAAS activation lead to further vasoconstriction and hypoxia (165, 499). Hypoxia, in turn, triggers L-FABP gene expression in the tubular cells and potentially in other

renal tissues as well, thereby increasing the urinary L-FABP concentrations (397). Another source of urinary L-FABP may be its expression in other tissues (e.g., liver) (500). Such extrarenal expression could be followed by increased L-FABP serum concentrations and subsequent urinary elimination. Passage through the glomerular barrier is possible due to L-FABP's low molecular weight (14.2 kD), which is much smaller than that of albumin (66 kD) (500). The L-FABP prediction of the onset of microalbuminuria is independent of AER. This means that the L-FABP increase at this stage is related more to tubular dysfunction (probably due to hypoxia) than to glycemic control or glomerular damage, since urinary L-FABP was poorly correlated with both the HbA_{1C} ($r = 0.06$ in non-diabetic subjects; $r = 0.11$ in individuals with T1DM and normal AER) and the AER ($r = 0.15$).

Albumin is bound to fatty acids when it passes the glomerular barrier. Thus, microalbuminuria may trigger fatty acid overload in the proximal tubules. The L-FABP expression in the tubular cells may therefore be further elevated, with simultaneous increase of free fatty acid transport into the mitochondria, when microalbuminuria appears (501, 502). On the other hand, at the microalbuminuric stage the L-FABP passage through the glomerular barrier becomes more probable. Thus, its expression in other tissues that elevate serum L-FABP concentrations could become more important for its urinary concentrations. Urinary L-FABP could accordingly be influenced by a high fat diet, non-alcoholic fatty liver disease, insulin resistance or gut permeability, all factors associated with the serum L-FABP concentrations (503-506).

Interestingly, the most important determinants of urinary L-FABP in individuals with microalbuminuria were urinary ADP, AER and history of smoking. Furthermore, urinary ADP alone explained 23% of the urinary L-FABP variability at this stage. Thus, factors such as BMI, HbA_{1C} or gender (most important determinants of urinary ADP at this stage in study II) could play a role for the urinary L-FABP elevation.

At the late stages, oxidative stress and hypoxia (accentuated by anemia) probably co-operate with the severe glomerular damage to cause a further increase in urinary L-FABP (165).

Urinary L-FABP was previously shown to be closely associated with structural and functional tubular kidney damage, which makes it mainly a tubular marker (405, 483). However, even if there are no studies regarding the glomerular passage of L-FABP, since L-FABP was initially discovered in the liver and its molecular weight is less than that of human albumin, a glomerular passage cannot be ruled out (507). Moreover, in study I AER was a strong determinant of urinary L-FABP at all stages, together with urinary ADP. Furthermore, urinary L-FABP was a strong independent predictor of AER-based progression of DN. Given these results, the link between tubular injury reflected by urinary L-FABP and early AER-based progression of DN may either reflect a tubular component of AER or a triggering role of the glomerular damage on tubular dysfunction.

Finally, both AER and L-FABP were strong independent predictors of progression to ESRD. Despite reflecting other factors also, progression to ESRD is correlated with the loss of eGFR (please see discussion on outcomes). These results collectively suggest that the rapid decline in kidney function at late stage DN is a complex process involving vascular, glomerular and tubular damage.

6.3.1.2. Perspective on stroke

Beyond the potential issue of study power, the independent prediction of stroke, but not of the other cardiovascular endpoints, could be interesting from the pathophysiological point of view. A few interpretations need to be discussed to understand the likely implications of these results.

Since the *L-FABP* gene is expressed in the proximal tubular cells, but not in the brain, urinary L-FABP may represent tubular injury. L-FABP production in the proximal tubular cells may be triggered by NEFAs overload secondary to injury in the glomerular barrier, oxidative stress or toxic insults (501). Thus, the NEFAs overload in the proximal tubules could mimic the effect of polyunsaturated fatty acids (PUFAs) on the astrocytes and endothelial cells, the two major components of the blood brain barrier (508). Another mechanism potentially linking brain atherosclerosis with L-FABP could be the ox-LDL or other modified lipoproteins. This is supported by the role of these lipoproteins in stroke development as well as tubular dysfunction (509). In addition, tubular and endothelial cells share a similar response to ox-LDL exposure, while statin therapy also has a positive effect on tubular dysfunction and urinary L-FABP concentrations (510). Furthermore, local expression of L-FABP in the kidney can be triggered by hypoxia due to a decrease in the peritubular capillary blood flow, which could mimic the effect of hypoxia on the astrocytes or endothelial cells (397).

Fatty acids are metabolized by FABP proteins in all organs, and new data have linked NEFAs with the blood pressure regulation. One relationship of urinary L-FABP with stroke could therefore come via regulation of the blood pressure (511). However, this is unlikely, as urinary L-FABP still predicted stroke after adjustments for AER and blood pressure.

Another link between urinary L-FABP and stroke may be heart failure. On one hand, urinary L-FABP was a strong independent predictor of worsening of kidney function in individuals with heart failure (512-514). On the other hand, heart failure was associated with increased risk of stroke (515). Thus, heart failure could increase the risk of both stroke and DN progression, or DN progression could lead to heart failure and secondarily to stroke, but this hypothesis could not be investigated in the present research.

Other mechanisms may also be implicated, but it is beyond the scope of this study to further explore the underlying mechanisms. The single, evidence-based conclusion of this study is that tubular injury predicted stroke, and this observation may lead to speculations about a kidney–brain axis. However, future studies should clarify if there is a specific kidney–brain relationship linking the FABPs with the kidneys and the brain.

It is significant that urinary L-FABP predicted the other three outcomes (CAD, PVD and CVD) independently of the cardiovascular risk factors and eGFR, but not when adjusted for AER. These results suggest that neither the well-known cardiovascular risk factors nor kidney dysfunction mediate urinary L-FABP's predictive ability regarding these cardiovascular endpoints. Urinary L-FABP's effect on these outcomes may, however, be dependent on endothelial dysfunction represented by the increased AER (516). Another explanation could be that the most important

triggers of *L-FABP* gene expression—hypoxia and NEFAs—play a smaller role in the processes leading to CAD or PVD compared to the acute coronary syndromes, while AER and endothelial dysfunction are more important for the chronic states (397). This theory could explain previous reports that have suggested that urinary L-FABP is a good identifier of individuals with acute coronary syndrome as well as a good predictor of future coronary restenosis after a first acute coronary event (517, 518).

6.3.2. Urinary ADP

Urinary ADP predicted the onset of microalbuminuria and the progression from microalbuminuria to macroalbuminuria independently of the basic models, but not independently of AER. One statistical explanation may be the lack of power despite the large number of subjects studied. While albuminuria is an important predictor of DN progression, another explanation may be an interaction between urinary ADP and AER (487). This interaction has a strong statistical argument, as AER was a constant determinant of urinary ADP at all stages. The AER association with the urinary ADP concentrations may reflect that these two molecules share common pathophysiological mechanisms. These similarities may explain urinary ADP's lack of independent predictive ability, with regard to the AER-based progression of DN.

Indeed, AER and urinary ADP share "common soil". Urinary ADP is a circulating plasma protein filtered at the glomerular level and suggested to represent vascular damage (420, 519). Urinary ADP has furthermore been related to glomerular barrier integrity (425). Thus, urinary ADP may capture the glomerular and vascular damage similar to albumin. In addition, since HbA_{1C}, L-FABP and KIM-1 are also constantly associated with urinary ADP, this could reflect tubular damage due to poor glycemic control, on top of glomerular damage. Thus, urinary ADP may reflect vascular, glomerular and tubular dysfunction, since others have shown that ADP is present in the normal kidney and then is gradually lost alongside the progression of DKD, first at the glomerular level and later from the intra-renal arterioles (424). Furthermore, urinary ADP may also reflect other intrarenal mechanisms, given that urinary ADP has been suggested to play a protective role in renal fibrosis (426).

In study II, low BMI was another significant determinant of the urinary ADP concentrations, but only in those with macroalbuminuria. This result suggests that the urinary ADP measurement could capture renal cachexia. In animal studies, high serum ADP was associated with increased energy expenditure and weight loss (520). In human studies, well-nourished individuals undergoing hemodialysis had significantly lower serum ADP compared with malnourished individuals (521). Furthermore, in T1DM the urinary ADP is mainly of low molecular weight, showing high homology with TNF (tumor necrosis factor or cachectin), while the serum ADP is mainly of high molecular weight ADP having a protective role (522, 523). Thus, one hypothesis behind the high serum ADP observed in those with macroalbuminuria could be its potential protective role against cachexia (424).

Another important determinant of urinary ADP, in the individuals with normal AER or macroalbuminuria enrolled in study II, was serum ADP. It is well accepted that serum ADP is associated with glycemic control and insulin resistance. In addition, factors such as physical exercise, hypoglycemia, caloric restriction, gut permeability

and hypoxia have been shown to influence the serum ADP concentrations and may be relevant also for the increased urinary ADP concentrations (524-526). However, neither were the ADP isoforms determined nor any data on exercise, hypoglycemia, caloric intake or hypoxia collected at baseline in study II.

6.3.3. Urinary KIM-1

Study III demonstrated that AER is a powerful confounder of the relationship between KIM-1 and eGFR. In addition, this study found a complex interaction between KIM-1, AER and eGFR, which is also supported by other studies (369, 527, 528).

This complex association unveiled one of its probable causes in study III, the first study to show the strong genetic determination of the urinary KIM-1 concentrations. Thus, in study III the increase in the urinary KIM-1 concentrations was driven by two factors: the genetic determination as well as acquired factors such as diabetes and glomerular damage (through HbA_{1C}, diabetes duration and AER). Consequently, the effect of these acquired factors needed to be extremely strong to overtake the effect of the genes in order to predict the AER-based progression of DN. Therefore, prediction of progression to ESRD independently of the other risk factors was seen only in those with macroalbuminuria in whom the urinary KIM-1 increase was strong enough due to acquired factors. However, even in those with macroalbuminuria, urinary KIM-1 was not independent of AER, due to their strong interaction.

One explanation for this complex interaction could be that AER is triggering the eGFR decrease and the KIM-1 increase or that *KIM-1* expression is triggered by different factors affecting both the AER and eGFR (529). Indeed, urinary KIM-1 concentrations are increased by albuminuria, being associated with podocytopenia and proteinuria and attenuated by anti-proteinuric treatment (414, 530). In addition, the KIM-1 concentrations mirrored the AER variation in individuals with non-diabetic proteinuric CKD (531). Furthermore, AER was demonstrated to alter the eGFR (532). Finally, elevated HbA_{1C} and blood pressure affect both the AER and the eGFR in individuals with T1DM (533).

A second explanation could be the heterogenous outcome in individuals with baseline macroalbuminuria, as progression to ESRD is also related to other factors than the eGFR decline (please see the discussion on outcomes).

A third explanation may reside in the complex roles of KIM-1 in the kidneys, which influence glomerular, tubular and interstitial processes (529). KIM-1 is expressed both in the glomeruli and the proximal tubular cells in response to hypoxia, and plays an important role in cell-cell and cell-matrix interactions such as preservation of podocyte integrity, development of interstitial fibrosis and regulation of inflammation, clearance of apoptotic and necrotic tubular cells, etc. (414, 534-536). Furthermore, KIM-1 is not only implicated in multiple processes but plays a dual role both in interstitial fibrosis and inflammation as well as in renal regeneration and tubular cell repairing (529, 536-538). Its soluble form neutralizes KIM-1 ligands such as ox-LDL or other yet undiscovered ligands, while statin therapy lowers AER and KIM-1, preserving the eGFR (536, 539, 540).

Finally, KIM-1 is also called TIM-1, having been initially discovered in the liver, where it functions as a receptor facilitating the cellular entry of hepatitis A virus (541). KIM-1 is activated by multiple factors such as toxic substances, hyperglycemia, viruses, etc. (538, 541). In addition, TIM-1 is involved in many immunological processes leading to T cell proliferation, activation and cytokine production (542). While cytokine production and infections have been linked to insulin resistance, urinary KIM-1 also has been linked to insulin resistance (543). Furthermore, serum KIM-1 was shown to predict the progression to ESRD in individuals with T1DM. Thus, the presence of KIM-1 in urine by filtration of serum KIM-1 – since intact KIM-1 has a molecular weight of 104 kDa and soluble KIM-1 of 90 kDa – could reflect its systemic triggers (viral infection, hypoxia, toxic substances) in the liver or different organs where it is abundantly expressed (colon, lymph nodes, gall bladder, appendix, bone marrow, testes, etc.) as well as insulin resistance. Finally, urinary KIM-1 could reflect the same trigger of its expression in the tubular cells as in the case of serum KIM-1.

All these complex mechanisms do confirm the complex interaction between urinary KIM-1, AER and eGFR and the complex systemic-glomerular-tubular interplay reflected by KIM-1's causal role in the loss of kidney function.

6.3.4. Common pathophysiological perspective

6.3.4.1. Tubular injury predicts glomerular damage progression and loss of renal function in DN

In studies I and II urinary L-FABP and urinary ADP predicted AER-based progression of DN after or before adjustment for AER, while in study III urinary KIM-1 was not a predictor in the observational study. Thus, by predicting the AER-based progression of DN, these biomarkers in fact predicted the progression of glomerular damage.

In studies I, II and III all three tubular biomarkers predicted the progression to ESRD. L-FABP predicted the progression independently of all common risk factors and AER, while urinary ADP predicted this independently of the common risk factors, AER and eGFR. Urinary KIM-1 in turn predicted the progression to ESRD independently of the common risk factors, but not independently of AER. Despite its heterogenous reflection of eGFR decline, the progression to ESRD mainly reflects the loss of eGFR at the late stages of DN. Accordingly, considering the fact that all three tubular biomarkers predicted the eGFR decline, it can be concluded that tubular dysfunction precedes and predicts the loss of kidney function.

6.3.4.2. Tubular injury plays a causal role in the loss of renal function in DN

Causality with respect to the loss of kidney function could not be tested using a MR approach for urinary L-FABP and ADP. However, when causality was tested for urinary KIM-1, this biomarker was indeed found to be associated with the loss of kidney function and the relationship was very likely to represent a causal link based on the MR analysis. Thus, tubular dysfunction as represented by urinary KIM-1 is causally linked to the loss of kidney function in individuals with T1DM.

6.3.4.3. The three biomarkers reflect both glomerular and tubular injury

The presence of the three discussed biomarkers in urine may be from several likely sources. One is the expression of the molecules in the proximal tubular cells and their shedding into the urine as a consequence of tubular injury. Second, considering their molecular weight a passage through the glomerular barrier cannot be excluded. In general, glomerular passage and reflection of systemic factors by urinary biomarkers is based on the main determinants of their urinary levels at each stage of DN (Table 5, Table 12, Table 19). An important argument for this route is that AER was a common determinant at almost all stages and of all biomarkers. Thus, since AER is a common determinant of the levels of the urinary biomarkers, one explanation could be that AER itself triggers tubular dysfunction. Another explanation could be that common systemic factors trigger both glomerular and tubular injury. Thus, there are valid reasons to argue that the presence of these biomarkers in urine may reflect not only injury to the tubular cells but also to the integrity of the glomerular barrier. In addition, these biomarkers may also mirror systemic processes or processes in the organs from where they originate (Figure 19).

Systemic factors reflected by these three tubular biomarkers may be represented by hepatic, muscle or adipocyte dysfunction or resistance to insulin in the target tissues, gut injury triggered by hyperglycemia (or hypoglycemia), hypoxia, immune factors, etc. Such a hypothesis is supported by the common determinants at each stage of DN as well as previous studies (see discussion above). The final argument for a glomerular component and reflection of systemic processes by these biomarkers is the prediction of stroke and other cardiovascular disease components as well as the prediction of mortality, in study IV.

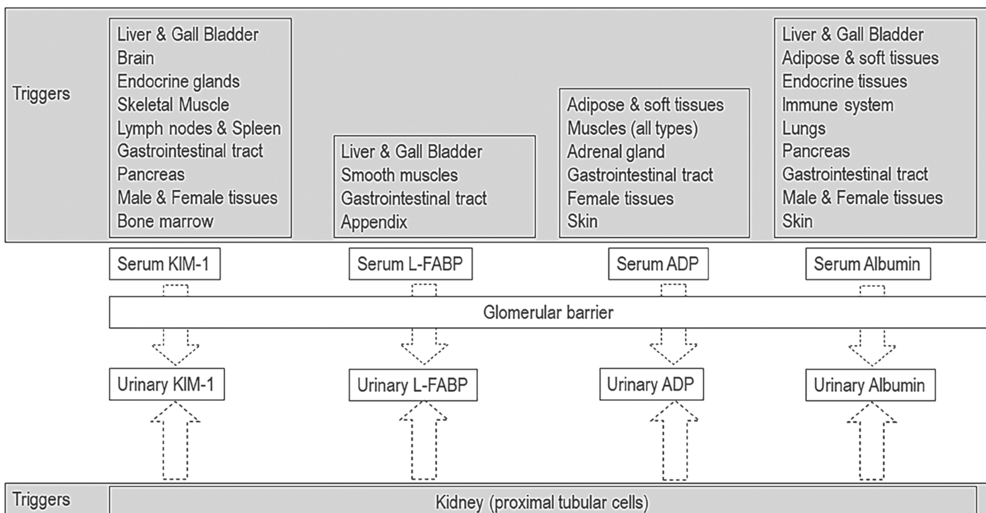

Figure 19. The probable common routes of urinary albumin and of the 3 urinary tubular biomarkers studied, for appearing in urine. L-FABP, KIM-1 and ADP gene expression according to www.proteinatlas.org and www.ncbi.nlm.nih.gov/gene.

Complicating the discussions even more, one important finding worth mentioning is that moderate to high albumin expression has been demonstrated in the tubular cells of the kidney (https://www.proteinatlas.org/ENSG00000163631-ALB/tissue), but not in glomeruli. Consequently, due to its proven expression in the tubules, even albumin may have a tubular secretion component, apart from its well-known tubular reabsorption, in case of tubular injury.

6.4. Therapeutic perspective of the results

All three studied biomarkers represent interesting therapeutic targets, beyond their clinical predictive abilities. Urinary L-FABP can be modulated by moderate-intensity physical exercise and dietary n3-PUFAs (544-547). However, the most interesting aspect may be the selective modulation of different FABPs [Adipocyte fatty-acid binding protein (A-FABP), L-FABP, intestinal fatty-acid binding protein (I-FABP)], which could have protective effects on the kidney beyond their direct effect. This indirect effect may originate from modulation of the total body fat or the visceral fat, serum triglycerides and cholesterol concentrations as well as of the general beta-oxidation/elimination of fatty acids (393, 548). In addition, L-FABP modulation and especially L-FABP silencing protected against obesity by diminishing the impact of a high-fat diet (549, 550).

KIM-1 plays an important role in the clearance of debris from damaged renal tubules. Thus, the potential therapeutic effect of KIM-1 modulation may be antifibrotic (551). In addition, it is worth mentioning that KIM-1 was first discovered in the liver as a hepatitis-A cellular receptor (HAVCR) and then as a T-cell immunoglobulin and mucin-containing molecule or Tim-1 with different expression between Th1 and Th2 lymphocytes (552, 553). Given these results, therapeutic modulation of KIM-1 through immunological mechanisms may have renoprotective effects, besides providing means of antiviral therapies.

Last but not least, ADP was a constant determinant of all biomarkers at almost all stages, and it is therefore possible that ADP per se could influence several mechanisms involved in the loss of kidney function. Such a view is supported by the fact that urinary ADP predicted the progression to ESRD independently of all the other biomarkers. Indeed, ADP modulation through lifestyle measures proved efficient in preventing DN progression (112, 554, 555). In addition, positive effects on DN progression were demonstrated by ADP modulation, at transcriptional or posttranslational levels, using thiazolidinediones, statins or ARBs (556-559). Other compounds may, in the future selectively modulate the ADP receptor with a much narrower action and more specific effects beyond insulin sensitivity or obesity.

7. CONCLUSIONS AND FUTURE DIRECTIONS

7.1. Conclusions

In study I, urinary L-FABP was an independent predictor of AER-based progression of DN. Progression to ESRD was also independently predicted by this biomarker. Urinary L-FABP used alone or together with AER did not improve the risk prediction of AER-based progression of DN or progression to ESRD in individuals with type 1 diabetes. Further studies are needed, however, to test its predictive ability as to eGFR decline.

In study II, before adjustment with AER, urinary ADP predicted AER-based progression of DN. In addition, urinary ADP was a strong independent predictor of progression to ESRD and added significant predictive benefit when used together with either AER or eGFR. Further studies are warranted to explore its ability to predict other DN outcomes in individuals with type 1 diabetes.

In study III, urinary KIM-1 did not predict progression to ESRD independently of AER. However, the MR analysis showed an independent causal association between increased urinary KIM-1 concentrations and a lower eGFR. Future studies are mandatory to explore its predictive potential with regard to loss of eGFR over time in individuals with type 1 diabetes as well as to replicate this causal association.

In study IV, urinary L-FABP was an independent predictor of stroke and mortality, but did not add predictive benefit on top of AER and eGFR. However, L-FABP was not a predictor of any other cardiovascular endpoints (coronary artery disease, peripheral vascular disease and overall CVD events) when adjusted for AER, whereas urinary ADP and KIM-1 also predicted different cardiovascular outcomes.

Urinary ADP and AER were common determinants of all the tested biomarkers at all stages, proving a complex interaction between different tubular, glomerular and systemic mechanisms. In addition, judging by the baseline determinants of their levels, the studied tubular biomarkers represent much more than tubular injury, capturing also glomerular damage and systemic factors.

7.2. Future directions

It is still unknown whether the discussed urinary biomarkers would predict other phenotypes of DN progression, such as the slopes of renal function decline.

Moreover, no data are available on the causal relationship between these biomarkers and other phenotypes of DN progression such as the slopes of renal function decline.

Furthermore, testing the potential causal relationship between urinary KIM-1 and the AER increase using a MR approach could clarify the complex relationship between urinary KIM-1 and AER.

Another way to test the causality of these urinary biomarkers on the loss of renal function and increase in AER would be using an econometric analysis with only the clinical data, without genetic data or a randomized controlled trial.

Further testing of the causality between these biomarkers and dichotomic cardiovascular outcomes is now possible using a MR approach or generalized econometric analysis.

One other important research aspect in regard to these urinary biomarkers is to clarify the potential for their use in clinical practice, but for this objective the important clinical cut-offs need to be determined.

Another research possibility related to these biomarkers would be to test if a new eGFR formula incorporating or based on at least one of these biomarkers may be suitable.

In the light of their pathophysiological roles, it may be attractive to study their utility, if any, together with other biomarkers, in establishing a liquid kidney biopsy.

Finally, investigation of the therapeutic potential of the three mentioned urinary biomarkers deserves greater attention.

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