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Determinants of vascular complications in type 2 diabetic South Asians

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Determinants of Vascular Complications in type 2 diabetic South Asians a focus on the complement system

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Determinants of Vascular Complications in type 2 diabetic South Asians a focus on the complement system

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Medische Wetenschappen

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“in die hooggeleerde kringen
voel ik mij vaak oliedom
ook al gaan de grootste dingen
buiten alle weten om”

Toon Hermans

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General introduction, study objectives and thesis-outline

Diabetes and cardiovascular disease

Type 2 diabetes is becoming a pandemic [1]. The prevalence of diabetes not only increases in western societies but also in developing countries [2]. Central in the pathogenesis of type 2 diabetes is reduced insulin-sensitivity of glucose-utilizing tissues (insulin resistance). Insulin resistance in its turn is strongly associated with - mainly visceral- obesity. Both genetic and environmental factors are involved in diabetogenesis.

Diabetic subjects have an increased incidence of both macrovascular (coronary artery events, stroke, peripheral artery disease) and microvascular (nephropathy, retinopathy) disease. Multiple factors contribute to vascular damage, including direct toxic effects of high glucose levels to the vasculature, dyslipidemia and hypertension. Several large-scale intervention trials targeting these factors have shown that the incidence of cardiovascular disease in type 2 diabetic subjects can be significantly reduced [3,4,5].

South Asians, central obesity and type 2 diabetes

In western societies, subjects of South Asian descent have an increased prevalence of insulin resistance and type 2 diabetes [6,7]. Besides exogenous factors like excessive energy ingestion [8] and diminished physical activity [9], genetic factors clearly play a role. Compared to Caucasians, body composition is different in South Asians. South Asians have a preferential truncal (deep adipose tissue) fat distribution [10], and even at the same body mass index the amount of -mainly visceral- fat is increased in South Asians [11]. Central obesity is associated with insulin resistance [7]. Compared to the relatively inactive superficial subcutaneous fat (primary compartment), deep subcutaneous and visceral fat (secondary compartment) is an active regulator of body metabolism [12].

Not only fat distribution is different in South Asians, adipose tissue function is also different [13]. The relationship between insulin sensitivity and adiposity is much stronger in South Asians than in Caucasians [10]. Even when central obesity has not (yet) developed, South Asian children have decreased insulin sensitivity compared to Caucasian children [14]. In South Asians, insulin resistance is already present at birth [15].

Several hypotheses try to explain the increased tendency to central obesity and the apparent adipose tissue dysfunction in South Asians. Chronic energy depletion and climatic influences may have favoured energy storage in the quickly mobilizable deep adipose tissue compartment, making fat metabolism

in South Asians more sensitive to fluctuations in energy supply (el-Nino hypothesis) [16]. Alternatively, differences in mitochondrial gene structure and function (favouring energy storage at the cost of heat production) might explain the tendency to central obesity in South Asians (the "mitochondrial efficiency hypothesis") [17].
Besides its function as an energy storage pool, adipose tissue -especially the deep compartment- plays an important role in immune system maintenance [18-20]. A high burden of - mainly gastrointestinal - infectious diseases may have resulted in the tendency to prioritize deep visceral adipose tissue depots to meet the immediate demands of the immune system in the defeat of gastrointestinal pathogens, as postulated by the "variable disease selection hypothesis" [21].

South Asians and cardiovascular disease

Besides an increased prevalence of central obesity and type 2 diabetes, South Asians also have an increased incidence of cardiovascular disease, especially coronary artery disease and stroke. This is especially true for South Asian immigrants in western societies [22-23], but also for South Asians living in the Indian subcontinent [24]. Although classical risk factors like smoking, hypertension and hypercholesterolemia do contribute to cardiovascular morbidity in South Asians [25], they are not excessive or more prevalent in comparison with Caucasians and hence do not account for the higher rate of cardiovascular disease. Moreover, the higher prevalence of type 2 diabetes in South Asians also fails to fully explain the excessive cardiovascular morbidity [26]. Several other potential cardiovascular risk factors have been studied, including hemostatic factors, inflammatory parameters and metabolic factors. South Asians have higher levels of plasminogen activator inhibitor 1 (PAI-1) , fibrinogen and homocystein, indicating an increased thrombotic tendency [27-29]. South Asians also have higher levels of high-sensitivity C-reactive protein [30], lipoprotein(a) [31] and decraesed levels of adiponectin [32]. However, all the above-mentioned factors were examined in cross-sectional studies, and prospective studies addressing their use in predicting cardiovascular disease are lacking.
Not only macrovascular complications are increased, South Asians with type 2 diabetes also have an increased rate of microvascular (renal and retinal) complications [33-35]. Type 2 diabetic South Asians have a 40-fold increased risk for end-stage diabetic nephropathy [36]. In non-diabetic South Asians, central obesity is an independent risk factor for albuminuria [37].

Central obesity, cardiovascular disease and the complement system

As recognized by the "variable disease selection hypothesis" (see above), adipose tissue function and immunity are closely related [18-20]. South Asian ancestors faced a high pressure of - mainly gastrointestinal- infectious diseases. This might have set the immune system at a higher level of activity, with adipose tissue as a potential mediator. Although protective when faced with infections, enhanced immune activity might turn out to be harmful with respect to cardiovascular disease in the context of excessive energy supply and diminished infectious pressure. Central obesity itself is associated with low grade inflammation, reflected by increased levels of C-reactive protein [27]. In South Asians however, complement factor C3 is even more closely related to the metabolic syndrome (a clustering of cardiovascular risk factors with central obesity as a central feature) than CRP [38], and gene expression and production of several complement factors has been described in -mainly visceral- adipose tissue [39,40].

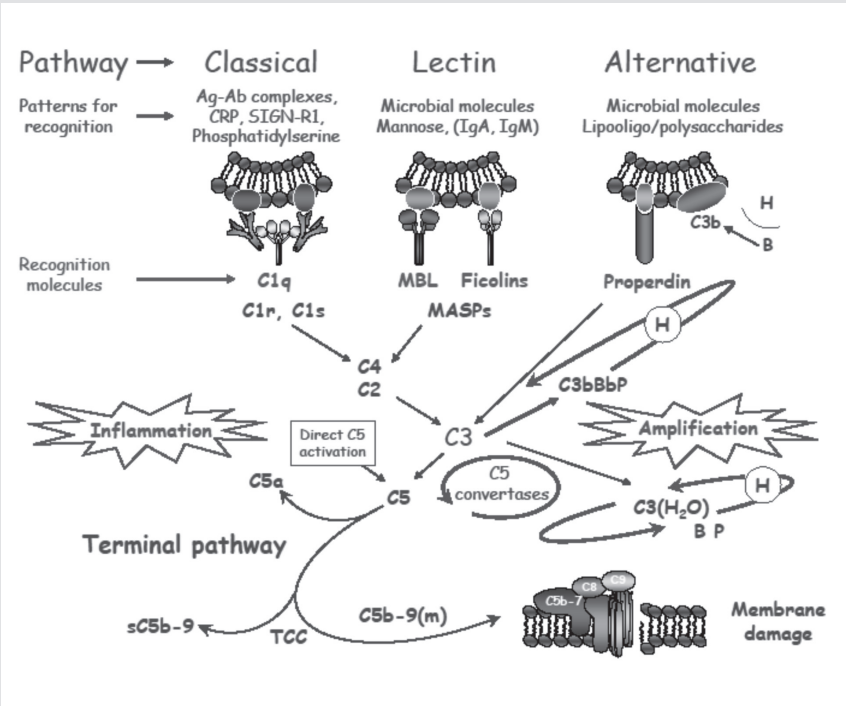
The complement system is a key player in the innate immune response, and its involvement in cardiovascular disease is increasingly being recognized [41,42]. The complement system refers to a cascade of proteins which are involved in opsonisation of harmful particles such as bacteria and facilitate the inflammatory response by attraction of inflammatory cells and enhancing vascular permeability.

The complement system can be activated via three pathways: the classical pathway, the alternative pathway and the lectin pathway. These three pathways converge into a C3 convertase, and from this point the cascade follows a final common pathway leading to the formation of C5b-9, also known as the Membrane Attack Complex (MAC). The Membrane Attack Complex induces cell damage by creating pores in the cellular membrane leading to osmotic cell swelling and subsequent cell death, but sublytic effects have also been described [43]

The classical pathway is activated by immune complexes and involves factor 1,2 and 4. The alternative pathway is dependent on the stabilisation of spontaneously hydrolysed C3. In addition, recent evidence suggests that properdin might act as the primary recognition molecule in the alternative pathway. The lectin pathway, which also involves factor 2 and 4, is activated when Mannan Binding Lectin (MBL) binds to carbohydrate residues present on various pathogenic surfaces. Activation products of the complement system have been detected in atherosclerotic plaques and in kidneys and urine of

diabetic subjects [44-46], and MBL has recently evolved as a cardiovascular and renal risk marker [47-49].

Figure 1 the complement system



Complement activation occurs via the classical pathway (initiated by the binding of immunoglobulins to antigens) , the lectin pathway (initiated by the binding of Mannose Binding Lectin (MBL) or ficolins to sugar residues), or the alternative pathway. The latter pathway is spontaneously activated by slow hydrolysis of C3. The alternative pathway also serves as an amplification loop for the classical and lectin pathway. Recent evidence suggests that binding of properdin to its ligand initiates the alternative pathway. All three pathways converge into a C3 convertase, and from this point the cascade follows a final common pathway leading to the formation of C5b-9, also known as Terminal Complement Complex (TCC) or Membrane Attack Complex (MAC), which induces damage to the cell membrane leading to cell death. Cleavage products of C3 and C5 facilitate the inflammatory response. (prof. T.E.Mollnes, reprinted with permission)

Scope of this thesis

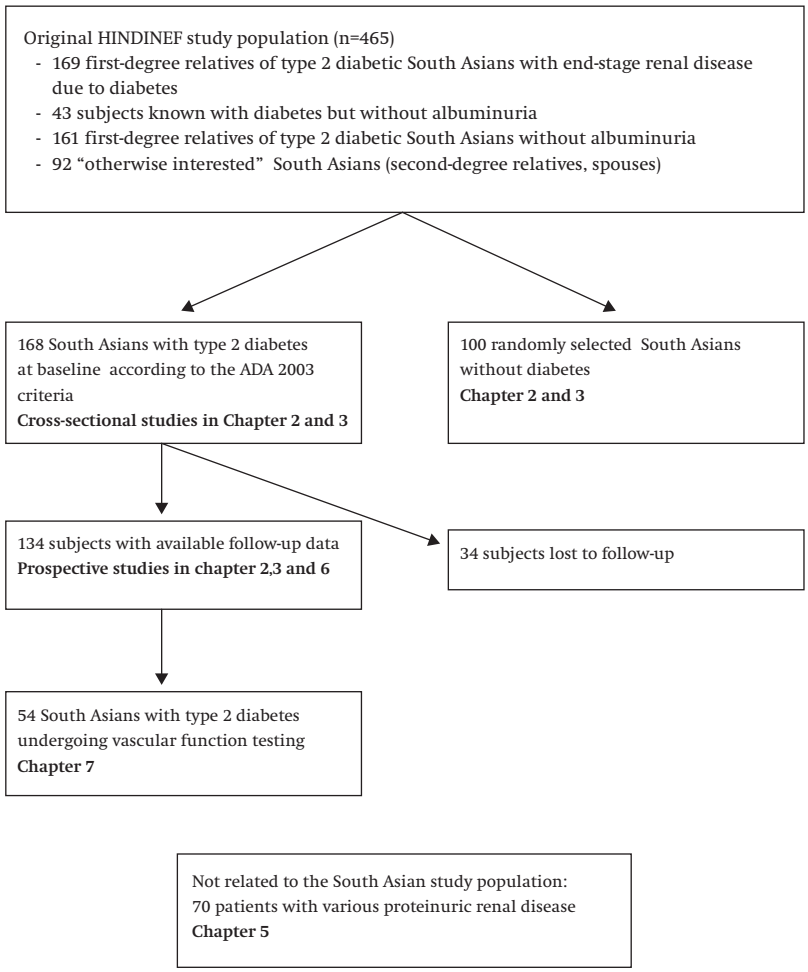
The high rate of cardiovascular complications in type 2 diabetic South Asians, the above-mentioned relationship between central obesity and immune function and the participation of the complement system in vascular damage led us to explore the association of the complement system and vascular complications in type 2 diabetic South Asians. We hypothesized that the activity of the complement system is enhanced in South Asians compared to Caucasians and that this contributes to the increased susceptibility to vascular and renal complications in type 2 diabetic South Asians.

Study population

The studies were accomplished in a population of immigrants of South Asian descent living in The Hague and surroundings. The ancestors of these South Asians originally came from the Indian Subcontinent, in a circumscriptive area of North India called Uttar Pradesh, Uttarakhand and West-Bihar [50]. In the late 19th century, some 30.000 inhabitants of this region migrated to Suriname to work on the plantations. The independence of Suriname in 1975 and the unstable political climate afterwards led many South Asians to migrate to the Netherlands where they took residence mainly in The Hague, Rotterdam and Amsterdam. In the South Asian population in the Hague, type 2 diabetes was shown to be very common, with a prevalence of 40% in those over the age of 60 [51].

The studies described in this thesis are an extension of the previously conducted HINDINEF study [52]. The study population comprised 169 first-degree relatives of South Asian subjects with end-stage renal disease due to diabetic nephropathy, 161 first-degree relatives of type 2 diabetic South Asians without diabetic nephropathy, 43 subjects already known with diabetes but without nephropathy, and 92 “otherwise interested” South Asians (second-degree relatives and spouses). The total population consisted of 465 South Asians. As stated above [51], in this population having a first-degree relative with type 2 diabetes is common rather than exceptional. Out of these 465 subjects, 168 subjects had type 2 diabetes at baseline according to the ADA 2003 criteria. Between 2007 and 2009, follow-up data of these 168 type 2 diabetic subjects were collected. Out of these 168 type 2 diabetic subjects, 54 subjects also underwent vascular function testing. The results of this follow-up study are described in this thesis

Figure 2 study population



Thesis outline

As the primary focus of this thesis is on the complement system, chapter 2 - 5 focus on the complement system as a determinant of cardiovascular and renal disease.

Chapter 2 consists of a cross-sectional study comparing levels of complement factor 3 and SC5b-9 –the soluble end product of complement activation- in South Asians and Caucasians and assessing the relation between C3, SC5b-9, Mannose-binding lectin (MBL) and renal damage as reflected by urinary albumin/creatinin ratio. In addition, in a prospective observational part the predictive value of complement factors for the occurrence of cardiovascular events (C3, SC5b-9) and progressive renal failure (C3, SC5b-9, MBL) is studied.

Chapter 3 is a prospective observational study assessing the relationship between Mannose-binding lectin, serum MBL levels and the occurrence of coronary artery events.

In chapter 4 and 5 we study the role of the complement system in the pathogenesis of progressive renal failure. The focus is on properdin, a promotor of the alternative pathway complement activation.

Chapter 4 describes experimental studies on the role of properdin in complement activation on cultured proximal tubular epithelial cells.

Chapter 5 assesses the association between urinary properdin excretion, intrarenal complement activation and renal function. This study was done in seventy patients with proteinuria, mainly Caucasians.

Chapter 6 is a prospective observational study assessing the predictive value of waist-to-hip ratio, a measure of central obesity, for cardiovascular events in type 2 diabetic South Asians.

Chapter 7 is a cross-sectional study addressing the relationship between plasma Connective Tissue Growth Factor (CTGF) – a pro-fibrotic cytokine – and vascular damage as assessed by vascular function testing. This study was performed in 54 type 2 diabetic South Asians.

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2

Complement activation and vascular complications in type 2 diabetic South Asians

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Abstract

Introduction

South Asian immigrants in Western societies exhibit a high burden of diabetes and subsequent vascular complications. Diabetic vascular complications are associated with vascular inflammation. We hypothesize that enhanced complement activation is involved.

Research desing and methods

Levels of complement C3, SC5b-9 -the soluble end-product of complement activation, and Mannose- Binding Lectin (the recognition molecule of the lectin pathway of complement activation) were measured in 268 South Asians (168 type 2 diabetic and 100 non-diabetic subjects) and compared to an age- and sex matched control-group of native Caucasians. In a cross-sectional study, the association between complement levels, diabetes and albuminuria was assessed. In a longitudinal study, the 168 type 2 diabetic South Asians were followed prospectively for a median duration of 7.66 years. The association of complement levels with future cardiovascular events and progressive renal failure was assessed.

Results

Compared to native Caucasians, South Asians had significantly higher levels of both serum C3 and plasma SC5b-9 and higher levels of MBL. A SC5b-9 level above the median was associated with albuminuria at baseline (univariate OR 2.3, 95%CI 1.18-4.55, P = 0.015) and with progressive renal failure (univariate HR 5.45, 95%CI 1.52-19.5, P = 0.009) Neither C3 nor SC5b-9 predicted cardiovascular events. Baseline MBL levels were associated with progressive renal failure (multivariate HR 3.59, 95%CI 1.30-9.93, P = 0.014)

Conclusion

These results suggest that complement activation is involved in the pathogenesis of microvascular complications in South Asians with type 2 diabetes.

Introduction

South Asian immigrants in Western societies have a high burden of ischemic heart disease, stroke and diabetes [1,2]. In addition to macro-vascular disease, South Asians also have an increased incidence and a faster rate of progression of diabetic nephropathy compared to Caucasians.[3]

Traditional cardiovascular risk factors do not completely explain the increased incidence of cardiovascular disease in South Asians [4]. Hence other factors must be involved.

Atherosclerosis, the pathologic substrate of macro-vascular disease, is recognized to be an inflammatory process [5-7]. Micro-vascular disease such as diabetic nephropathy has also been linked to inflammatory markers [8,9]. As a key-player in the inflammatory response, the complement system has been implicated in this vascular inflammation [10-12]. Deposition of complement components has been demonstrated in atherosclerotic plaques, retinae and kidneys of diabetic subjects [13-15] . Complement activation products also have been detected in urine of subjects with diabetic nephropathy [16].

The complement system can be activated via the classical, alternative or lectin pathway. All three pathways converge into the generation of a C3 convertase, which activates the central molecule C3. The final activation product, C5b-9, exerts lytic and non-lytic harmful effects to its target cells. The lectin pathway is activated when Mannose-Binding Lectin (MBL) binds to its target molecule. MBL binds carbohydrate moieties on microorganisms, but endogenous MBL ligands, such as glycosylated immunoglobulins or cells exposed to oxidative stress, have also been identified [17]. MBL serum levels are primarily determined by 3 polymorphisms (B,C and D genotypes, commonly referred to as O-alleles) in the MBL gene (*mbl2*). Subjects with wild type MBL genotype (A/A) have the highest serum MBL levels, subjects with 1 variant allele (A/O) have intermediate levels and subjects with 2 variant alleles (O/O) have the lowest levels. In addition, polymorphisms in the promoter region influence the MBL level [18]. MBL is synthesized in the liver. Although intra-individual levels are relatively stable over time [19], a two- to threefold increase occurs during acute phase reactions [20]. MBL has recently been implicated in the pathogenesis of diabetic vascular complications [21].

We hypothesized that in South Asians the complement system contributes to the increased susceptibility for vascular and renal disease. We therefore examined complement, as judged by the level of the main component C3, the

level of the final activation product SC5b-9 and MBL in South Asian subjects with and without type 2 diabetes living in the Netherlands, and compared these to complement levels in an age- and sex-matched group of native Caucasian volunteers without diabetes. In addition, we examined the predictive value of serum C3, plasma SC5b-9 and serum MBL level for the occurrence of cardiovascular events and progressive renal failure.

Materials & methods

Study design and study population

This study consists of 2 parts: a cross-sectional case-control study and an observational follow-up study.

Cross-sectional study

At baseline, 268 South Asians were studied. All subjects were participants in a previously conducted study and had been recruited as described earlier [22]. At baseline, 168 subjects had type 2 diabetes according to the ADA 2003 criteria. In addition, a sample of 100 non-diabetic subjects out of the original study population (n=465) was studied. Levels of complement C3, which plays a pivotal role in complement activation, and SC5b-9, the soluble end-product of complement activation, were measured and compared to levels in a group of native Caucasians without diabetes, recruited from healthy personnel from the dialysis ward and laboratory (n=60). Complement levels in South Asians with diabetes were compared to South Asians without diabetes. In the diabetic South Asian subgroup, the association between complement levels and albuminuria was assessed.

Follow-up study

After a median duration of 7.66 (IQR 7.48-8.10) years, follow-up data from the 168 type 2 diabetic subjects were collected. Study-patients were followed up by letter and subsequently by phone. When subjects could not be traced by address or phone number in our database, general practitioners or participating family members were involved.

Follow-up data consisted of medical history with regard to cardiovascular events. Subjects were sent a questionnaire and were invited for a visit to our out-patient clinic. During this visit the questionnaire was reviewed by the

main investigator (M.A.S.). Subjects not willing to visit the out-patient clinic were asked permission to collect medical data from their general practitioner. For subjects who had died during the follow-up period, cause of death and cardiovascular history was retrieved from the general practitioner. All (self-) reported events were verified by contacting the hospital in which the event had occurred. In addition, renal data (serum creatinine, urinary albumin/creatinin ratio) were measured or obtained from the general practitioner. The association of baseline complement levels with cardiovascular events and renal deterioration was assessed.
The study protocol was approved by the Institutional Medical Ethics Committee. All subjects provided informed consent.

Measurement of baseline parameters
The study protocol has previously been published in detail [22]. Briefly, from all subjects morning urine and fasting blood samples were taken and immediately put in ice. Ethylenediaminetetraacetic acid (EDTA) plasma was obtained after centrifugation (10 minutes at 4,000 rpm at 4 degrees Celsius) and the samples were stored in aliquots at -80º Celsius within 1 hour after collection. Serum was prepared by coagulation at room temperature, and after centrifugation the samples were stored at -80º Celsius. All study participants, except known diabetic subjects, were subjected to an oral glucose tolerance test (75 gram glucose load). Diabetes was diagnosed based on the American Diabetes Association 2003 criteria. A brief physical examination (blood pressure, length, weight, hip- and waist circumference) was performed. Clinical information concerning medical history and medication was obtained from a questionnaire. Laboratory measurements including creatinin, fasting lipid profile, HbA1c, and urinary albumin/creatinine (ACR) ratio were measured according to standard methods. High-sensitivity C-reactive protein (hsCRP) was measured with a fully automated Cobas Integra 800, according to the manufacturers protocols (Roche, Almere, the Netherlands).

Quantification of SC5b-9
SC5b-9 levels were assessed by sandwich ELISA. In brief, 96-well ELISA plates were coated with the monoclonal antibody aE11 (mouse IgG2a anti-C5b-9 3mg/ml), described in detail previously [23]. Plasma samples were diluted 1/5 and 1/20 and incubated in the coated wells. Bound SC5b-9 was detected with a biotin labeled monoclonal anti C6 antibody (9C4), followed by detection with

streptavidin-poly horse radish peroxidase (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO). The optical density was measured at 415nm using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using zymosan activated serum with a known concentration of SC5b-9 of 1000 U/ml.

Quantification of C3

Serum C3 was quantified using radial immunodiffusion according to Mancini, using a polyclonal rabbit anti human C3 antiserum as described earlier [24].

Quantification of MBL

At baseline, serum MBL levels were assessed by sandwich ELISA as described previously [25]. In brief, 96-well ELISA plates (Greiner, Frickenhausen, Germany) were coated with the monoclonal antibody 3E7 (mouse IgG1 anti-MBL at 2.5 µg/ml), kindly provided by Dr. T. Fujita (Fuhushima, Japan). Serum samples were diluted 1/50 and 1/500 and incubated in the coated wells. MBL was detected with Dig-conjugated 3E7. Detection of binding of Dig-conjugated antibodies was performed using HRP-conjugated sheep anti-Dig Abs (Fab fragments, Roche, Mannheim, Germany). Enzyme activity was detected using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO)). The optical density was measured at 415nm using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using human serum from a healthy donor with a known concentration of MBL. Earlier studies indicated that this assay primarily detects wildtype MBL in serum and plasma and that there is a direct association with the MBL genotype and with MBL function.

MBL genotyping

DNA was isolated routinely from peripheral blood leucocytes. MBL single nucleotide polymorphisms at codons 52, 54 and 57 of the *mbl2* gene were typed by pyrosequencing. The detailed methodology has been published separately [26]. The MBL genotype of only wild-type allele carriers is designated as A/A and the presence of 1 or 2 variant alleles(s) (B, C, or D) is designated as A/O or O/O, respectively.

Definitions and endpoints

Albuminuria was defined as a urinary albumin/creatinine ratio > 2.5 mg/mmol in men and > 3.5 mg/mmol in women.

Cardiovascular events were pre-defined as the occurrence of either a myocardial infarction, Percutaneous Transluminal Coronary Angioplasty (PTCA), Coronary Artery Bypass Grafting (CABG), stroke, Carotid Artery Desobstruction, peripheral vascular angioplasty, bypass or amputation, or sudden cardiac death. The latter was defined as a witnessed sudden circulatory arrest. The primary end-point was the time to the first cardiovascular event.

Progressive renal failure was arbitrarily pre-defined as a 50% increase in serum creatinine or the initiation of renal replacement therapy (dialysis or kidney transplantation)

Statistical analysis

Normally distributed variables are expressed as mean ± 1 standard deviation and skewed distributed variables as median and interquartile range. Differences between two groups were assessed by Student's t-test or Mann-Whitney-U test as appropriate. Correlations were assessed by Pearson's or Spearman's correlation as appropriate. Associations between complement levels and albuminuria were assessed with logistic regression . Associations between complement levels, cardiovascular events and progressive renal failure were assessed with Cox proportional hazards regression. All test were two-sided and the level of significance was 0.05. All analyses were performed using SPSS for windows, version 17.

Results

Cross-sectional study

Two hundred sixty-eight South Asians (168 diabetic and 100 non-diabetic subjects) were studied. Of the 168 diabetic subjects, 121 were already known with diabetes at the time of the study, and 47 subjects were newly diagnosed with diabetes based on oral glucose tolerance testing. In the diabetic group, 53 subjects had albuminuria, and in the non-diabetes group 2 subjects had albuminuria. Characteristics of the South Asian study population are shown in Table 1.

Table 1 baseline characteristics of 268 South Asians

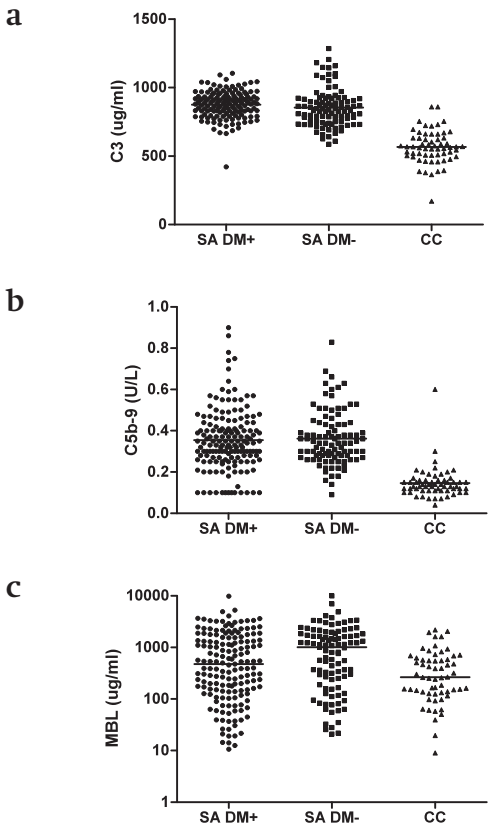
	Diabetic SA (n=168)	Non-diabetic SA (n=100)	P-value
Age (yrs)	50.5 ± 11.3	46 ± 7.3	< 0.001
% male sex	46	47	0.97
Diabetes duration (yrs)	7.0 (0.0-13.0)	-	
HbA1c (%)	7.7 ± 1.9	-	
Current or former smoker (%)	44	31	0.037
Body Mass Index	28.1 ± 4.5	26 ± 4.0	< 0.001
Waist circumference (cm)	97.2 ± 14.5	91.0 ± 10.3	< 0.001
Waist-to-hip ratio	0.98 ± 0.08	0.92 ± 0.08	< 0.001
Systolic blood pressure (mm Hg)	139 ± 25	128 ± 17	< 0.001
Diastolic blood pressure (mm Hg)	84 ± 11	81 ± 10	0.016
Total cholesterol (mmol/L)	5.1 ± 0.97	5.3 ± 0.97	0.082
Fasting triglycerides (mmol/L)	1.5 (1.2-2.2)	1.2 (0.84-1.88)	< 0.001
Ratio total: HDL cholesterol	4.33 ± 1.22	4.17 ± 1.24	0.318
Cockroft-Gault creatinin clearance (ml/min)	93 ± 31	86 ± 17	0.012
Urinary albumin/creatinine ratio (mg/mmol)	1.0 (0.39-4.75)	0.32 (0.20-0.63)	< 0.001
hs CRP (mg/L)	3.5 (1.8-8.3)	Not available	
Serum C3 (μg/ml)	875 ± 97	854 ± 139	0.184
Plasma SC5b-9 (U/ml)	0.35 ± 0.15	0.36 ± 0.13	0.708
Serum MBL (μg/L)	476 (143-1563)	1009 (239-1847)	0.043

Age and sex were not significantly different between the whole South Asian and Caucasian group (mean age 48.8 ± 10.2 versus 46 ± 8 , P = 0.10 and % male sex 46 versus 45, P = 0.9, respectively) .

Complement levels in South Asians compared to native Caucasians

Serum C3 in the whole South Asian group was significantly higher compared to native Caucasians (867 ± 115 μg/ml versus 580 ± 128μg/ml, P<0.001) (Figure 1). When considering only non-diabetic South Asians, they still had higher C3 levels than native Caucasians (854 ± 139 μg/ml versus 580 ± 128 μg/ml, P<0.001).

Figure 1



Serum concentration of C3 (upper panel), plasma concentration of SC5b-9 (middle panel) and serum concentration of Mannose-Binding Lectin (lower panel, Logarithmic scale) in South Asians with type 2 diabetes (SA DM+, n=168), South Asians without type 2 diabetes (SA DM-, n=100), and a control group of native non-diabetic Caucasians (CC, n=60). Horizontal bars represent mean (C3, SC5b-9) and median (MBL)

South Asians also had significantly higher plasma levels of SC5b-9, the soluble end-product of complement activation (mean 0.358 ± 0.14 U/ml versus 0.149 ± 0.07 U/ml, P<0.001) (Figure 1b). This was also the case when considering only non-diabetic South Asians (0.362 ± 0.13 U/ml versus 0.149 ± 0.07 U/ml, P< 0.001). MBL levels in the whole South Asian group were higher than in the Caucasian

group (median 558 (IQR 156-1680 μg/L versus 263 (IQR 133-613, P = 0.002). Non-diabetic South Asians had even higher MBL levels compared to Caucasians (1009 (IQR 239-1847) versus 263 (IQR 133-613), P < 0.001)

Diabetic versus non-diabetic South Asians

The diabetic South Asian group was older than the non-diabetic South Asian group (mean age 50.5 ± 11 versus 46 ± 7, P < 0.001). Sex distribution was not different (% male sex 46 versus 47, P = 0.97)

Both C3 and SC5b-9 levels were not different in diabetic South Asians compared to non-diabetic South Asians (875 μg ± 97 versus 854 ± 139, P = 0.184 and 0.355 ± 0.15 versus 0.362 ± 0.13, P = 0.708, respectively).

Median MBL level was higher in non-diabetic South Asians compared to diabetic South Asians (1009 (IQR 239-1847) versus 476 (IQR 143-1536), P = 0.043). Genotype distribution was not different between non-diabetic and diabetic South Asians (A/A 63 versus 57%, A/O 31 versus 35%, O/O 6 versus 8%, P = 0.58)

Correlations of complement levels and clinical parameters in type 2 diabetic South Asians

In the 168 type 2 diabetic South Asians, serum C3 levels correlated with age, sex, hsCRP, Body Mass Index, hip circumference, waist circumference and Cockroft-Gault creatinine clearance. SC5b-9 correlated only with sex and with fasting triglycerides. MBL level correlated with HbA1c and hip circumference but not with C3, SC5b-9, and hsCRP (table 2).

Complement levels and albuminuria in diabetic South Asians

To investigate whether increased complement levels are associated with renal damage, complement levels in diabetic South Asians with albuminuria (n=53) were compared to diabetic South Asians without albuminuria (n=112). A SC5b-9 level above the median was associated with the presence of albuminuria (sex adjusted OR 2.31, 95% CI 1.18-4.55, P = 0.015) whereas a C3 level above the median was not (OR 0.83, 95% CI 0.42-1.65, P = 0.60).

MBL level above the median was not associated with albuminuria (OR 1.2, 95%CI 0.63-2.13, P = 0.580). There was no statistically significant difference in median MBL level between diabetic subjects with and without albuminuria (528 μg/L (193-1308)versus 388 μg/L (130-1713), P = 0.740). Genotype distribution was also not different between diabetic South Asians with or without albuminuria (A/A 60 versus 55%, A/O 32 versus 37%, O/O 8 versus 8%, P = 0.873)

Table 2 Association between baseline clinical parameters and complement levels in 168 type 2 diabetic South Asians

	C3		SC5b-9		MBL[#]	
	Pearson's r	P-value	Pearson's r	P-value	Pearsons's r	P-value
Age	-0.285	< 0.001	0.139	0.078	-0.21	0.785
Sex	0.463	< 0.001	0.213	0.006	-0.141	0.071
Serum C3	1		0.097	0.247	-0.059	0.474
Plasma SC5b-9	0.97	0.247	1		0.027	0.731
Serum MBL[#]	-0.059	0.474	0.027	0.731	1	
High-sensitivity C-reactive protein[#]	0.479	< 0.001	0.006	0.936	0.042	0.596
Diabetes duration[#]	-0.79	0.430	0.079	0.403	-0.055	0.562
HbA1c	0.049	0.554	0.046	0.564	0.165	0.034
Systolic blood pressure	-0.061	0.463	0.078	0.325	-0.148	0.059
Diastolic blood pressure	-0.033	0.689	-0.012	0.875	-0.133	0.090
Body Mass Index	0.264	0.001	0.079	0.319	-0.155	0.470
Waist circumference	0.180	0.029	0.009	0.913	-0.124	0.113
Hip circumference	0.218	0.008	0.038	0.627	-0.169	0.030
Waist-to-hip ratio	-0.050	0.547	-0.037	0.641	0.042	0.594
HOMA-IR[†]	0.014	0.862	0.015	0.853	0.141	0.072
Total cholesterol	0.035	0.675	0.084	0.287	-0.108	0.170
Fasting triglycerides[#]	0.026	0.752	0.168	0.033	-0.081	0.304
Urinary albumin/creatinine ratio[#]	0.072	0.382	0.093	0.236	-0.001	0.986
Cockroft-Gault creatinine clearance	0.307	< 0.001	-0.016	0.843	-0.084	0.286

Not normally distributed parameters(#) were log-transformed
[†]HOMA-IR = Homeostatic Model Assessment for assessing insulin resistance

Follow-up study

Out of 168 type 2 diabetic subjects at baseline, 21 could not be traced and 13 subjects refused to participate. Eighty-six subjects visited the out-patient clinic, 31 subjects did not visit the out-patient clinic but medical information was

obtained from the general practitioner, and 17 subjects had died (see below). The median duration of follow-up was 7.66 (IQR 7.48-8.10) years. Participants lost to follow-up did not differ in baseline characteristics from participants in whom follow-up data were available. Renal follow-up data were missing in 4 subjects which were excluded from analysis with respect to renal endpoints.

Complement and cardiovascular events

During follow-up, 39 cardiovascular events occurred in 30 subjects (16 men, 14 women): 3 sudden cardiac deaths, 2 fatal and 5 non-fatal myocardial infarction, 13 percutaneous coronary interventions, 8 coronary artery bypass graft procedures, 2 fatal and 5 non-fatal strokes, 1 lower extremity amputation. Eleven of these 30 subjects had already experienced a cardiovascular event at baseline. Ten subjects died due to non-cardiovascular causes. Two of these subjects reached the primary end-point before they died, eight subjects did not and these were censored.

Baseline levels of complement did not predict cardiovascular events during follow-up (table 3). Serum C3 above the median was not associated with cardiovascular events (HR 1.28, 95% CI 0.58-2.84, P = 0.538). A SC5b-9 level above the median was also not associated with cardiovascular events (HR 1.79, 95% CI 0.84-3.78, P = 0.132). In addition, complement levels were not associated with total cardiovascular events at the end of follow-up. Baseline MBL levels were not associated with cardiovascular events (data not shown). The association between MBL genotype and cardiovascular events is described in more detail in chapter 3 of this thesis.

Complement and progressive renal failure

At the end of follow-up, 14 subjects had progressive renal failure (11 subjects had a 50% increase in serum creatinine level, 1 initiated hemodialysis and 2 had a kidney transplantation).

A baseline SC5b-9 level above the median was associated with progressive renal failure (sex-adjusted HR 6.18, 95%CI 1.69-22.64, P = 0.006). After adjusting for MBL level, a SC5b-9 level above the median was still associated with progressive renal failure (HR 5.24, 95% CI 1.44-19.05, P = 0.012). After adjusting for age, sex, systolic blood pressure, HbA1c, fasting triglycerides, and urinary ACR, the association was statistically insignificant (multivariate HR 4.38, 95%CI 0.88-22.0, P = 0.072).

Serum C3 was not associated with progressive renal failure.

Table 3 Association between clinical parameters, cardiovascular events and progressive renal failure

	Cardiovascular events		Progressive renal failure	
	Univariate HR	**95% CI**	**Univariate HR**	**95%CI**
Serum C3	1.002	0.997-1.006	0.997	0.991-1.003
Serum C3 > median	1.28	0.58-2.84	0.66	0.235-1.861
Plasma SC5b-9	1.31	0.12-14.22	16.6	1.15-240
Plasma SC5b-9 > median	1.78	0.84-3.80	5.45	1.53-19.5
Serum MBL[$]	1.18	0.68-2.05	3.19	1.23-8.23
Serum MBL > median	1.48	0.71-3.09	2.61	0.90-7.54
Age	1.02	0.99-1.06	1.037	0.993-1.083
Male sex	1.51	0.74-3.12	0.79	0.29-2.11
Diabetes duration	1.03	0.99-1.07	1.043	0.994-1.094
HbA1c	1.22	1.02-1.46	1.26	0.993-1.607
RR systolic	1.01	0.999-1.027	1.027	1.008-1.045
RR diastolic	1.02	0.989-1.058	1.029	0.983-1.077
Cockroft-Gault creatinin clearance	0.99	0.98-1.006	0.973	0.951-0.996
Urinary albumin/creatinine ratio[$]	1.97	1.36-2.83	4.44	2.61-7.54
Current or former smoking	1.27	0.61-2.62	1.04	0.384-2.814
Total cholesterol	1.20	0.81-1.80	1.28	0.74-2.21
Waist circumference	1.032	0.999-1.067	1.034	0.991-1.08
Hip circumference	0.96	0.94-099	1.012	0.957-1.070
Waist circumference > median	2.99	1.33-6.75	1.67	0.61-4.56
Waist-to-hip ratio > median	3.84	1.62-9.01	2.69	0.93-7.77
Body Mass Index	0.995	0.914-1.08	1.043	0.947-1.148
Fasting Triglycerides[$]	1.84	0.37-9.19	7.08	1.25-40.13
Hing-sensitivity C-reactive protein[$]	1.37	0.60-3.13	1.53	0.52-4.51
Previous cardiovascular event	5.09	2.33-11.14	1.74	0.49-6.19
			Multivariate HR[#]	**95% CI**
Serum SC5b-9 > median			4.38	0.88-22.0
Serum MBL[$]			3.59	1.30-9.93

[$] skewed-distributed variables are log-transformed

[#] included as covariates are age, sex, systolic blood pressure, ACR, triglycerides, HbA1c.

Baseline MBL levels were significantly associated with progressive renal failure (univariate HR 3.19, 95%CI 1.23-8.23, P = 0.017), while MBL genotype was not. Adjustment for SC5b-9 did not change this association (HR 3.1, 95%CI 1.26-7.64, P = 0.014), nor did adjustment for hsCRP (HR 3.01, 95%CI 1.17-7.77, P = 0.023). In a multivariate analysis with sex, age, systolic blood pressure, hsCRP, HbA1c, fasting triglycerides, and urinary ACR as covariates, MBL levels remained associated with progressive renal failure (multivariate HR 3.59, 95%CI 1.30-9.93, P = 0.014) Baseline Cockroft-Gault creatinine clearance, urinary albumin/creatinine ratio, systolic blood pressure and fasting triglycerides were also associated with progressive renal failure (table 3).

Discussion

South Asians have a high incidence of diabetes and subsequent vascular complications. Since traditional cardiovascular risk factors do not completely explain the increased incidence of vascular disease in South Asians, other factors must be involved. There is increasing evidence that the complement system is involved in both macro- and micro-vascular complications [10-12]. Complement activation products are detected in atherosclerotic aortic plaques and in kidney biopsies and urine of subjects with diabetic nephropathy. In addition, experimental evidence supports a pathophysiologic role for SC5b-9 in the progression of atherosclerosis [27,28]

We hypothesized that increased activity of the complement system might be involved in the enhanced rate of vascular complications in diabetic South Asians. In the cross-sectional study, we found that not only C3 – the central molecule in complement activation – is increased in South Asians compared to native Caucasians, but also SC5b-9 – the effector phase of complement activation- , supporting our hypothesis that complement activation in South Asians is occurring at a higher level of activity. In addition, a high SC5b-9 level was associated with albuminuria, suggesting that complement activation is related to renal damage.

C3 levels were closely related to measures of adipose tissue mass (BMI, waist circumference, hip circumference). Indeed, C3 mRNA expression has been observed in –mainly omental- adipose tissue [29]. Adipocyte-derived C3 serves as a precursor of C3adesarg , also called Acylation Stimulating Protein (ASP) [30]. C3adesarg is generated through interaction of C3 with complement factor

B and the enzyme adipsin (also known as complement factor D). C3adesarg is inactive with respect to complement activation. Its main function is to increase triglyceride synthesis in fat-storing cells through stimulation of fatty acid incorporation. Increased C3 levels in South Asians thus might simply reflect increased central adipose tissue mass, although it is currently unknown to what extent adipocyte-derived C3 contributes to serum total C3 levels. Secondary mechanisms involving hepatic C3 production might also be involved.

A key question is whether an increased C3 level predisposes to enhanced complement activation. As C3 did not correlate with SC5b-9, we did not find evidence that increased C3 levels result in enhanced complement activation, although enhanced complement activation at tissue level cannot be ruled out.

In the prospective study, C3 level did not predict the occurrence of cardiovascular events, which is in contrast with observations in other populations [31]. However, the predictive value of a certain risk marker depends on the population being studied. Given the increased C3 level in the South Asian population, the inter-individual predictive value with respect to cardiovascular disease might be attenuated, which has also been observed with other cardiovascular risk markers [32].

Baseline SC5b-9 levels also failed to predict cardiovascular events. Although this does not rule out a role for complement activation in atherosclerosis, plasma SC5b-9 level is not sensitive enough to serve as a risk marker for cardiovascular events.

There is firm evidence that the complement system is involved in the progression of chronic renal failure. Experimental studies in various proteinuric models show complement activation on tubular cell brush border [33-37]. Inhibition of complement, either by administration of a complement inhibitor or by knock-out of complement components, results in attenuation of tubulointerstitial damage [33,36,37]. Complement activation also has been linked to the induction of renal fibrosis [38,39].

In our study, a high baseline SC5b-9 level was associated with both albuminuria and progressive renal failure. In contrast to the above mentioned experimental studies, which studied urinary complement activation in overtly proteinuric animals, we measured blood levels of complement activation in subjects with various degrees of proteinuria. An association between blood levels of complement activation products and renal failure has not been reported previously. However, progressive renal damage has been linked to serum levels

of other inflammatory markers such as interleukines and Tumor Necrosis factor [40], suggesting that inflammation plays an important role in the pathogenesis of diabetic nephropathy. Whether increased SC5b-9 level in progressive renal failure reflects leakage of intra-renally formed SC5b-9, or whether it reflects systemically formed SC5b-9 due to generalized vascular inflammation is unknown.

More recently, the MBL pathway of complement activation has come into focus as a contributor to diabetic vascular complications [10]. Cross-sectional studies found increased MBL levels in type 1 diabetic subjects compared to healthy controls [41]. In contrast, we found lower MBL levels in our type 2 diabetic South Asians compared to non-diabetic South Asians. This is probably explained by hormonal and metabolic differences between type 1 and type 2 diabetes, since MBL levels are lowered by insulin [42], and type 2 diabetes but not type 1 diabetes is characterized by hyperinsulinemia.

In type 1 diabetic Caucasians, MBL levels predict future development or progression of diabetic nephropathy [43, 44]. Experimental data show that MBL deficient streptozotocin-treated mice develop less diabetic nephropathy [45], which strongly suggests a pathophysiologcal role of MBL in the pathogenesis of diabetic nephropathy. Although earlier studies suggested that a high MBL genotype conferred an increased risk to diabetic nephropathy [10, 42], later studies with greater patient numbers could not confirm this [46]. In line with this, we found that high MBL levels at baseline were associated with progressive renal failure, whereas MBL genotype was not. High MBL levels thus may reflect an inflammatory state. In experimental ischemia/reperfusion models, high MBL levels exacerbate tissue damage [47].Oxidative stress induces a change on the cellular surface [48], which results in binding of MBL leading to enhanced complement mediated injury. Since in our study the association of MBL and progressive renal failure was independent of plasma SC5b-9, we found no evidence that high MBL levels result in increased complement activation, although enhanced complement activation at tissue-level cannot be ruled out. Whether increased MBL levels itself results in enhanced complement activation, or whether MBL levels are just a marker of inflammation thus remains to be resolved.

In contrast to progressive renal failure, baseline MBL levels were not associated with cardiovascular events (see chapter 3 of this thesis).

In conclusion, C3 levels are increased in South Asians compared to Caucasians, but do not predict future cardiovascular events or progressive renal failure.

MBL levels and a high SC5b-9 were associated with progressive renal failure (micro-vascular disease) but not with cardiovascular events (macro-vascular disease), suggesting that complement activation primarily affects the micro-circulation.

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3

Low Mannose-Binding Lectin (MBL) genotype is associated with future cardiovascular events in type 2 diabetic South Asians

A prospective cohort study

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Abstract

Objective

South Asians have a high burden of type 2 diabetes and vascular complications. Vascular inflammation is considered central in the pathophysiology of atherosclerosis, and the complement system is thought to play an important role. Mannose-Binding Lectin (MBL), which activates the lectin pathway of complement activation, has been introduced as a risk marker of vascular damage. The present study explores the association of MBL levels, genotype and cardiovascular events in type 2 diabetic South Asians.

Research design and methods

We conducted a prospective observational study. A cohort consisting of 168 type 2 diabetic South Asians was followed for a median duration of 7.66 years. At baseline, MBL levels and genotype were determined. The association with future cardiovascular events was assessed by Cox proportional hazard regression.

Results

During follow-up, 31 cardiovascular events occurred in 22 subjects (11 men, 11 women). Compared to wild-type, the O/O genotype was significantly associated with the occurrence of cardiovascular events (hazard ratio 3.42, 95%CI 1.24-9.49, P = 0.018). However, MBL levels were not associated with the occurrence of cardiovascular events (hazard ratio 0.93, 95% CI 0.50-1.73).

Conclusion

In type 2 diabetic South Asians, the O/O MBL genotype is associated with cardiovascular events , although single serum MBL levels are not.

Introduction

South Asian immigrants in Western societies have a high burden of diabetes and vascular complications [1]. Traditional cardiovascular risk factors only partially explain this increased risk [2]. Hence other factors must be involved.
Atherosclerosis, the pathologic substrate of macrovascular disease, is recognized to be an inflammatory process [3]. As a player in the inflammatory response, the complement system is thought to be involved in this vascular inflammation [4] Indeed, complement activation products have been demonstrated in atherosclerotic plaques [5].
The complement system can be activated via the classical, alternative or lectin pathway, which is activated when Mannose-Binding Lectin (MBL) binds to its target molecule. MBL binds carbohydrate moieties on microorganisms. However, endogenous MBL ligands, such as glycosylated immunoglobulins or cells exposed to oxidative stress, have also been identified [6]. MBL serum levels are primarily determined by 3 polymorphisms (B,C and D genotypes, commonly referred to as O-alleles) in the MBL gene (*mbl2*). Subjects with wild type MBL genotype (A/A) have the highest serum MBL levels, subjects with 1 variant allele (A/O) have intermediate levels and subjects with 2 variant alleles (O/O) have the lowest levels. In addition, polymorphisms in the promoter region influence the MBL level [7]. MBL is synthesized in the liver. Although intraindividual levels are relatively stable over time [8], a two- to threefold increase occurs during acute phase reactions [9].
It has recently been suggested that MBL is involved in the pathophysiology of cardiovascular damage in high-risk populations [10]. In a group of type 1 diabetic Caucasians, MBL levels were significantly higher in subjects with either a history of cardiovascular disease or diabetic nephropathy compared to subjects without these vascular complications [10]. In type 2 diabetic Caucasians high MBL levels were associated with increased mortality [11]. However, others found an association between low MBL levels and cardiovascular events [8,12]. Data on MBL in South Asians are lacking.
We hypothesized that MBL might be involved in the high incidence of cardiovascular complications in type 2 diabetic South Asians. The current study aims to explore the association of MBL levels and genotype with cardiovascular complications in type 2 diabetic South Asians.

Research design and methods

Design of the follow-up study

We conducted a prospective cohort study. All studied subjects were recruited from a previously published study [13]. The original study population comprised 465 South Asians. At baseline, subjects that were not known with diabetes underwent a 75 g oral glucose tolerance test. Diabetes was diagnosed based on the ADA 2003 criteria. Out of 465 subjects, 168 subjects had type 2 diabetes at baseline (122 already known with diabetes, 46 newly diagnosed), and from these subjects follow-up data were collected. The study protocol was approved by the Institutional Medical Ethics Committee. All subjects provided informed consent.

Study-patients were followed up by letter and subsequently by phone. When subjects could not be traced by address or phone number in our database, general practitioners or participating family members were involved.

Follow-up data consisted of medical history with regard to cardiovascular events. Subjects were sent a questionnaire and were invited for a visit to our out-patient clinic. During this visit the questionnaire was reviewed by the main investigator (M.A.S.). Subjects not willing to visit the out-patient clinic were asked permission to collect medical data from their general practitioner. For subjects who had died during the follow-up period, cause of death and cardiovascular history was retrieved from the general practitioner. All (self-) reported events were verified by contacting the hospital in which the event had occurred.

Measurements at baseline

Laboratory measurements at baseline included lipids, creatinin, fasting glucose, urinary albumin/creatinine ratio, high-sensitivity C-reactive protein (hsCRP), and plasma SC5b-9, the soluble end product of complement activation. Lipids, creatinin, glucose and urinary albumin/creatinine ratio were measured according to standard methods. High-sensitivity C-reactive protein was measured with a fully automated Cobas Integra 800, according to the manufacturers proceedings (Roche, Almere, the Netherlands). The variation coefficients (VC) were below 3 %. Plasma levels of SC5b-9 were measured with an ELISA as described earlier [14].

MBL genotyping

DNA was isolated routinely from peripheral blood leucocytes. MBL single

nucleotide polymorphisms at codons 52, 54 and 57 of the *mbl2* gene were typed by pyrosequencing. The detailed methodology has been published separately [15]. The MBL genotype of only wildtype allele carriers is designated as A/A and the presence of 1 or 2 variant alleles(s) (B, C, or D) is designated as A/O or O/O, respectively.

Serum MBL levels

At baseline, serum MBL levels were assessed by sandwich ELISA as described previously [16]. In brief, 96-well ELISA plates (Greiner, Frickenhausen, Germany) were coated with the monoclonal antibody 3E7 (mouse IgG1 anti-MBL at 2.5 µg/ml), kindly provided by Dr. T. Fujita (Fuhushima, Japan). Serum samples were diluted 1/50 and 1/500 and incubated in the coated wells. MBL was detected with Dig-conjugated 3E7. Detection of binding of Dig-conjugated antibodies was performed using HRP-conjugated sheep anti-Dig Abs (Fab fragments, Roche, Mannheim, Germany). Enzyme activity was detected using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO)). The optical density was measured at 415nm using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using human serum from a healthy donor with a known concentration of MBL. Earlier studies indicated that this assay primarily detects wild-type MBL in serum and plasma and that there is a direct association with the MBL genotype and with MBL function [17]

Definitions and endpoint

Cardiovascular events were defined as the occurrence of either a myocardial infarction, Percutaneous Transluminal Coronary Angioplasty (PTCA), Coronary Artery Bypass Grafting (CABG), or sudden cardiac death. The latter was defined as a witnessed sudden circulatory arrest. The primary end-point was the time to the first cardiovascular event.

Statistical analysis

Normally distributed variables are expressed as arithmetic mean ± 1 standard deviation. Skewed distributed variables are expressed as median with interquartile range.

Differences between groups were assessed with the independent samples t-test or the Mann-Whitney-U test for normally and not-normally distributed variables, respectively. Comparison between multiple groups was performed

with analysis of variance. Correlations were assessed by using Pearson's correlation and Spearman's correlation as appropriate. Associations with cardiovascular events were assessed by Cox proportional hazard regression. The mean difference in log transformed MBL levels per MBL genotype (A/A, A/O,O/O) was calculated with linear regression. Hazard ratios (HR) were calculated by Cox regression analysis. The association between MBL genotype and cardiovascular events with the A/A genotype as reference genotype was calculated, and the HR for cardiovascular events per number of variant alleles. Subsequently, the association between MBL level and cardiovascular events was analyzed. In a Mendelian randomization approach following Fisher [18], we examined the estimated causative effect of MBL level on cardiovascular events using the genotype as instrumental variable (figure 2). All tests were two-sided and the level of significance was 0.05.
All analyses were performed using SPSS Statistical Software Package (version 17.0; SPSS, Chicago, IL)

Results

Baseline analysis

At baseline, serum MBL levels and MBL genotype were determined in 168 diabetic subjects (122 already known with diabetes, 46 newly diagnosed). DNA was not available in 5 diabetic patients. Baseline characteristics of the study population are shown in table 1.
The median MBL level was 476 μg/L (IQR 143-1536 μg/L). Genotype distribution in South Asians was the same as the reported genotype distribution in Caucasians [7] (table 2). MBL levels differed significantly per genotype ($P < 0.001$): subjects with the A/A genotype had the highest MBL levels (median 1300 μg/L, IQR 535-2258) with the A/O genotype had intermediate MBL levels (median 160 μg/L , IQR 75-295) and subjects with the O/O genotype had the lowest MBL levels (median 74 μg/L , IQR 38-101).
MBL levels correlated weakly with Body Mass Index ($r = -0.155$, $P = 0.014$), HbA1c ($r = 0.165$, $P = 0.034$) and hip circumference (-0.169, $P = 0.030$) , but not with sex, age, blood pressure, high-sensitivity C-reactive protein (hsCRP), smoking status, total cholesterol, fasting triglycerides, and plasma SC5b-9.

Table 1 baseline characteristics of the type 2 diabetic South Asian study population

	Follow-up (n=134)	Lost to follow-up (n=34)	P-value
Age (years)	50.7 ± 11.2	48.9 ± 11.2	0.392
% male sex	46	45	0.886
Diabetes duration (years)	7.0 (0-13)	5.0 (0-11)	0.519
HbA1c (%)	7.7 ± 1.8	7.7 ± 2.0	0.976
Systolic blood pressure (mm Hg)	138 ± 24	140 ± 27	0.638
Diastolic blood pressure (mm Hg)	84 ± 11	84 ± 11	0.937
Urinary albumin/creatinine ratio (mg/mmol)	1.0 (0.4-4.6)	1.0 (0.4-5.0)	0.991
High-sensitivity C-reactive protein (mg/L)	3.5 (1.8-8.0)	4.8 (1.7-8.4)	0.851
Cockroft-Gault creatinine clearance (ml/min)	86 ± 27	93 ± 20	0.205
Total cholesterol (mmol/L)	5.1 ± 1.0	5.0 ± 0.9	0.666
Fasting triglycerides (mmol/L)	1.59 (1.16-2.32)	1.46 (1.24-2.13)	0.869
HDL-cholesterol (mmol/L)	1.23 ± 0.3	1.28 ± 0.3	0.954
Ratio total cholesterol: HDL-cholesterol	4.14 (3.45-5.05)	4.20 (3.10-5.0)	0.432
Body Mass Index	28.0 ± 4.7	28.4 ± 4.0	0.551
Waist circumference (cm)	96.4 ± 15.4	100 ± 10.1	0.154
Waist-to hip ratio	0.97 (0.93-1.03)	0.99 (0.95-1.04)	0.223
% previous cardiovascular event	14	11	0.663
% current or previous smoker	45	39	0.528

normally distributed variables are expressed as mean +/- SD, skewed distributed variables as median and interquartile range

Longitudinal analysis

Out of 168 type 2 diabetic subjects at baseline, 21 could not be traced and 13 subjects refused to participate and thus were excluded from analysis. Eighty-six subjects visited the out-patient clinic, 31 subjects did not visit the out-patient clinic but medical information was retrieved from the general practitioner, and 17 subjects had died (see below). The median duration of follow-up was 7.66

Table 2 median MBL level (interquartile range in brackets), genotype distribution and cardiovascular events

	Median MBL level	MBL genotype (%)		
		A/A	A/O	O/O
Cross sectional				
South Asians (n=168)	476 µg/L (143-1536)	57	35	8*
Caucasians [7]		60	36	4
Longitudinal				
cardiovascular event† (n=22)	390 µg/L (77-1348)	50	20	30‡
no cardiovascular event (112)	466 µg/L (139-1545)	56	37	7

† Cardiovascular event: see text for definition
* P = 0.226 (% O/O in South Asians compared to Caucasians)
‡ P = 0.005 (% O/O in subjects with cardiovascular event compared to subjects without cardiovascular event)

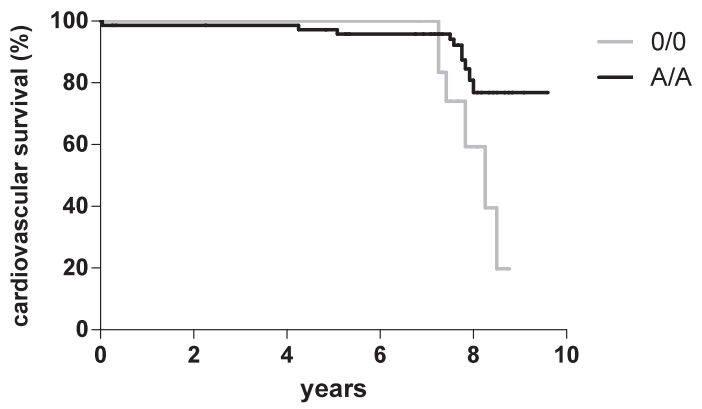
Table 3 MBL and risk for cardiovascular events

	Hazard Ratio	95% CI
MBL genotype		
A/A (n=74)	1	
A/O (n=42)	0.65	0.20-2.07
O/O (n=13)	3.43	1.24-9.49
Combined A/O and O/O	1.26	0.52-3.04
Log MBL level (per log MBL increase)		
Crude	0.93	0.50-1.73
Adjusted[a]	1.19	0.61-2.30

[a] Adjusted for urinary albumin-to-creatinin ratio, log high sensitivity CRP, waist-to-hip ratio, smoking status, ratio total cholesterol: HDL-cholesterol, systolic blood pressure, age, sex, HbA1c, diabetes duration

(IQR 7.48-8.10) years. Participants lost to follow-up did not differ in baseline characteristics from participants for whom follow-up data were available (table 1).
During follow-up, 31 cardiovascular events occurred in 22 subjects (11 men, 11 women): 3 sudden cardiac deaths, 2 fatal and 5 non-fatal myocardial infarction, 13 percutaneous coronary interventions, and 8 coronary artery bypass graft procedures. Eight of these 22 subjects had already experienced a cardiovascular event at baseline.
Twelve subjects died due to non-cardiovascular causes. These patients were censored, none of them reached the primary end-point before dying.
Compared to the wild-type genotype, the O/O genotype was significantly associated with the occurrence of a cardiovascular event (hazard ratio 3.42, 95%CI 1.24-9.48, P = 0.018) (figure 1). Subjects with the O/O genotype did not differ in lipid parameters or blood pressure compared to subjects with the A/A or A/O genotype. The A/O genotype was not associated with cardiovascular events (HR 0.65, 95% CI 0.20-2.07, P = 0.456). Cardiovascular events were also associated with a previous cardiovascular events (HR 4.3, 95% CI 1.2-10.3) and log urinary albumin/creatinine ratio (HR 1.58, 95% CI 1.0-2.48).

Figure 1



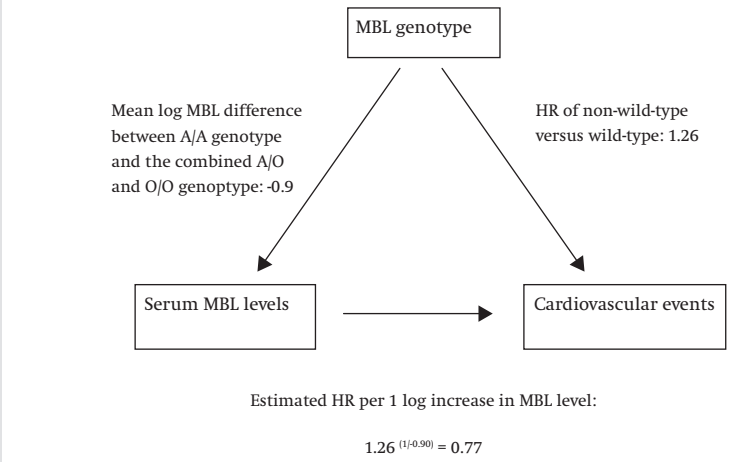
Unadjusted Kaplan-Meier survival curves according to MBL genotype (black line = A/A (wild type) genotype, grey line = O/O genotype. The O/O genotype has a worse event-free survival compared to the A/A genotype (Log rank test P = 0.011).

There was no statistically significant difference in baseline median MBL level between subjects experiencing a cardiovascular event during follow-up and subjects without a cardiovascular event (390 μg/L (IQR 77-1348 μg/L) versus 466 μg/L (IQR 139-1545 μg/L), P = 0.674). Log-transformed MBL levels were not associated with the occurrence of cardiovascular events (hazard ratio 0.93, 95% CI 0.50-1.73). MBL levels above the median were not associated with cardiovascular events (hazard ratio 0.94, 95% CI 0.40-2.20). Using other MBL cut-off levels also failed to show an association with cardiovascular events (data not shown).

We used the MBL genotype as an instrumental variable to estimate the causal effect of MBL levels on cardiovascular events (Mendelian Randomization [19], figure 2). The rationale in Mendelian randomization is that, if an exposure is causally related to an outcome, a genetic variant, which is associated with the exposure, should have a similar relation to the outcome as the supposedly causal exposure itself. If the association with the outcome differs between genetic variant and genetic product, residual confounding or reverse causation (the outcome influencing the exposure) is present.

Because of the small number in the O/O genotype and the relatively small difference in mean log MBL level between the A/O and O/O genotype, we combined the A/O and the O/O genotype and compared this to the A/A genotype. Given the mean log MBL difference of 0.9 μg/L between the A/A and the combined A/O and O/O genotype and the HR for cardiovascular events of 1.26 for the combined A/O and O/O genotype with the A/A genotype as reference, the causative effect of MBL level on cardiovascular events was estimated, resulting in an HR of 0.77 per log MBL increase (Figure 2)

Figure 2



Calculation of the estimated causative hazard ratio of MBL levels on cardiovascular events, given the observed mean difference in log MBL level (between the A/A genotype and the combined A/O and O/O genotype) and the observed association between the genotype and cardiovascular events

Discussion

The main finding of the present study is that in type 2 diabetic South Asians, the O/O MBL genotype was significantly associated with the occurrence of cardiovascular events compared to wild-type.

The association between low MBL genotype and cardiovascular events was previously reported in the Strong Heart Study [12]. This study included American Indians, which - like South Asians- have a high burden of diabetes and subsequent vascular complications. A low MBL genotype was associated with a threefold increased risk for coronary heart disease.

With respect to serum MBL levels and cardiovascular events, data are more controversial. Cross sectional studies found higher MBL levels in type 1 and type 2 diabetic Caucasians with a previous cardiovascular event compared to

diabetic subjects without cardiovascular disease [10,11]. In non-diabetic Caucasian males but not in females, high MBL levels were associated with future cardiovascular events [20]. In type 2 diabetic Caucasians high MBL levels were associated with increased all cause mortality, although data with respect to cardiovascular events were not reported [11]. In contrast, the prospective Reykjavik study found that in type 2 diabetic subjects low rather than high MBL levels were associated with increased incidence of myocardial infarction [8]. Recently, the Strong Heart Study provided data on MBL levels, confirming that low baseline MBL levels indeed were associated with future cardiovascular events [21]. In our study, MBL levels were not associated with future cardiovascular events.

A possible explanation for an association between low MBL levels and cardiovascular events might be a defective clearance of atherogenic particles. MBL binds N-acetylglucosamine moieties, which are expressed on several lipoproteins and oxidized LDL [22], and this may facilitate their phagocytic clearance. This hypothesis is supported by a recently published study showing that MBL deficient subjects have impaired clearance of triglyceride-rich lipoproteins [23]. Additionally, MBL deficiency might influence the susceptibility and course of infection with *Chlamydia pneumoniae*, which is associated with coronary artery disease [24]. On the other hand, MBL levels may increase in the setting of an inflammatory response [9]. Experimental studies show that in the setting of ischemia/reperfusion injury high MBL levels are detrimental rather than protective [25]. Oxidative stress induces a change on the cellular surface [26], which results in binding of MBL leading to enhanced complement mediated injury. However, since MBL levels were not correlated with plasma SC5b-9 levels in our study, we found no evidence that high MBL levels result in increased complement activation.

Summarizing the above, whereas most cross-sectional studies found an association between cardiovascular disease and high MBL levels, most prospective studies show an association between low MBL levels and cardiovascular events.

In our study, low MBL genotype was associated with cardiovascular events and MBL genotype corresponds with MBL level. One would therefore expect low MBL levels to be associated with cardiovascular events, which however was not the case in our study. MBL genotype probably is a more accurate estimate of cumulative MBL exposure than a single serum MBL level. In addition, the contribution of MBL to vascular disease might differ according to the pathophysiologic phase: early in the course low MBL levels might promote

atherosclerosis, and once a vascular inflammatory response is established MBL levels might secondarily become increased and - perhaps - subsequently promote vascular inflammation. A recent experimental study demonstrated local MBL synthesis in early atherosclerotic plaques [27], supporting the hypothesis that MBL levels might become increased due to atherogenesis. Based on the assumption of time-dependency of the association between MBL and cardiovascular disease, our single baseline sample might have been too late in the pathophysiologic course to detect the effect of low MBL level on cardiovascular outcome. In addition, variations of the MBL assay might contribute to the discordant findings between MBL genotype and MBL level. Furthermore, because we did not include the MBL promoter genotype, the genotype-phenotype relation might be attenuated. Finally, at least theoretically, the association of low MBL genotype with cardiovascular events might be based on an association with other susceptibility genes for cardiovascular events.

Noteworthy, although the O/O genotype was associated with cardiovascular events, the A/O genotype was not, although the difference in median MBL level between the O/O and A/O genotype was relatively small. The deleterious effect of low MBL level may only become apparent below a critical MBL value.

To further assess the causative effect of low MBL levels on cardiovascular events, we estimated the effect of low MBL levels on cardiovascular events based on the relation between MBL genotype and cardiovascular events using a Mendelian Randomization approach [19]. In this approach, it is expected that the association between the genetic variant and the outcome (cardiovascular events) is equal to the association between exposure levels (MBL levels) and outcome. Differences between the expected and observed association are caused by residual confounding or reverse causation (that is, the disease influences the exposure levels). In our data, the expected HR per log MBL increase was 0.77, whereas the observed HR was 0.93. This suggests that, based on a single serum value, the association of low MBL levels and cardiovascular events is attenuated. Reverse causation (MBL levels being increased because of vascular inflammation) might account for this.

In conclusion, low MBL genotype is associated with cardiovascular events in type 2 diabetic South Asians, suggesting that MBL is involved in the pathogenesis of cardiovascular events. However, single serum MBL concentrations were not associated with cardiovascular events and therefore a single MBL level is not a clinically useful risk marker for cardiovascular events in type 2 diabetic South Asians.

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4

Complement activation by tubular cells is mediated by properdin binding

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Abstract

Activation of filtered complement products on the brush border of the tubular epithelium is thought to be a key factor underlying proteinuria-induced tubulointerstitial injury. However, the mechanism of tubular complement activation is still unclear. Recent studies on mechanisms of complement activation indicate a key role for properdin in the initiation of alternative pathway. We hypothesized that properdin serves as a focal point for complement activation on the tubulus.

We observed a strong staining for properdin on the luminal surface of the tubules in kidney biopsies from patients with proteinuric renal disease. In vitro experiments revealed dose-dependent binding of properdin to PTEC whereas no significant binding to endothelial cells was detected. Exposure of PTEC with normal human serum as a source of complement resulted in complement activation with deposition of C3 and generation of C5b-9. These effects were virtually absent with properdin deficient serum. Pre-incubation of PTEC with properdin before addition of properdin-depleted serum fully restored complement activation on the cells, strongly suggesting a key role for properdin in the activation of complement at the tubular surface.

In proteinuric renal disease, filtered properdin may bind to PTEC and act as a focal point for alternative pathway activation. We propose that this contribution of properdin is pivotal in tubular complement activation and subsequent damage. Interference with properdin binding to tubular cells may provide an option for the treatment of proteinuric renal disease.

Introduction

Worldwide, the number of patients suffering from chronic kidney disease (CKD) is increasing dramatically [1]. The two most important factors contributing to the global rise in CKD are ageing of the population and the epidemic of type 2 diabetes mellitus [2]. It has been well established now that in chronic kidney disease, regardless of the aetiology, proteinuria is a strong and independent predictor for the progression of chronic renal failure to end-stage renal disease (ESRD) [3,4]. Anti-proteinuric treatment is associated with preservation of renal function [5,6].

Several pathophysiologic mechanisms have been proposed to account for proteinuria-induced tubulointerstitial injury. These include lysosomal rupture due to reabsorbed proteins, oxidative damage induced by transferrin reabsorption, and the stimulatory effects of various plasma proteins on the expression of proinflammatory and profibrotic mediators in renal tubular epithelial cells [7-9]. There is accumulating evidence for complement activation as a powerful mechanism underlying the progression of proteinuric renal disease.

In the setting of proteinuria, plasma complement components may enter the tubular lumen [10]. If these complement components are then locally activated this would lead to cell activation and resulting tubular damage and interstitial fibrosis [11,12]. Indeed, proximal tubular epithelial cells (PTEC) activate serum complement *in vitro* via the alternative pathway [13,15]. Also *in vivo*, both in human chronic proteinuric disease and in experimental models, evidence of complement activation can be detected on the apical surface of the renal tubules [14,16,17]. The protective effect of C6 deficiency in the puromycin model of nephrotic syndrome, as well as in the remnant kidney model, provides further evidence for the role of complement in mediating tubulointerstitial injury [16,18]. Targeting complement inhibitory molecules to the proximal tubules in a rat model of proteinuric kidney disease protects against renal dysfunction [19].

However, the exact mechanism of the unique complement activating property of the proximal tubules has not yet been elucidated. Previous studies reported a role for local ammonium (NH_4) in initiating alternative complement pathway activity [20,21]. We hypothesize that besides ammonium, other mechanisms might be involved in triggering tubular complement activation.

The alternative pathway of complement is triggered by spontaneous hydrolysis

of C3, which generates C3a and C3b. Cleavage of C3 results in the formation of a positive feedback loop to produce a rapid local response [22]. Properdin, discovered in 1954 by Pillemer *et al.*[23], is the only known positive regulator of the complement system and consists of dimers, trimers and tetramers arranged in a head-to-tail orientation[24,25]. Properdin binds to C3b and enhances complement activation by stabilizing the alternative pathway C3 convertase[26]. Lately, there has been renewed interest in properdin. It was shown that target-bound properdin may serve as a focal point for amplification of C3 activation. Each subunit in the oligomer provides a ligand-binding site and the unoccupied ligand-binding sites can assemble the alternative pathway convertase on target surfaces [27,28]. It has recently been re-emphasized that properdin may act as a focal point in the activation of the alternative pathway of complement [27-30]. It was suggested already in 1954 that properdin might interact directly with cell surfaces [23,31].
In this study, we show that properdin binds to viable tubular epithelial cells and via this mechanism initiates complement activation.

Materials and methods

Immunohistochemical staining

Frozen 4 μm tissue sections were used to determine the presence of properdin in cortical tissue of human kidneys. After the sections were fixed with acetone, endogenous peroxidase activity was blocked with 0.1% H_2O_2 and 0.1% NaN_3 for 30 min at room temperature (RT). Then the slides were washed and subsequently blocked with phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA) and 5% heat-inactivated normal human serum for 45 min at RT. Next, sections were incubated with a polyclonal rabbit anti-human properdin antibody (Laboratory of Nephrology, Leiden, the Netherlands) in PBS, 1% BSA and 1% normal human serum in a humid atmosphere overnight at RT. After washing with PBS, antibody binding was detected with horseradish peroxidase (HRP)-labeled goat anti-rabbit Ig (DAKO, Glostrup, Denmark) in PBS, 1% BSA and 1% normal human serum (60 min RT) followed by washing with PBS, incubation with Tyramide-fluorescein isothiocyanate in tyramide buffer (NEN™ Life Science Products, Boston, MA, USA; 20 min RT), washing with PBS, incubation with HRP-conjugated rabbit anti-fluorescein isothiocyanate (DAKO) for 60 min at RT, washing with PBS and development with DAB (Sigma, St Louis, MO, USA).

Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany) and mounted with imsol (Klinipath, Duiven, The Netherlands).

Cell culture

The immortalized renal proximal tubular epithelial cell-line HK-2 was kindly provided by M. Ryan, University College Dublin, Ireland [32]. Cells were grown in serum-free DMEM/HAMF12 (Bio-Whittaker, Walkersville, MD) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Breda, the Netherlands), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), tri-iodothyronine (40 pg/ml), epidermal growth factor (10 ng/ml), hydrocortisone (36 ng/ml, all purchased from Sigma). Primary human proximal tubular epithelial cells (PTEC) were isolated from pre-transplant biopsies or from kidneys not suitable for transplantation and cultured as described earlier (33). HUVEC were isolated from umbilical cords as described previously [34]. Cells were cultured on a matrix of fibronectin in M199 medium containing 20% heat-inactivated FCS, 100 U/ml penicillin, 100 ug/ml streptomycin, 50 ug/ml Bovine Pituitary Extract (all from Invitrogen) and 10 U/ml heparin (LEO Pharma B.V., Breda, the Netherlands). The cell lines ECRF-24, Jurkat, HL-60 and U937 were cultured as described earlier [35,36].

Isolation of properdin

Properdin was isolated from pooled human donor serum. First, a precipitation step was performed by dialyzing the serum against water containing 5 mM EDTA, pH 6.0. The resulting precipitate was dissolved in Veronal-buffered saline (2x VBS, 1.8 mM Na-5,5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl), dialyzed against 0.01 M NaAc containing 2mM EDTA, pH 6.0 and applied to a Sulphopropyl Sephadex C50 cation exchange colomn (Pharmacia Biotech, Uppsala, Sweden). Properdin was eluted from the column with a linear salt gradient. Properdin-containing fractions, as determined by enzyme-linked immunosorbent assay (ELISA), were pooled, concentrated, and subsequently applied to a Sephacryl S-300 gel filtration column (Pharmacia), after which properdin-containing fractions were pooled. In order to remove contaminating C1q from the preparation, the properdin-pool was dialyzed against PBS, 2 mM EDTA and further purified using human IgG coupled to a Biogel A5 column (Bio-Rad, Hercules, CA). Purity of the properdin preparation was confirmed by analysis on 10% non-reducing SDS-PAGE gel. A single band of 220 kDa was observed.

Serum preparation

Normal human serum was depleted of properdin by immune adsorption using Biogel-coupled anti-human properdin monoclonal antibodies (a gift of State Serum Institute, Copenhagen, Denmark). The properdin-depleted serum showed normal classical and lectin pathway activity in hemolytic assay. C4-depleted serum, which lacks both classical and lectin pathway activity, was prepared by affinity adsorption using goat anti-human C4 IgG coupled to CNBr-activated Sepharose 4 Fast Flow (Amersham Bioscience Europe, Roosendaal, the Netherlands). After C4 depletion, the serum was free of C4 antigen and classical pathway hemolytic activity could be restored fully by purified hemolytically active C4.

FACS analysis

Deposition of complement on cells was determined by flow cytometry. Properdin binding to the cells was visualized using a polyclonal rabbit anti-human properdin antibody followed by RPE-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, US). Deposition of C3, C5b-9, C1q, and MBL on the cells was detected using a mouse monoclonal anti-human C3 antibody (RFK22, Laboratory of Nephrology, Leiden, the Netherlands), anti-human C5b-9 (mAb AE11, kindly provided by Dr. T.E. Mollnes, Nordland Central Hospital, Bodo, Norway), anti-human C1q (mAb 2204, kindly provided by Dr. C.E. Hack, Sanquin Research, Amsterdam) and anti-human MBL (mAb 3E7, kindly provided by Dr. T. Fujita, Medical University School of Medicine, Fukushima, Japan) respectively, followed by RPE-conjugated polyclonal goat anti-mouse Ig (DAKO). All antibody incubations were performed on ice for 30 min. Cell surface staining was assessed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Propidium iodide (1μg/ml, Molecular Probes, Leiden, the Netherlands) was used for exclusion of dead cells.

Properdin binding and complement activation assays

For FACS experiments, cells were grown to confluence in 48-well tissue culture plates. HK-2 cells and HUVEC were exposed to 20% normal human serum diluted in serum-free DMEM/HAMF12 for 2 h at 37°C. C3, C5b-9, C1q, MBL and properdin were assessed on the cell surface by FACS analysis. Alternative pathway mediated complement activation by HK-2 was tested by incubating the cells with 20% normal human serum in the presence of 5 mM Mg EGTA. Properdin binding to HK-2, primary PTEC, HUVEC, ECRF-24, U937, HL-60 and

Jurkat was assessed by incubating the cells with purified human properdin (20 μg/ml) diluted in serum-free DMEM/HAMF12 for 1 h at 37°C. Dose-dependent properdin binding to HK-2 and HUVEC was tested by incubating the cells with increasing concentrations of human properdin (10 to 40 μg/ml). The functional consequences of properdin binding were determined by incubating the cells with 5% properdin-depleted, normal human serum or C4-depleted human serum as a complement source, diluted in serum-free DMEM/HAMF12 culture medium, for 2 h at 37°C after pre-incubation with properdin. Following properdin and/or serum incubation, the cells were washed twice in PBS, harvested by scraping and resuspended in FACS-buffer (1% BSA and 0.02 % sodium azide in PBS) for FACS staining.

Results

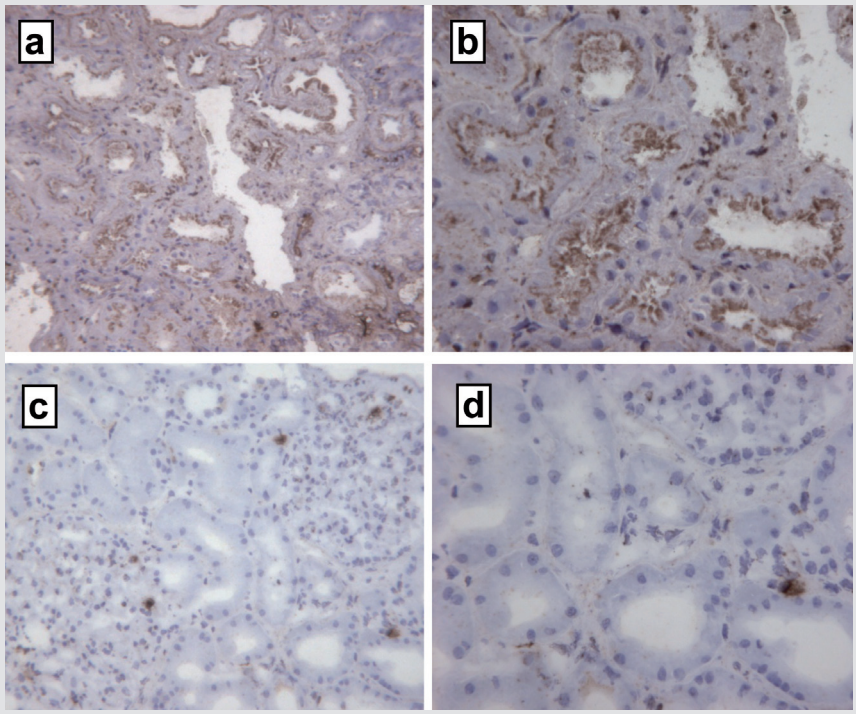
Properdin is present on the tubular brush border in proteinuric kidneys

The presence of properdin on the brush border of the proximal tubules was determined in renal biopsies of three patients with membranous nephropathy and in pretransplant renal biopsies of three living related kidney donors. Properdin could be detected along the brush border of the tubules in diseased kidneys, whereas properdin was absent in the tubules of healthy kidney tissue (Figure 1). Since the presence of properdin on the tubular brush border of proteinuric kidneys does not distinguish where in the cascade of complement activation properdin comes in, we proceeded to *in vitro* studies to determine whether properdin is an initiating factor in tubular complement activation.

Complement activation by HK-2 cells

Incubation of Human Kidney-2 (HK-2) cells with normal human serum resulted in fixation of complement products on the cell surface. C3, C5b-9 and properdin, but not C1q and mannan-binding lectin (MBL) could be detected (Figure 2a). The complement system was activated on the cell surface via the alternative pathway since deposition of C3 and C5b-9 was unaffected by Mg EGTA, which interferes with the classical and lectin pathway of complement by chelating calcium (Figure 2b). In contrast, complement fixation was completely blocked by EDTA, which inhibits all three pathways of complement activation. C3 and C5b-9 deposition was also detected on HK-2 cells after exposure to C4-depleted

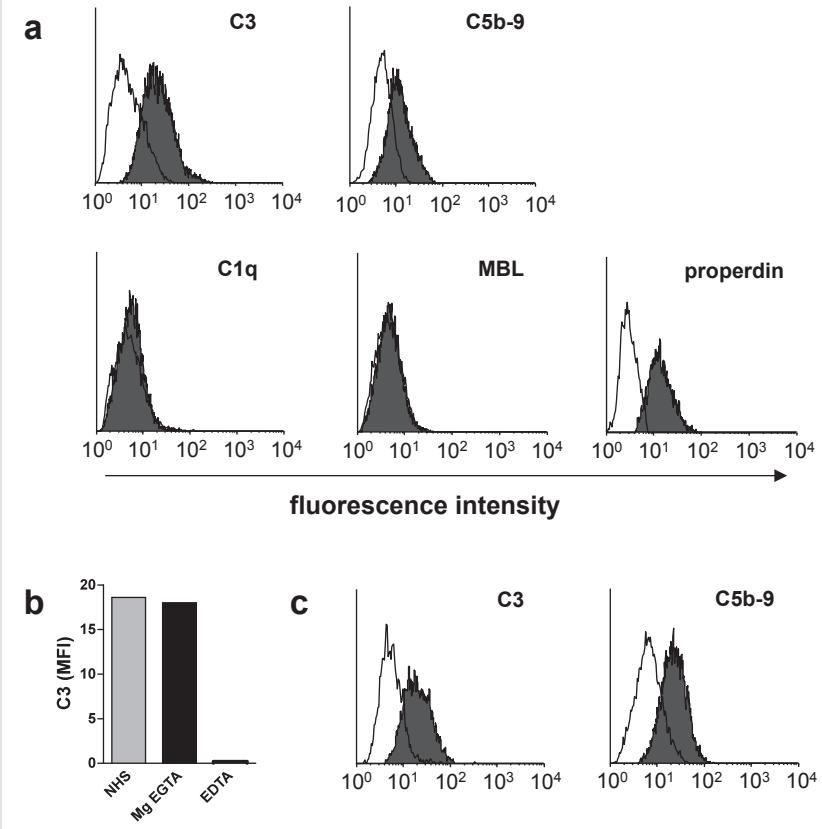
Figure 1 Properdin staining on the tubular brush border in proteinuric kidneys



Cryosections of (**a** and **b**) a renal biopsy of a patient with membranous nephropathy and (**c** and **d**) a pretransplant biopsy of a healthy donor were stained immunohistochemically for properdin. (**a** and **c**) Original magnifications were either × 100 or (**b** and **d**) × 250. Pictures are representative for three patients with membranous nephropathy and three healthy kidneys donors.

human serum, which excludes involvement of the classical or lectin pathway (Figure 2c). To assure that complement activation was localized to the apical surface, serum incubations were performed on cells that were grown to confluence in a tissue culture plate. Human umbilical vein endothelial cells (HUVEC) were used as a control. No complement deposition was observed on these cells after treatment with normal human serum.

Figure 2 Complement activation by HK-2 cells



(**a**) HK-2 cells were incubated with 20% normal human serum (NHS). C3, C5b-9, C1q, mannan-binding lectin (MBL) and properdin binding (filled histograms) were assessed on the cells using the mAbs RFK22, AE11, 2204, 3E7 and a polyclonal rabbit anti-properdin antibody, respectively. Open histograms show staining on cells that were not exposed to serum. (**b**) C3 deposition was assessed on HK-2 cells after incubation with 20% human serum in the presence or absence of 5mM Mg EGTA or 10 mM EDTA. Results are expressed as the mean fluorescence intensity, MFI. (**c**) C3 and C5b-9 deposition on HK-2 cells after exposure to 20% C4-depleted human serum.

Binding of properdin to HK-2 cells

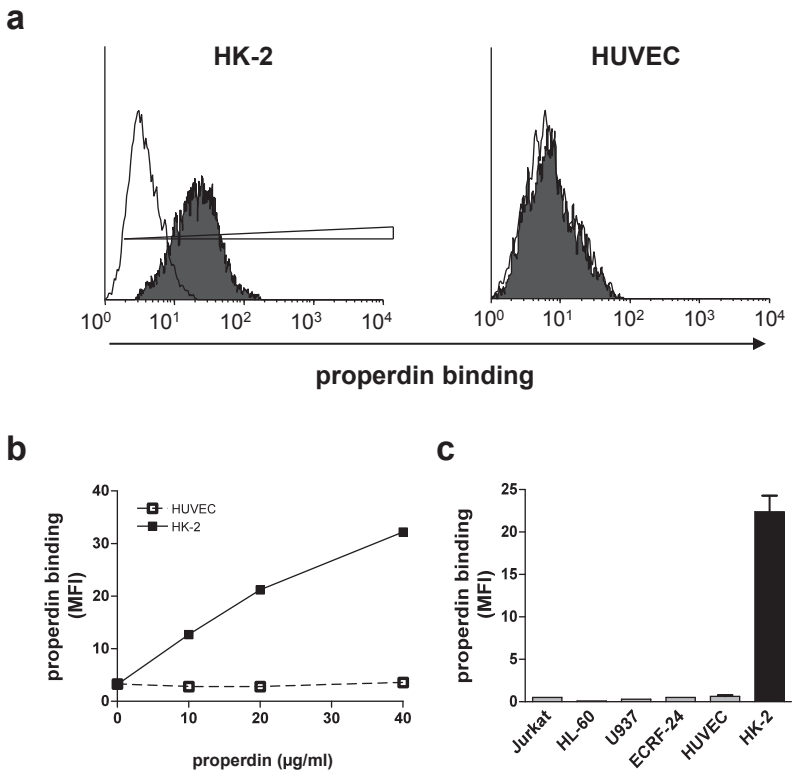
We then questioned whether properdin could bind to tubular cells prior to the activation of complement and the deposition of its known ligand C3b. In order to study binding of properdin to the cell surface, confluent cells in a tissue culture plate were incubated with purified human properdin at a concentration of 20 μg/ml. Properdin binding was analysed by flow cytometry. Only cells which were negative for propidium iodide staining were analysed in order to exclude properdin binding to dead cells. As shown in Figure 3a, strong binding of properdin to viable HK-2 cells was detected, whereas no significant binding was shown on HUVEC. As a negative control, the fluorescence intensity of cells incubated with detection antibody only, i.e., without pre-incubation with properdin, is shown. Properdin binds to viable HK-2 cells in a dose-dependent manner (Figure 3b). The cell lines HL-60, U937 (monocytes), Jurkat (T-cell leukaemia) and ECRF-24 (immortalized HUVEC) were all negative for properdin binding (Figure 3c).

Properdin binding is a focal point for alternative pathway activation on HK-2 cells

Next, we investigated whether properdin, after binding to the tubular surface, acts as a focal point for local amplification of the alternative pathway of complement. To demonstrate properdin-dependent complement activation, deposition of C3 and C5b-9 was assessed on HK-2 cells and HUVEC incubated with properdin-deficient normal human serum, with and without pre-incubation of the cells with purified properdin. HK-2 cells incubated with properdin-depleted serum show a strongly reduced C3 deposition compared to cells exposed to normal human serum. This is accompanied by a strong reduction of C5b-9 deposition. Complement activation was restored completely on cells that had been pre-incubated with properdin, prior to exposure to properdin-deficient serum (Figure 4a and b). HUVEC showed no significant complement activation, both with and without pre-incubation with purified properdin. This indicates that properdin, bound to the cellular surface of HK-2, initiates and targets the amplification of the complement cascade to the surface of tubular cells.

To confirm that complement activation on HK-2 is properdin-dependent, cells were pre-exposed to different concentrations of purified properdin, ranging from 2,5 to 40 μg/ml, before incubation with 5% normal human serum. Properdin was shown to increase the deposition of both C3 and C5b-9 on HK-2

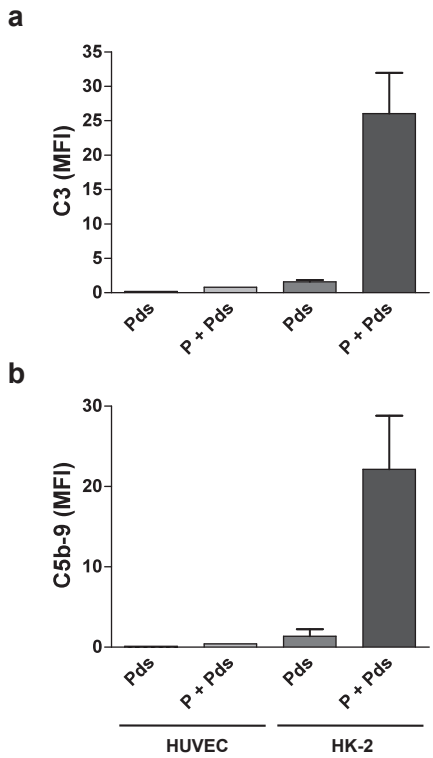
Figure 3 Properdin binding to HK-2 cells



(**a**) HK-2 cells and human umbilical vein endothelial cells (HUVEC) were incubated with 20 µg/ml purified human properdin. Binding of properdin (filled histograms) was detected with a polyclonal rabbit anti-human properdin antibody followed by goat anti-rabbit conjugated with PE. As a negative control, staining with both primary and secondary antibody was performed on cells that were not exposed to properdin (open histograms). (**b**) Dose-dependent binding of properdin to HK-2 and HUVEC is shown as the mean fluorescence intensity (MFI). Data are representative for two individual experiments. (**c**) Binding of properdin (shown as the mean fluorescence intensity, MFI) to the cell lines Jurkat, HL-60, U937, ECRF-24, HUVEC and HK-2. Data are expressed as the mean ± SD of three independent experiments.

in a dose-dependent way (Figure 5a). Similar dose-dependent effects were detected when increasing concentrations of properdin were added prior to incubation with C4-depleted human serum (Figure 5b).

Figure 4 Properdin-dependent complement activation

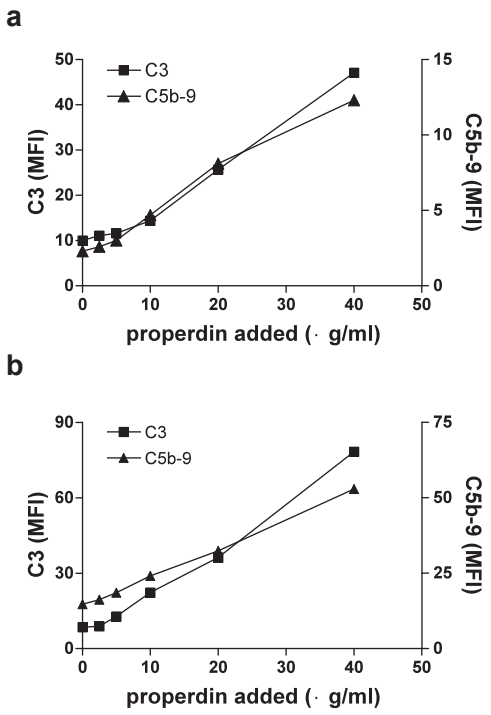


HK-2 cells and human umbilical vein endothelial cells (HUVEC) were pre-incubated with properdin (P, 20 μg/ml), washed and subsequently exposed to 5% properdin-depleted human serum (Pds). (**a**) C3 and (**b**) C5b-9 deposition (shown as the mean fluorescence intensity, MFI) was detected on the cells using the mAbs RFK22 and AE11, respectively. The results are expressed as the mean ± SD of three independent experiments.

Properdin binding and complement activation on PTEC

In order to test whether the PTEC cell line HK-2 is representative for primary PTEC lines, properdin binding and properdin-dependent complement activation was assessed on primary PTEC cultures. As shown in Figure 6a, properdin binding on PTEC is comparable to HK-2 (Figure 3a). The six tested PTEC cell lines showed variability in properdin binding (Figure 6b). None of the three

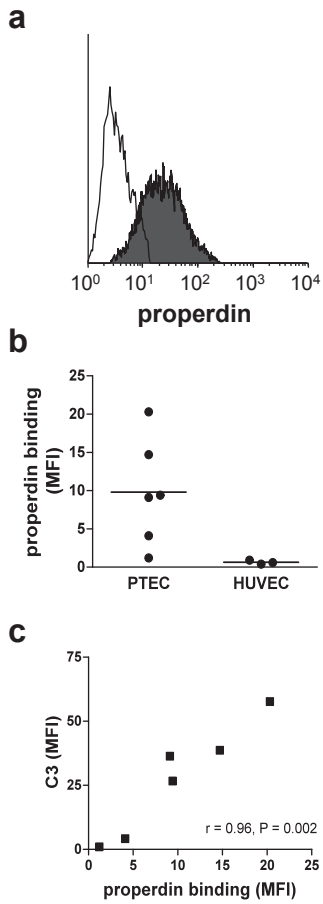
Figure 5 Dose-dependent effect of properdin on complement deposition



HK-2 cells were pre-incubated with increasing concentrations of human properdin. After extensive washing, cells were exposed to (**a**) 5% normal human serum or (**b**) 5% C4-depleted human serum. Complement deposition (expressed as the mean fluorescence intensity, MFI) was assessed by flow cytometry using mAbs RFK22 and AE11 for staining of C3 and C5b-9, respectively. Results represent one out of two experiments.

HUVEC cell lines showed significant binding of properdin. However, the extent of properdin binding to PTEC was strongly correlated with the level of C3 deposition on these cells, r = 0.96 / p = 0.002 (Figure 6c).

Figure 6 Properdin-mediated complement fixation on primary PTEC



(**a**) Primary proximal tubular epithelial cell (PTEC) lines were analysed for properdin binding (filled histogram) by flow cytometry after incubation with 20 μg/ml purified human properdin. The open histogram shows staining on cells that were not incubated with properdin. (**b**) Binding of properdin to different PTEC and human umbilical vein endothelial cell (HUVEC) lines. Properdin binding is expressed as the mean fluorescence intensity (MFI). The background fluorescence (primary and secondary antibody without properdin pre-incubation) is subtracted for each cell line individually. (**c**) Properdin binding and resulting properdin-dependent complement activation was tested by incubating the cells with 5% properdin-depleted human serum (Pds) after pre-exposure to 20 μg/ml purified properdin. Properdin binding and C3 deposition is shown as the mean fluorescence intensity (MFI). The association between properdin binding and the level of C3 deposition was analysed by calculating the Pearson correlation coefficient.

Discussion

In the present study, we show that properdin binds to the surface of viable PTEC. Properdin binding serves as a focal point for local amplification of the alternative pathway of complement on PTEC and explains the complement activating capacity of these cells.

It has been known for a long time that the apical surface of human proximal tubular epithelial cells activates the complement system *in vitro* and *in vivo* via the alternative pathway [13,14]. In patients suffering from chronic proteinuric renal disease, deposition of complement along the tubular brush border is accompanied by tubulointerstitial injury and progressive loss of renal function. Experimental models of non-selective proteinuria provide further evidence for the role of tubular complement activation in mediating tubulointerstitial injury [17,19]. C6 deficiency protects kidney function in the remnant kidney model as well as in the puromycin-induced model of nephrotic syndrome [16,18].

Although in physiological conditions complement components are not filtered through the glomerular barrier, several studies demonstrated the presence of complement activation products (CAP) in the urine of patients with nephrotic syndrome due to a variety of causes [10,37-39]. These studies showed a positive correlation between tubular C3 fixation and the excretion of complement components as well as complement activation products (including iC3b, Bb and C5b-9) in the urine. Interestingly, the level of urinary CAP excretion was significantly decreased after two weeks of oral sodiumbicarbonate administration (38;40). The protective effect of bicarbonate was suggested to be due to lowering of the tubular ammonium concentration but may also be explained by a direct effect of increasing the urinary pH [41].

Despite extensive research, the mechanism of complement activation on the tubular brush border has not yet been fully elucidated. It was suggested that local ammonium reacts biochemically with the thioester of C3 and thereby acts as a C3 activator [20,21]. However, the addition of ammonium to serum only resulted in 15% increase in lysis of rabbit erythrocytes. This weak effect of ammonium on complement activation was only present in the lower concentration range. At higher concentrations, ammonium inhibited the alternative pathway. Recently, the activation of complement in proximal tubule cells was studied using proteinuric urine [41]. Increasing concentrations of ammonium resulted in an inhibition of complement activation. Ammonium

excretion obviously does not fully explain the propensity of the renal tubule cells to activate the complement system.

Others have suggested that the lack of complement regulatory molecules on the apical surface of PTEC may explain the capacity of these cells to activate complement. Indeed, CD46 (membrane cofactor protein, MCP) only seems to be expressed on the basolateral surface of PTEC and CD55 (decay accelerating factor, DAF) could not be detected at all [42,43]. On the other hand CD59 is expressed abundantly on PTEC and surface expression of both CD46 and CD55 were detected on a PTEC cell line [44].

We suggest that the unique properdin binding capacity of PTEC critically controls the tubular complement activation in proteinuric states. In 1974, Sato *et al.* described that the damaging effect of intraluminally perfused normal rat serum on the rat kidney proximal tubule could be abolished by pre-incubating the serum with a brush border membrane fraction [45]. Possibly the effect of pre-inbcubation with the brush border membrane fraction is explained by its capacity to absorb properdin.

It was recently re-emphasized that properdin, the only known naturally occurring positive regulator of complement, can act as a focal point for alternative pathway amplification [27,28], thereby directing complement activation to the cell surface of apoptotic and necrotic cells [29,46]. Several decades before, Pillemer *et al.* suggested that properdin might also interact directly with target surfaces [23,31] Likewise, we hypothesized that properdin might be the activator of the alternative pathway on the tubular brush border by interacting with molecules present on the cell membrane.

At the moment, the ligand on PTEC that mediates the interaction with properdin has not yet been identified. Properdin has been shown to bind to surface-bound C3b via one of its subunits followed by the assembly of the alternative pathway convertase at the ligand-binding sites of the adjoining subunits [27]. At the moment we can not fully exclude that properdin binds to PTEC via cell-bound C3b that is derived from endogenously produced and activated C3. Although C3b is undetectable by flow cytometry on PTEC, it might be present below the detection limit. On the other hand, it seems unlikely that significant amounts of C3b are present on quiescent cells. Recent data suggest that properdin also binds to the glycosphingolipid sulfatide [47]. The presence of sulfatide on the brush border of the tubules has been demonstrated in the rat kidney [48]. It is likely that these molecules are also expressed on the tubules in the human kidney, where they may mediate properdin binding to PTEC.

The mechanism by which a sublytic dose of C5b-9 on PTEC leads to tubular damage and subsequent tubulointerstitial fibrosis is thought to be via activation of proinflammatory and fibrogenic pathways [4]. Insertion of C5b-9 into the cell membrane of PTEC results in the production of proinflammatory cytokines and collagen synthesis. Interestingly, PTEC have been shown to synthesize a functional alternative pathway of complement, which is capable of activating the cells [49]. This intratubular complement activation is tightly regulated and probably plays a role in protecting the kidney from urinary tract infections. Since the apical tubular surface does not come into contact with high concentrations of plasma proteins in normal physiology, protection against circulating complement is of less importance compared to circulating cells and the endothelium. However, in proteinuric renal disease, the tubules are exposed to filtered complement components. In these circumstances, the complement activating capacity of PTEC is harmful, especially since the apical surface has virtually no protection against complement attack [42].
Our data show that properdin binding to the brush border is the rate-limiting step in tubular complement activation. Targeting the interaction between properdin and the tubular brush border might be a therapeutic approach for controlling tubulointerstitial injury, thereby preventing progressive loss of kidney function in patients with chronic proteinuric renal disease.

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5

Urinary Properdin excretion is associated with intrarenal complement activation and poor renal function

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Abstract

Background

Proteinuria predicts progressive renal failure. Next to being a progression marker, non-selective proteinuria itself is thought to be toxic to the tubulointerstitium. In proteinuric states, activation of filtered or locally produced complement is toxic for renal tubular cells and likely contributes to the progression of renal failure. Recent experimental evidence suggests an important role for properdin in promoting intrarenal complement activation. We measured properdin in proteinuric urine and assessed its relation with urinary SC5b-9 levels, the soluble form of the effector phase of complement activation.

Methods

Seventy patients with renal disease of different origin but all with a protein excretion of at least 1 gram/day were studied. Urinary properdin and SC5b-9 levels were measured using an ELISA technique.

Results

Properdin was detectable in the urine of 37 patients (53%). These subjects had higher urinary SC5b-9 levels (median 0.50 U/ml (IQR 0.13-1.81) versus 0.049 U/ml (IQR 0.024-0.089), P<0.001). When adjusted for proteinuria and renal function, properdin excretion was strongly associated with increased urinary SC5b-9 levels (Odds Ratio 16.2, 95%CI 3.6-74.4) Properdin excretion was associated with worse renal function.

Conclusion

Our results suggest that urinary properdin excretion enhances intrarenal complement activation and thus may contribute to the progression of renal damage in proteinuric states.

Introduction

Proteinuria is a prognostic marker in renal disease. Besides being a marker of renal damage, non-selective proteinuria is thought to be toxic to the tubulointerstitium [1-4]. Activation of filtered or locally produced complement components is likely involved in tubulotoxicity of proteinuria [5;6]. Complement activation products indeed are detectable in urine of patients with different proteinuric renal disease [7-9].

The complement cascade is activated by binding of recognition molecules to their respective target. Immunoglobulins and Mannan Binding Lectin (MBL) are the recognition molecules of the classical and lectin pathway, respectively. The alternative pathway is characterized by spontaneously occurring low-grade complement activation [10], and renal proximal tubular cells have long been known to activate complement via the alternative pathway [11-14]. Properdin enhances alternative pathway complement activation by stabilizing the alternative pathway C3 convertase [15]. More recent data suggest that properdin also acts as a recognition molecule of the alternative pathway [16-19].

We recently demonstrated a pivotal role for properdin as a mediator of complement activation by proximal tubular epithelial cells (PTECs) [20]. After incubation with normal human serum, complement activation on PTECs was observed, whereas complement activation was absent when PTECs were incubated with properdin deficient serum. Pre-incubation of PTECs with purified properdin before addition of properdin-deficient serum restored the complement activating capacity by PTECs in a dose-dependent manner, indicating that properdin acts as a focal point for complement activation. However, data regarding involvement of properdin in proteinuric renal disease are lacking.

We therefore studied the excretion of properdin in proteinuric kidney disease and assessed its association with urinary excretion of SC5b-9 and renal damage. We show that properdin is present in proteinuric urine, and that its presence is associated with increased SC5b-9 excretion and worse renal function.

Subjects & methods

Between february 2006 and november 2007 all adult patients attending the renal out-patient clinic with a protein excretion of at least 1 gram per day during the last visit were included. All patients gave informed consent. Ten ml

of freshly voided urine (to which 400 µl of a protease inhibitor solution (25 x concentrated solution of Complete Protease Inhibition, Roche, Mannheim, Germany) was added), 10 ml of EDTA blood and 10 ml of serum were collected and immediately put in ice. After 10 minutes centrifugation at 2500 rounds per minute at 4º Celsius, aliquots of the samples were stored at -80º Celsius for later complement measurements. Serum creatinin was measured by Jaffé's method [21]. Urinary albumin was measured by an immunoturbidimetric assay [22], and total protein was measured by a colorimetric method [23].
To secure that at the time of inclusion the patient indeed was excreting at least 1 gram of protein per day, the actual urinary protein excretion was calculated as follows:

$$\frac{\text{creatinin (mmol/24 hour) in 24 h urine collection}}{\text{creatinin (mmol/L) in aliquot}} \times \text{total protein (gr/L) in aliquot}$$

When calculated actual protein excretion was less than 1 gram per day, the patient was excluded from further analysis.

Quantification of SC5b-9

SC5b-9 levels were assessed by sandwich ELISA. In brief, 96-well ELISA plates (Nunc Bioscience, Belgium) were coated with the monoclonal antibody aE11 (mouse IgG2a anti-C5b-9 3mg/ml), which was kindly provided by dr. T. Mollnes and described in detail previously [24]. Plasma and urine samples were diluted 1/5 and 1/20 and incubated in the coated wells. Bound SC5b-9 was detected with a biotin labeled monoclonal anti C6 antibody (9C4), followed by detection with streptavidin-poly horse radish peroxidase (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO). All assays were performed on ice. The optical density was measured at 415nm using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using zymosan activated serum with a known concentration of SC5b-9 of 1000 U/ml

Quantification of properdin

Properdin levels were assessed by a previously described sandwich ELISA with a detection limit of 1.5 ng/ml. [25]. In brief, 96-well ELISA plates (Nunc Bioscience, Belgium) were coated with a polyclonal rabbit anti-human properdin solution generated by immunizing a rabbit with purified human properdin. Serum

(diluted 1:1000) and urine (diluted 1:5) were incubated in the coated wells. Bound properdin was detected with digotonin labeled rabbit anti human properdin, followed by detection with anti digotonin conjugated with horse radish peroxidase (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO). The optical density was measured at 415nm using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using a normal human serum-properdin-standard with a known properdin-concentration of 20 μg/ml.

Statistical analysis

Normally distributed variables are expressed as mean ± standard deviation and skewed distributed variables as median and interquartile range. Differences between groups are assessed by Student's t-test or Mann-Whitney-U test as appropriate. Spearman's correlation was determined for skewed distributed variables. Associations between properdinuria and urinary SC5b-9 were assessed with logistic regression. All tests were two-sided and the level of significance was set at 0.05. All analyses were performed using SPSS for windows, version 15.0.

Results

Seventy patients were suitable for analysis. In 5 of these patients, data on 24 hour protein excretion were missing, but the total protein to creatinin ratio was > 0.33 gr/mmol, confirming that the actual protein excretion is > 1 gram/24 hours and therefore they were also included.

The mean age of the patient population was 55 ± 16 years. The median endogenous creatinin clearance was 39 ml/min (IQR 21-75) and the median actual calculated protein excretion was 3.4 gr/24 hours (IQR 2.1-6.0). There were 18 type 2 and 9 type 1 diabetic subjects suffering from diabetic nephropathy, as diagnosed by a characteristic course of proteinuria and renal deterioration in the presence of diabetic retinopathy. Twenty seven patients had glomerular disease (7 membranous nephropathy, 2 IgA nephropathy, 7 lupus nephritis, 2 secondary Focal Segmental Glomerulosclerosis, 2 ANCA associated vasculitis, 3 Membranoprolipherative glomerulonephritis, 2 minimal change nephropathy, 2 Monoclonal Immunoglobulin Deposition Disease /

amyloidosis). In all these patients diagnosis was based on renal biopsy. Sixteen subjects were categorized as "other": they did not have a renal biopsy and only a presumptive diagnosis was made, mostly nephrosclerosis.
The characteristics of the study population are summarized in table 1.

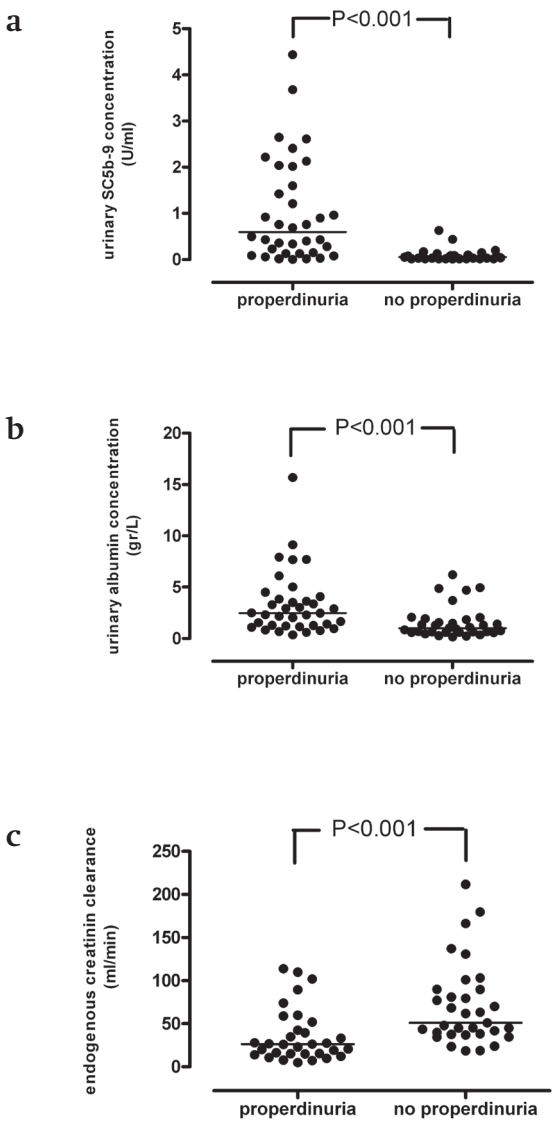
Table 1 Patient characteristics

	All (n=70)	Diabetic nephropathy (n=27)	Glomerular disease (n=27)	Other (n=16)
Age (years)	55 ±16	56 ± 14	51 ± 18	59 ± 4
Endogenous creatinine clearance (ml/min)	39 (21-75)	26 (15-45)	52 (33-103)	40 (26-78)
Calculated protein excretion (g/24h)[a]	3.4 (2.1-6.0)	4.8 (1.9-7.0)	3.7 (2.8-6.9)	2.2 (1.5-2.6)
Urinary SC5b-9 (U/ml)	0.11 (0.003-0.71)	0.36 (0.003-2.01)	0.125 (0.06-0.63)	0.05 (0.03-0.12)
% of subjects with properdinuria	53%	74%	44%	31%

Normally distributed variables are represented as mean ± standard deviation, skewed distributed variables are represented as median (interquartile range in brackets)
[a] see text for details

In 37 patients (53%), properdin (up to 0.39 μg/ml) was detected in the urine. Properdin was not detectable in the urine of 25 healthy subjects without proteinuria. Compared to patients without detectable urinary properdin excretion, patients with urinary properdin had significantly higher urinary SC5b-9 levels (median 0.50 U/ml (IQR 0.13-1.81) versus 0.049 U/ml (IQR 0.024-0.089), P<0.001) (Figure 1a). By logistic regression analysis it was demonstrated that the presence of properdinuria was associated with urinary SC5b-9 levels above the median (OR 16.3, 95%CI 5.0-53.2). When only subjects with properdinuria were considered, urinary properdin and SC5b-9 levels were correlated (Spearman's ρ 0.338, P=0.041)
Compared to subjects without properdinuria, subjects with properdinuria also had more proteinuria (urinary albumin concentration 2.48 gr/L(IQR 1.2-4.0) versus 1.0 gr/L (IQR 0.6-1.9), P<0.001) and worse renal function (endogenous

Figure 1



Subjects with properdinuria have significantly higher median levels of urinary SC5b-9 (figure 1a), a higher degree of proteinuria (figure 1b) and worse renal function (figure 1c) compared to subjects without properdinuria.

creatinin clearance 26 ml/min (IQR 15-50) versus 51 ml/min (IQR 38-90), P<0.001) (figure 1b and 1c, respectively). However, the presence of properdinuria remained strongly associated with urinary SC5b-9 excretion above the median when adjusted for urinary albumin concentration and renal function in a multivariate analysis (OR 16.2, 95%CI 3.6-74.4).

Discussion

In this study, we show that properdin is present in proteinuric urine, and that the presence of urinary properdin is associated with increased SC5b-9 excretion and worse renal function.

Intratubular complement activation is thought to be an important pathway of toxicity to the tubulointerstitium [5;6]. Experimental studies in various proteinuric models show complement activation on tubular cell brush border [26-29]. Inhibition of complement, either by administration of a complement inhibitor or by knock-out of complement components, results in attenuation of tubulointerstitial damage [26;29;30]. In vitro studies show alternative pathway mediated complement activation on cultured proximal tubular cells [11-14], and complement activation has been linked to the induction of renal fibrosis [31;32].

The complement cascade is activated by binding of recognition molecules to their respective target. Immunoglobulins and Mannan Binding Lectin (MBL) are the recognition molecules of the classical and lectin pathway, respectively. The alternative pathway is characterized by spontaneously occurring low-grade complement activation [10], but also serves as an important amplification loop for the classical and lectin pathway [15;33]. Properdin acts as a promoter of complement activation by stabilizing the alternative pathway C3 convertase [15], but more recent data suggest that properdin also acts as a recognition molecule of the alternative pathway [16-19]. Properdin is mainly synthesized by inflammatory cells like monocytes and leucocytes which release their properdin-containing granules upon activation [34,35].

Although the capacity of cultured tubular cells to activate the alternative complement pathway has long been known, the exact mechanism has not been determined. We recently demonstrated a pivotal role for properdin in complement activation by cultured proximal tubular cells [20]. In the present cross sectional study, properdinuria was associated with higher urinary SC5b-9 levels, suggesting that properdin is an important determinant in intratubular

complement activation. Interestingly, our results show that the association of properdinuria with urinary SC5b-9 levels was independent of the degree of proteinuria. Properdinuria was also associated with worse renal function suggesting a role for properdin in proteinuria mediated renal damage.

There are several possible explanations for the observed association of properdinuria and renal dysfunction. Properdin, filtered together with other complement components in glomerular protein leakage, may initially bind to tubular cells with subsequent activation of the alternative pathway. This tubular complement activation would then lead to tubulointertistial damage induced by complement activation products like C3a, C5a and C5b-9 [31;32]. Alternatively, as properdin is mainly synthesized by inflammatory cells such as polymorphonuclear cells, properdinuria might be related to intrarenal recruitment of inflammatory cells, that has been shown to be correlated with renal dysfunction [36]. Diminished tubular properdin reabsorption reflecting tubular damage might also contribute to the presence of urinary properdin and its association with worse renal function.

In serum, properdin is present as dimers, trimers, and tetramers, but not as monomers [37]. Discrimination between these forms might help in determining its main source (multimeric properdin from blood, monomeric properdin from intrarenal inflammatory cells). However, the polyclonal anti-properdin antibody we used recognizes all forms of properdin, so we can only speculate about its source.

It is noteworthy that properdinuria was frequent in our diabetic subjects. An increasing body of evidence suggests that in diabetic nephropathy- although traditionally considered a non-immune mediated disease- the immune system actually is involved, at least in the progression towards renal failure. In the present study as in previous ones, patients with diabetic nephropathy have relatively high levels of urinary SC5b-9 [7;8], indicating the contribution of complement mediated damage.

We are aware of the limitations of our study: first, because of the cross-sectional nature, cause and effect of the observed associations cannot be defined. Second, urinary levels of either properdin and SC5b-9 might not accurately reflect what is really going on in the kidney since tubular binding may alter urinary excretion.

In conclusion, we show that properdin is present in urine of proteinuric patients and that its presence is associated with worse renal function. The presence of urinary properdin excretion is associated with higher urinary levels of SC5b-9, the terminal product of complement activation, independent

of the degree of proteinuria and renal function. We hypothesize that properdin promotes intratubular complement activation. Further research on the relation between urinary properdin excretion and complement activation in the kidney is needed.

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6

Waist-to-hip ratio is an independent predictor of cardiovascular events in South Asians with type 2 diabetes

A prospective study

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Submitted

Abstract

Background and aims

South Asians have a high prevalence of diabetes and cardiovascular complications. Since traditional risk factors only partially explain this excessive risk, the identification of non-traditional risk factors is warranted. Among these, central obesity might be an important determinant of cardiovascular disease in this specific group.

Methods

We conducted a prospective observational study. A cohort consisting of 168 type 2 diabetic South Asians was followed for a median duration of 7.66 (IQR 7.48-8.1) years. The primary endpoint was the time to the first cardiovascular event, which was defined as the occurrence of either cardiovascular death, myocardial infarction, revascularization procedure, stroke, carotid artery desobstruction, peripheral artery revascularisation or amputation. To examine the effect of central obesity on cardiovascular events , Cox proportional hazards regression was performed.

Results

Out of 134 subjects for whom follow-up data were available, 30 subjects reached the primary endpoint. Waist-to-hip ratio was the principal predictor of cardiovascular events. Multivariate analysis for the occurrence of a cardiovascular event during follow-up revealed a hazard ratio of 3.77 (95% CI 1.09-13.1), P = 0.037) for the highest versus the lowest tertile of waist-to-hip ratio.

Conclusion

Waist-to-hip ratio is a principal predictor of future cardiovascular events in type 2 diabetic South Asians. The underlying pathophysiologic mechanism probably involves adipogenic inflammation, dyslipidemia and adipokine dysbalance. Our findings urges to explore the use of lifestyle intervention in this specific group.

Introduction

South Asians have a high incidence of diabetes and subsequent vascular complications [1]. Traditional risk factors do not fully explain this higher incidence [2], so non-traditional risk factors must be involved. Among these, research has focused on central obesity, which is highly prevalent in South Asians. At similar body mass index (BMI), South Asians have a higher percentage of body fat compared to Caucasian [3]. Moreover, fat distribution is different, with South Asians having a relatively high truncal fat mass [4]. Histomorphology of adipocytes in South Asians also differs from that in Caucasians [5], with South Asians having larger adipocytes which are considered dysfunctional compared to normal sized adipocytes [6].

Cross-sectional studies in South Asians demonstrate that central obesity is associated with several cardiovascular risk markers, such as increased C-reactive protein [7], insulin resistance and dyslipidemia [1], and urinary albumin/creatinine ratio [8]. Although these latter factors have been shown to be predictive for future cardiovascular events, prospective data on the role of central obesity itself in predicting cardiovascular events in diabetic South Asians are lacking.

We performed a prospective study in a cohort of 168 diabetic South Asians to examine the effect of central obesity on cardiovascular events.

Methods

Design of the follow-up study

We conducted a prospective follow-up study. All studied subjects were recruited from a previously published study [9]. The original study population comprised 465 South Asians. At baseline, subjects that were not known with diabetes underwent a 75 g oral glucose tolerance test. Diabetes was diagnosed based on the ADA 2003 criteria. Out of 465 subjects, 168 subjects had type 2 diabetes at baseline (122 already known with diabetes, 46 newly diagnosed), and from these subjects follow-up data were collected. The study protocol was approved by the Institutional Medical Ethics Committee. All subjects provided informed consent.

Study-patients were followed up by letter and subsequently by phone. When subjects could not be traced by address or phone number in our database, general practitioners or participating family members were involved.

Follow-up data consisted of medical history with regard to cardiovascular events. Subjects were sent a questionnaire and were invited for a visit to our out-patient clinic. During this visit the questionnaire was reviewed by the main investigator (M.A.S.). Subjects not willing to visit the out-patient clinic were asked permission to collect medical data from their general practitioner. For subjects who had died during the follow-up period, cause of death and cardiovascular history was retrieved from the general practitioner. All (self-) reported events were verified by contacting the hospital in which the event had occurred.

Measurements at baseline

Anthropometric measures at baseline included weight, height and waist- and hip circumference. Hip circumference was measured at the maximum hip circumference, waist circumference was measured at the point midway between the lower costal margin and the anterior superior iliac crest.

Laboratory measurements at baseline included lipids, creatinin, fasting glucose, fasting insulin, high-sensitivity C-reactive protein (hsCRP), adiponectin and serum complement factor 3 (C3) Lipids, creatinin, glucose an insulin were measured according to standard methods. High-sensitivity C-reactive protein was measured with a fully automated Cobas Integra 800, according to the manufacturers proceedings (Roche, Almere, the Netherlands). The variation coefficients (VC) were below 3 %. Adiponectin was measured with a radio-immunoassay after dilution. The interassay VC's ranged from 6.9 to 9.2 % at different levels (Millipore Corporation, Billerica, MA 01821, USA). Serum C3 concentration was quantified using radial immunodiffusion according to Mancini, using a polyclonal rabbit anti-human C3 anti-serum as described earlier [10]. Homeostatic model assessment for assessing insulin resistance (HOMA-IR) was performed as follows: [fasting insulin concentration (mU/L) x fasting glucose concentration (mmol/L)] / 22.5 [11].

Definition of endpoint

Cardiovascular events were defined as the occurrence of either a myocardial infarction, Percutaneous Transluminal Coronary Angioplasty (PTCA), Coronary Artery Bypass Grafting (CABG), stroke, Carotid Artery Desobstruction, peripheral vascular angioplasty, bypass or amputation, or sudden cardiac death. The latter was defined as a witnessed sudden circulatory arrest. The primary end-point was the time to the first cardiovascular event.

Statistical analysis

Normally distributed variables are expressed as mean ± 1 standard deviation and skewed distributed variables as median and interquartile range. Differences between two groups were assessed by Student's t-test or Mann-Whitney-U test as appropriate. Differences between more than two groups were assessed by analysis of variance, adjusting for multiple comparisons according to Bonferroni. Correlations were assessed using Pearson's correlation, skewed distributed variables being log-transformed. To identify predictors of cardiovascular events and to adjust for potential confounding factors, Cox proportional hazards regression was performed. Survival curves were compared using the Log-Rank test. All test were two-sided and the level of significance was set at 0.05. All analyses were performed using SPSS for windows, version 17.

Results

Out of 168 type 2 diabetic subjects at baseline, 21 could not be traced and 13 subjects refused to participate. Eighty-six subjects visited the out-patient clinic, 31 subjects did not visit the out-patient clinic but medical information was retrieved from the general practitioner, and 17 subjects had died (see below). The median duration of follow-up was 7.66 (IQR 7.48-8.10) years. Participants lost to follow-up did not differ in baseline characteristics from participants in whom follow-up data were available (table 1).

During follow-up, 39 cardiovascular events occurred in 30 subjects (16 men, 14 women): 3 sudden cardiac deaths, 2 fatal and 5 non-fatal myocardial infarction, 13 percutaneous coronary interventions, 8 coronary artery bypass graft procedures, 2 fatal and 5 non-fatal strokes, 1 lower extremity amputation. Eleven of these 30 subjects had already experienced a cardiovascular event at baseline. Ten subjects died due to non-cardiovascular causes. Two of these subjects reached the primary end-point before they died, the other 8 were censored.

The characteristics of the 134 subjects in whom follow-up data were available are shown in relation to waist-to-hip ratio (WHR) tertile in table 2. As expected, there were more women in the lowest WHR tertile and more men in the highest WHR tertile. Compared to subjects in the lowest WHR tertile, subjects in the highest WHR tertile had higher fasting triglycerides, lower HDL cholesterol levels, higher urinary albumin/creatinine ratio, and a higher prevalence of baseline cardiovascular events. Men and women differed only with respect to

Table 1 baseline characteristics of the study population

	Follow-up (n=134)	Lost to follow-up (n=34)	P-value
Age (years)	50.7 ± 11.2	48.9 ± 11.2	0.392
% male sex	46	45	0.886
Diabetes duration (years)	7.0 (0-13)	5.0 (0-11)	0.519
HOMA-IR*	7.4 (4.8-12.3)	7.7 (5.3-12.1)	0.841
HbA1c (%)	7.7 ± 1.8	7.7 ± 2.0	0.976
Systolic blood pressure (mm Hg)	138 ± 24	140 ± 27	0.638
Diastolic blood pressure (mm Hg)	84 ± 11	84 ± 11	0.937
Urinary albumin/creatinine ratio (mg/mmol)	1.0 (0.4-4.6)	1.0 (0.4-5.0)	0.991
High-sensitivity C-reactive protein (mg/L)	3.5 (1.8-8.0)	4.8 (1.7-8.4)	0.851
Cockroft-Gault creatinine clearance (ml/min)	86 ± 27	93 ± 20	0.205
Total cholesterol (mmol/L)	5.1 ± 1.0	5.0 ± 0.9	0.666
Fasting triglycerides (mmol/L)	1.59 (1.16-2.32)	1.46 (1.24-2.13)	0.869
HDL-cholesterol (mmol/L)	1.23 ± 0.3	1.28 ± 0.3	0.954
Ratio total cholesterol: HDL-cholesterol	4.14 (3.45-5.05)	4.20 (3.10-5.0)	0.432
Body Mass Index	28.0 ± 4.7	28.4 ± 4.0	0.551
Waist-to-hip ratio	0.97 (0.93-1.03)	0.99 (0.95-1.04)	0.223
Adiponectin (mg/L)	7.6 (5.3-10.6)	6.8 (4.6-9.4)	0.124
Serum C3 (μg/ml)	871 ± 100	895 ± 78	0.226
% previous cardiovascular event	14	11	0.663
% current or previous smoker	45	39	0.528

*HOMA-IR = Homeostatic model assessment for assessing insulin resistance

HDL cholesterol level (mean 1.11 ± 0.25 versus 1.32 ± 0.34 mmol/L in men and women, respectively, P<0.001). All other parameters were not different between men and women (data not shown).

Waist-to-hip ratio correlated weakly with adiponectin concentration (r = -0.213, P = 0.016), HDL cholesterol (r = -0.248, P = 0.004), HOMA-IR (r = 0.180, P = 0.040), fasting triglycerides (r = 0.211, P = 0.016) and urinary albumin/creatinine ratio (r = 0.180, P = 0.039) but not with hsCRP, C3, total cholesterol and BMI.

Table 2 baseline characteristics of 134 type 2 diabetic South Asians according to WHR tertile

WHR tertile	1	2	3
Age (years)	48.9 ± 12.2	50.8 ± 10.2	52.5 ± 11.0
% male sex	16	55*	64*
Diabetes duration (years)	6.0 (0.7-13.0)	4.0 (0.0-10.0)	10.5 (0.0-16.3)
HOMA-IR‡	6.26 (4.38-9.93)	7.73 (4.33-10.46)	8.6 (6.0-11.8)
HbA1c (%)	7.3 ± 1.6	7.7 ± 2.2	8.0 ± 1.6
Systolic blood pressure (mm Hg)	138 ± 27	133 ± 16	145 ± 27
Diastolic blood pressure (mm Hg)	85 ± 10	81 ± 11	87 ± 13
Urinary albumin/creatinine ratio (mg/mmol)	0.80 (0.34-2.76)	0.73 (0.33-3.20)	2.74 (0.7-14.9)*†
High-sensitivity C-reactive protein (mg/L)	3.9 (2.2-9.3)	3.5 (1.7-9.4)	3.2 (1.3-5.9)
Cockroft-Gault creatinine clearance (ml/min)	92 ± 31	90 ± 27	94 ± 37
Total cholesterol (mmol/L)	5.22 ± 0.98	5.13 ± 1.02	5.10 ± 0.97
Fasting triglycerides (mmol/L)	1.28 (1.03-1.61)	1.70 (1.10-2.63)	1.79 (1.37-2.61)*
HDL-cholesterol (mmol/L)	1.36 ± 0.37	1.17 ± 0.25*	1.16 ± 0.29*
Ratio total cholesterol: HDL-cholesterol	4.04 ± 1.24	4.57 ± 1.41	4.51 ± 0.92
Body Mass Index	28.3 ± 5.0	26.0 ± 3.6	28.6 ± 5.1
Adiponectin (mg/L)	8.8 (6.5-13.0)	7.0 (4.8-9.3)*	7.6 (5.7-11.7)
Serum C3 (µg/ml)	887 ± 83	869 ± 90	859 ± 124
% previous cardiovascular event	5	7	27*†
% current or previous smoker	39	48	52
Follow-up duration (years)	7.7 (7.5-8.1)	7.8 (7.5-8.1)	7.7 (7.3-8.2)

In the analysis of variance, skewed distributed variables were log-transformed.
*/† Significant difference (P < 0.05) compared to tertile 1 (*) or tertile 2 (†)
‡HOMA-IR = Homeostatic model assessment for assessing insulin resistance

Cox proportional hazard regression

In univariate analysis an increased waist-to-hip ratio was a predictor of cardiovascular events (hazard ratio 6.4 (95% CI 1.9-21.9), P = 0.003 for the highest versus the lowest WHR tertile, see table 3). In figure 1, the event-free survival curves for the lowest and the highest WHR tertile are shown for 8

Table 3 predictors of cardiovascular events

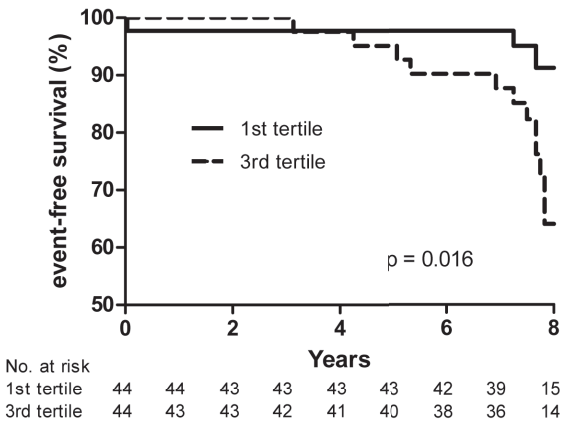
	Hazard ratio for future cardiovascular events (95%CI)	
	Univariate	**Multivariate***
Waist-to-hip ratio tertiles		
Lowest tertile	1.0 (reference)	1.0 (reference)
Middle tertile	2.46 (0.6-9.6)	2.3 (0.6-9.6)
Highest tertile	6.4 (1.9-21.9)	4.87 (1.25-18.93)
Age (years)	1.02 (0.99-1.06)	1.0 (0.95-1.05)
Male sex	1.51 (0.74-3.12)	0.88 (0.37-2.13)
Systolic blood pressure per 10 mm Hg	1.14 (0.98-1.32)	1.01 (0.98-1.03)
HbA1c (%)	1.22 (1.02-1.46)	1.17 (0.91-1.51)
Diabetes duration (years)	1.03 (0.995-1.07)	1.0 (0.94-1.06)
Total cholesterol (mmol/L)	1.20 (0.81-1.80)	1.33 (0.86-2.05)
Current or former smoker	1.27 (0.61-2.62)	1.08 (0.44-2.63)
Cardiovascular event at baseline	5.1 (2.3-11.1)	
urinary albumin/creatinine ratio (mg/mmol)#	1.97 (1.36-2.83)	
plasma adiponectin concentration (mg/L)#	4.96 (0.73-33.72)	
Body Mass Index	1.0 (0.91-1.08)	
HOMA-IR†#	0.93 (0.26-3.27)	
high-sensitivity C-reactive protein (mg/L)#	1.37 (0.60-3.13)	
fasting triglycerides (mmol/L)#	1.84 (0.37-9.19)	
HDL-cholesterol (mmol/L)	1.85 (0.57-6.03)	
Ratio total cholesterol:HDL-cholesterol	0.93 (0.69-1.27)	
Cockroft-Gault creatinine clearance (ml/min)	0.99 (0.98-1.01)	
Serum C3 (μg/ml)	1.002 (0.997-1.006)	

* Included as covariates are waist-to-hip ratio, age, sex, systolic blood pressure, HbA1c, diabetes duration, total cholesterol, and smoking status.

skewed distribuited variables were log-transformed

† HOMA-IR = Homeostatic model assessment for assessing insulin resistance

Figure 1



Unadjusted Kaplan-Meier survival curves according to waist-to-hip-ratio tertile (solid line = first / lowest WHR tertile, dashed line = third / highest WHR tertile). The highest WHR tertile has a worse event-free survival compared to the first WHR tertile (Log rank test P = 0.016).

years of follow-up. In addition to WHR, urinary albumin/creatinine ratio, a cardiovascular event at baseline and HbA1c were associated with the occurrence of cardiovascular events.

In multivariate analysis with age, sex, systolic blood pressure, HbA1c, diabetes duration, total cholesterol and smoking as covariates, only waist-to-hip ratio remained independently associated with the occurrence of cardiovascular events (multivariate HR 3.77(95% CI 1.09-13.1), P = 0.037 for the highest versus the lowest WHR tertile). The association remained after adjusting for a cardiovascular event at baseline (HR 4.84 (95%CI 1.36-17.2) for the highest versus the lowest WHR tertile). When only subjects without a prior cardiovascular event were analyzed, the association remained significant (univariate HR 8.04, 95% CI 1.79-36.04. P = 0.006, multivariate HR 7.1, 95% CI 1.24-40.9, P = 0.028). When men and women were analyzed separately, WHR above the median was associated with cardiovascular events (univariate HR 5.9 (95%CI 1.32-26.7, P = 0.02) and 3.6 (95%CI 1.0-13.0, P = 0.05), in men and women respectively. Multivariate HR with age, HbA1c, diabetes duration, systolic blood

pressure, smoking status and total cholesterol was not statistically significant (multivariate HR 4.56 (95%CI 0.89-23.3, P = 0.069) and 2.9 (95%CI 0.71-12.0, P = 0.137), in men and women respectively.
To identify possible mediators by which central obesity might influence cardiovascular risk, we separately adjusted for adiponectin concentration, hsCRP, complement C3, HOMA-IR, fasting triglycerides, total cholesterol, and HDL-cholesterol. However, the association of WHR and cardiovascular events was not attenuated by any of these covariates (data not shown).

Discussion

The current study demonstrates that waist-to-hip ratio, as a measure of central obesity, is a principal predictor of cardiovascular events in type 2 diabetic South Asians. Studies from different parts in the world report an increased prevalence of insulin resistance and diabetes in South Asians. South Asians develop diabetes 10 years earlier than Caucasians and therefore are exposed to a greater degree of cumulative glycemic damage to the vasculature [12]. Indeed, cardiovascular complications are more frequent in South Asians [13]. We previously studied South Asians living in the Netherlands, showing that central obesity is associated with albuminuria, even in the non-diabetic state [8]. These findings underscore the importance of developing effective preventive strategies for this specific population.
Instead of being an inert energy storage pool, visceral adipose tissue is an active regulator of metabolism [14]. Central obesity is associated with insulin resistance and dyslipidemia [15,16]. In addition, adipose tissue produces cytokines, complement factors and over 30 so-called adipokines [6]. Adipose tissue, as a source of pro-inflammatory cytokines, might drive chronic-vascular-inflammation ("adipogenic inflammation") [17]. Several studies show increased CRP levels in South Asians compared to Caucasians [7,18,19], indicating that an increased pro-inflammatory state is present in this ethnic group. However, so far, data on the predictive role of hsCRP for cardiovascular events in this specific group were lacking. In our type 2 diabetic South Asians, hsCRP was not a predictor of cardiovascular events.
Complement factor C3, which is also produced by adipose tissue [20], is the central molecule in the complement cascade. Compared to Caucasians, South Asians have increased levels of complement C3 [21,22]. As the complement

system is involved in atherogenesis [23], enhanced complement activation might contribute to increased atherogenicity in South Asians [21]. However, while in South Asians serum C3 is strongly associated with the metabolic syndrome [24], we did not find such a relationship with cardiovascular events in our study.

A previous cardiovascular event and a high urinary albumin/creatinine ratio were both associated with future cardiovascular events. In contrast to WHR, which likely has a pathophysiological link to cardiovascular events, this association likely reflects established end-organ damage. In addition, diabetes duration and HbA1c predicted cardiovascular events, pointing at the importance of cumulative glycemic damage to the vasculature.

Adjustment for hsCRP, complement C3, adiponectin, HOMA-IR, and various lipid parameters did not attenuate the strong predictive value of WHR for cardiovascular events. This suggests that neither insulin resistance, dyslipidemia, increased inflammation, complement or adiponectin levels alone can explain the association between central obesity and cardiovascular events. These factors likely act in concert.

A limitation of our study is the relatively small sample size which may limit the detection of predictors with smaller effects in this ethnic group.

In conclusion, we show that in patients with type 2 diabetes of South Asians descent living in a western society, waist-to-hip ratio is a principal predictor of future cardiovascular events. This warrants interventions aimed at weight loss and reduction of central abdominal fat mass to determine whether this will lead to a decline in cardiovascular events.

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7

Plasma level of Connective Tissue Growth Factor is associated with vascular and renal damage in patients with type 2 diabetes of South Asian descent

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Abstract

Objective

South Asians have a high burden of cardiovascular disease. Connective Tissue Growth Factor (CTGF), a profibrotic cytokine, has evolved as a risk marker for cardiovascular disease. The current study explores the relation between CTGF and vascular damage in type 2 diabetic South Asians.

Research design and methods

We performed a cross-sectional study in 54 type 2 diabetic South Asians. Vascular function testing (measurement of aortic Pulse Wave velocity an carotid artery Intima Media Thickness) was performed and plasma CTGF level and clinical parameters were assessed. The correlation between plasma CTGF level and measures of vascular damage was determined.

Results

Plasma CTGF correlated with pulse wave velocity ($r = 0.424$, $P = 0.002$), carotid artery intima-media thickness ($r = 0.324$, $P = 0.021$) and urinary albumin-to-creatinin ratio ($r = 0.376$, $P = 0.005$).

Conclusion

Plasma CTGF level is associated with vascular and renal damage in type 2 diabetic South Asians.

Introduction

Subjects of South Asian descent have a high burden of type 2 diabetes and cardiovascular disease. Type 2 diabetes presents at a younger age and once present, the risk of diabetic nephropathy is increased compared to Caucasians [1] Connective Tissue Growth Factor (CTGF) is a pro-fibrotic cytokine that is linked to development of vascular and renal disease [2,3].
Our aim was to determine the potential role of CTGF as a marker of vascular and renal dysfunction in subjects with type 2 diabetes of South Asian descent.

Methods and materials

Patient population and measurement of clinical parameters

All subjects belonged to a group of 465 South Asians that was previously studied [4] Patients with type 2 diabetes at baseline (n = 168) were contacted. Fifty-four patients agreed to participate in the current study. Data concerning medication, cardiovascular history and smoking habits were collected. Anthropometric measurements included weight, length, waist- and hip circumference. Laboratory parameters including lipids, creatinine, glucose, and urinary albumin/creatinine ratio (ACR) were measured according to standard methods. High sensitivity C-reactive protein (hsCRP) was measured with a fully automated Cobas Integra 800, according to the manufacturers protocol (Roche, Almere, the Netherlands). CTGF was measured in EDTA plasma drawn at study entry that had been stored at -80°C. CTGF was determined by a sandwich ELISA using monoclonal antibodies against two distinct epitopes on the N-terminal part of human CTGF (FibroGen, San Francisco, CA) detecting both full-length and N-terminal CTGF as described previously (2).
The study was approved by the Institutional Medical Ethics Committee. All subjects provided informed consent.

Assessment of pulse wave velocity and intima-media thickness

Arterial stiffness was assessed noninvasively by determination of the pulse wave velocity (PWV). Aortic PWV was calculated as the distance of the pulse wave traveling from the base of the neck to the right femoral artery per time frame (in m/s) as described previously [5]. For assessment of intima-media thickness (IMT) of the common carotid artery as a measure of atherosclerosis

the mean of eight ultrasound measurements (four measurements at different angles at each side of the neck) was taken as described previously [6]. All measurements were performed with subjects in the fasting state.

Statistical analysis

Normally distributed variables are expressed as mean ± 1 standard deviation and skewed distributed variables as median and interquartile range. Correlations were assessed using Pearson's correlation, with skewed distributed variables being log-transformed. Partial correlation was used to adjust for covariates. All test were two-sided and the level of significance was 0.05. All analyses were performed using SPSS for windows, version 17.

Results

Fifty-four type 2 diabetic South Asians were studied. Baseline characteristics are shown in table 1.

Median plasma CTGF level was 183 pmol/L (IQR 103-335). Plasma CTGF level positively correlated with PWV (r = 0.424, P = 0.002), carotid artery IMT (r = 0.324, P = 0.021), and log transformed urinary ACR (r = 0.376, P = 0.005). Plasma CTGF also positively correlated with age (r = 0.310, P = 0.023) but not with glycemic control (HbA1c r = 0.028, P = 0.841), systolic blood pressure (r = 0.259, P = 0.059), markers of inflammation (hsCRP r = 0.102, P = 0.461), or lipid parameters.

Adjustment for age and sex did not affect the relation between plasma CTGF and PWV and urinary albumin/creatinine ratio (r = 0.376, P = 0.007 and r = 0.396, P = 0.004, respectively) but the relation between plasma CTGF and IMT was no longer statistically significant (r = 0.260, P = 0.071). After addition of systolic blood pressure as a covariate to age and sex, only the relationship between CTGF and urinary albumin/creatinine ratio remained significant (r = 0.367, P = 0.009)

Pulse wave velocity additionally correlated with systolic blood pressure (r = 0.504, P < 0.001), BMI (r = 0.307, P = 0.027), waist circumference (r = 0.331, P = 0.017), hip circumference (r = 0.292, P = 0.036), HbA1c (r – 0.344, P = 0.013), Cockroft-Gault creatinin clearance (r = -0.282, P = 0.043), fasting triglycerides (r = 0.345, P = 0.013) and LDL-cholesterol (r = -0.354, P = 0.011), but not with age, sex, smoking status, urinary albumin/creatinine ratio, and high-sensitivity C-reactive protein. After adjustment for age, sex, systolic blood pressure, fasting

triglycerides, LDL cholesterol, HbA1c and Cockroft-Gault creatinin clearance, waist circumference remained independently associated with pulse wave velocity (r = 0.308, P = 0.045).

Table 1 Baseline characteristics of the study population

Age	56.8 ± 6.9
male sex (%)	48
Diabetes duration (yrs)	14 ± 6.3
HbA1c (%)	7.5 ± 1.4
Systolic bloodpressure (mm Hg)	147 ± 20
Diastolic bloodpressure (mm Hg)	88 ± 11
Body Mass Index	28.8 ± 4.4
Waist circumference (cm)	102 ± 12.8
Total cholesterol (mmol/L)	4.17 ± 0.88
LDL-cholesterol (mmol/L)	2.52 ± 0.82
HDL-cholesterol (mmol/L)	1.30 ± 0.35
Fasting triglycerides (mmol/L)	1.21 (0.98-1.80)
Previous cardiovascular event (%)[$]	26
Retinopathy (%)	74
Current or former smoking (%)	44
Oral anti-hyperglycemic agents withour insulin (%)	52
Insulin (%)	11
Oral anti-hyperglycemic agents with insulin (%)	37
Urinary albumin/creatinine ratio (mg/mmol)	1.7 (0.7-5.3)
Cockroft clearance (ml/min)	101 ± 35

[$] cardiovascular event is defined as a myocardial infarction, percutaneous transluminal coronary angioplasty, coronary artery bypass graft or stroke

Discussion

The current study demonstrates that in patients with type 2 diabetes of South Asian descent, plasma level of CTGF correlates with vascular and renal damage. These data suggest that the profibrotic cytokine CTGF may play a role in diabetic vascular and renal disease.

CTGF is produced by several cell types (including endothelial cells, vascular smooth muscle cells, renal mesangial and tubular epithelial cells) and its biological functions include proliferation, angiogenesis, migration, adhesion, and extracellular matrix production [7]

An increasing body of evidence points to the importance of vascular fibrosis in the pathogenesis of cardiovascular complications [8]. Decreased vascular elasticity results in systolic hypertension leading to an increased cardiac workload.

Our data indicate that CTGF, independent of age, may be a mediator of these changes given its profibrotic actions. Supporting this hypothesis, FG-3019, a CTGF inhibiting recombinant antibody, counteracted vascular fibrosis in diabetic rats [9].

Diabetic nephropathy occurs early in patients with type 2 diabetes of South Asian descent and blood pressure was shown to be a progression factor also in this subgroup [10]. Independent of blood pressure, plasma CTGF was strongly associated with urinary albumin/creatinin ratio as a measure of renal disease. Our data contribute to the observation that CTGF plays an important role in the pathophysiology of diabetic nephropathy [11]. A recent study showed reduction in albuminuria after administration of the CTGF-blocker FG-3019 to albuminuric patients with diabetes [12].

PWV was independently associated with waist circumference. The association between vascular stiffness and -mainly abdominal- obesity has been previously reported, and a beneficial effect of weight reduction on vascular stiffness has been shown [13]. Compared to Caucasians, South Asians have an increased prevalence of central obesity. We recently showed that in South Asians a high waist-to-hip ratio is a principal predictor of cardiovascular events [14].Vascular stiffness therefore might be an important link between central obesity and increased cardiovascular events in South Asians.

In conclusion, plasma CTGF is associated with vascular stiffness, increased intima-media thickness and albuminuria in patients with type 2 diabetes of South Asian descent and may be a relevant mediator of vascular and renal damage.

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Summary and General discussion

Subjects of South Asian descent have an increased prevalence of type 2 diabetes [1,2]. Reduced insulin-sensitivity of glucose-utilizing tissues (insulin resistance) is central in the pathogenesis of type 2 diabetes. Insulin resistance in its turn is strongly associated with - mainly visceral- obesity. South Asians have a preferential truncal (deep adipose tissue) fat distribution [3], and even at the same body mass index the amount of -mainly visceral- fat is increased in South Asians [4]. Compared to the relatively inactive superficial subcutaneous adipose tissue (primary compartment), deep subcutaneous and visceral adipose tissue (secondary compartment, central obesity) is an active regulator of body metabolism [5]. Function and activity of the immune system is also influenced by the adipose tissue compartment [6-8]. Central adiposity is associated with chronic low-grade inflammation and activation of the immune system, apparently related to production of pro-inflammatory cytokines by infiltrating macrophages [9]. It is well-known that the inflammatory response is central in vascular diabetic complications, like atherosclerosis, ischemic heart disease, stroke, and nephropathy [10-14]. As a key player in the immune response, the involvement of the complement system is increasingly being recognized [15,16]. The complement system refers to a cascade of proteins which are involved in opsonisation of harmful particles such as bacteria and facilitate the inflammatory response by attraction of inflammatory cells and enhancing vascular permeability. This thesis explores the possible role of the complement system in diabetic vascular complications in type 2 diabetic subjects of South Asian descent.

Study population

The HINDINEF cohort offers an unique opportunity to study determinants of vascular complications in a population known for its high rate of renal and cardiovascular disease. At baseline the cohort was very well phenotyped, and both serum , plasma, urine and DNA were available to perform additional studies. As expected, we observed a high rate of cardiovascular complications (39 events in 30 out of 134 subjects after 7.66 years of follow-up).

Our study was performed in a selected population. Subjects had been selected based on either having a first-degree family member with end-stage diabetic renal disease (48 out of 168), or having a first-degree family member with type 2 diabetes but without albuminuria (115 out of 168). A small number (5 out of 168) was a second-degree relative of a subject with type 2 diabetes or had no familial relation with the other study participants (spouses). So most of the

study participants had a first-degree relative with type 2 diabetes. However, as the prevalence of type 2 diabetes in this specific population approaches 40% in those over the age of 60 [17], the chance of having a first-degree relative with type 2 diabetes is very high. Therefore, this selection-criterium is not a strong discriminator between our study population and type 2 diabetic subjects in the general South Asian population in The Hague. The other selection-criterium (having a first-degree family member with end-stage diabetic nephropathy) is theoretically more discriminating, although a familial predisposition to diabetic nephropathy was not found previously [18]. Furthermore, the 168 subjects described in this thesis originated from 89 different families. Therefore, taken together, we feel that our observations can be extrapolated at least to type 2 diabetic subjects in the general South Asian population in The Hague and surroundings. We lost 34 subjects to follow-up because they were unreachable. However, baseline clinical characteristics of subjects with and without available follow-up data were not different. Therefore, it is unlikely that an important selection bias was present in the recruitment of follow-up data. Of note, our population size was relatively small, which limits the detection of predictors of vascular complications with smaller effects.

Functional meaning of increased C3 levels

In **Chapter 2**, we found increased levels of complement C3 -the central molecule in complement activation- in South Asians compared to a Caucasian control group. A key question is whether increased C3 levels predispose to enhanced complement activation. Although we found increased levels of SC5b-9 - the effector molecule of complement activation – in South Asians compared to Caucasians, C3 levels were not correlated with SC5b-9 levels. Thus C3 levels do not per se result in enhanced complement activation at the systemic level, although enhanced complement activation at tissue level cannot be ruled out. C3 levels were not associated with albuminuria, cardiovascular events or progressive renal failure and hence C3 in itself is not a useful cardiovascular risk marker in type 2 diabetic South Asians. Given the increased C3 level in the South Asian population, the inter-individual predictive value with respect to cardiovascular disease might be attenuated. This phenomenon has also been observed with other cardiovascular risk markers like adiponectin [19]

C3 levels were closely related to measures of adipose tissue mass (BMI, waist circumference, hip circumference). Indeed, C3 mRNA expression has been observed in –mainly omental- adipose tissue [20]. Adipocyte-derived C3 serves

as a precursor of C3adesarg , also called Acylation Stimulating Protein (ASP) [21]. C3adesarg is generated through interaction of C3 with complement factor B and the enzyme adipsin (also known as complement factor D). C3adesarg is inactive with respect to complement activation. Its main function is to increase triglyceride synthesis in fat-storing cells through stimulation of fatty acid incorporation. Increased C3 levels in South Asians thus might simply reflect increased central adipose tissue mass, although it is currently unknown to what extent adipocyte-derived C3 contributes to serum total C3 levels. Secondary mechanisms involving hepatic C3 production might also be involved.

Complement activation and nephropathy

In the cross-sectional part of **chapter 2**, high plasma SC5b-9 levels were associated with albuminuria, and in the prospective part with progressive renal failure. This suggest that complement activation is linked to diabetic nephropathy, although the pathophysiologic mechanism remains to be clarified. We found no other data in the literature concerning plasma SC5b-9 levels and renal disease. Plasma SC5b-9 not necessarily reflects intrarenal complement activation but rather might reflect complement activation due to general vascular inflammation and atherosclerosis, confounding the relation with nephropathy.

Fibrosis is the ultimate final common pathway in the progression of chronic renal disease. Interestingly, both SC5b-9 and split products of C3 have been reported to induce renal fibrosis [22,53]. Histopathological studies show SC5b-9 deposition in diabetic kidney biopsies, and a correlation between C5b-9 deposition and interstitial lesions has been reported [23]. Complement activation might thus be associated with renal fibrosis. SC5b-9 has also been detected in urine of subjects with macroproteinuric diabetic nephropathy [24], but its potential in predicting progressive renal failure has not been determined.

Mannose-binding lectin (MBL): a two-edged sword

Mannose-binding lectin (MBL) is the primary recognition molecule of the lectin pathway of complement activation. MBL binds carbohydrate moieties on microorganisms, but endogenous MBL ligands, such as glycosylated immunoglobulins or cells exposed to oxidative stress, have also been identified [25]. MBL serum levels are primarily determined by 3 polymorphisms (B,C and D

genotypes, commonly referred to as O-alleles) in the MBL gene (*mbl2*). Subjects with wild type MBL genotype (A/A) have the highest serum MBL levels, subjects with 1 variant allele (A/O) have intermediate levels and subjects with 2 variant alleles (O/O) have the lowest levels. In addition, polymorphisms in the promoter region influence the MBL level [26]. MBL has been introduced as a risk marker of cardiovascular complications in type 1 and type 2 diabetes [27,28]. However, the exact role of MBL, which previously has been called "the Dr. Jekyll and Mr. Hide of the innate immune system" [29] remains controversial. On the one hand, low MBL levels have been related to increased cardiovascular events [30,31], while on the other hand increased MBL levels were associated with diabetic nephropathy and enhanced ischemia/reperfusion injury [32,33]. In our study we found the same apparent paradox. In **chapter 2**, we found high MBL levels to be independently associated with progressive renal failure. Whether and how increased MBL levels result in increased renal damage remains to be clarified. In an experimental diabetic mouse model, MBL was associated with increased renal collagen expression [34], suggesting that high MBL levels might be related to renal fibrogenesis. However, the exact pathophysiological pathway is currently unresolved, although *in vitro* studies suggest that oxidative stress, which indeed is enhanced in the diabetic state, might be an important trigger for MBL mediated complement activation [35].

In **chapter 3** however, we found that the O/O MBL genotype, which results in low MBL levels, are associated with increased cardiovascular events, as was previously found by others [30,31]. A possible explanation for this observation might be a defective MBL mediated clearance of atherogenic particles [36]. MBL binds N-acetylglucosamine moieties, which are expressed on several lipoproteins and oxidized LDL [37], and this may facilitate their phagocytic clearance. Additionally, MBL deficiency might influence the susceptibility and course of infection with *Chlamydia pneumoniae*, which is associated with coronary artery disease [38]. Therefore, the role of MBL in micro- and macrovascular disease might involve different mechanisms: in the former, direct complement-mediated injury might be involved, whereas in the latter enhanced atherogenesis caused by diminished anti-atherogenic or anti-infectious activity seems to be responsible. As genotype distribution was not different between South Asians and Caucasians, genetically determined differences in MBL levels are unlikely to explain the increased rate of vascular complications in South Asians.

Complement and progressive renal failure

Proteinuria is a prognostic marker in renal disease. Besides being a marker of renal damage, non-selective proteinuria is thought to be toxic to the tubulointerstitium [39-42]. Activation of filtered or locally produced complement components is likely involved in tubulotoxicity of proteinuria [43,44]. Complement activation products are indeed detectable in urine of patients with different proteinuric renal disease [45-47]. Experimental studies in various proteinuric models show complement activation on tubular cell brush border [48-52]. Inhibition of complement, either by administration of a complement inhibitor or by knock-out of complement components, results in attenuation of tubulointerstitial damage [48,51,52]. Complement activation also has been linked to the induction of renal fibrosis [22,53]. Although the capacity of cultured tubular cells to activate the alternative complement pathway has long been known, the mechanism had not been determined.

Properdin acts as a positive regulator of the alternative pathway of complement activation by stabilizing the alternative pathway C3-convertase, but more recent data suggest that properdin also acts as a recognition molecule of the alternative pathway [54-57]. In **chapter 4**, we demonstrate that properdin acts as a focal point for alternative pathway complement activation on cultured proximal tubular epithelial cells. In **chapter 5**, we demonstrate that properdin is excreted in proteinuric renal disease and that "properdinuria" is associated with urinary SC5b-9 level and renal dysfunction. These results suggest that properdin promotes intrarenal complement activation and might contribute to proteinuria mediated renal damage.

Central obesity as a risk factor for cardiovascular disease

South Asians have an increased prevalence of central obesity. Cross-sectional studies in South Asians demonstrate that central obesity is associated with several cardiovascular risk markers, such as increased C-reactive protein level [58], insulin resistance and dyslipidemia [59], and urinary albumin/creatinine ratio [60], but the predictive value for cardiovascular events of central obesity itself had never been assessed. In **chapter 6**, we show that a high waist-to-hip ratio, as a measure of central obesity, is a principal predictor of cardiovascular events in type 2 diabetic South Asians. Instead of being an inert energy storage pool, visceral adipose tissue is an active regulator of metabolism [5] that produces cytokines, complement factors and over 30 so-called adipokines [61]. Adipose tissue, as a source of pro-inflammatory cytokines, might drive chronic

-vascular- inflammation ("adipogenic inflammation") [9,62]. However, we could not find a single mediator (insulin resistance, inflammation, adiponectin, dyslipidemia, complement levels) that explained the effect of central obesity on cardiovascular events. All these factors likely act in concert.
In addition, central obesity was independently associated with arterial stiffness (see chapter 7 below) . Arterial stiffness leads to an increased cardiac workload resulting in left ventricular hypertrophy. Therefore, increased arterial stiffness might be an important link between central obesity and cardiovascular complications in type 2 diabetic South Asians.

Connective Tissue Growth Factor and vascular and renal damage
In **chapter 7** we studied the association of Connective Tissue Growth Factor (CTGF, a pro-fibrotic cytokine) with vascular and renal damage in 54 type 2 diabetic South Asians. We found that plasma CTGF level is associated with pulse wave velocity (PWV, a measure of arterial stiffness), carotid artery Intima-Media Thickness (IMT, a measure of atherosclerosis) and urinary albumin/creatinine ratio (a measure of renal damage). CTGF level also predicts progressive renal failure in South Asian with type 2 diabetes (own observations, unpublished). Given its pro-fibrotic actions, CTGF might be an important mediator in vascular and renal fibrosis.

Concluding remarks and future perspectives
In this thesis we studied several non-traditional risk factors for the progression of renal and cardiovascular disease in subjects with type 2 diabetes of South Asians descent. In the epidemiological studies incorporated in this thesis we focused on the role of adipose tissue and complement as mediators of damage. Additionally, we specifically studied the role of complement in the progression of proteinuric renal disease.
Our studies raise several clinical perspectives. First, as central obesity is the principal predictor of cardiovascular events in this specific population, weight reduction should be of primary importance in the reduction of cardiovascular risk. Although it has to be tested in an prospective intervention study, weight reduction might prove to be more effective than pharmacological interventions.
One intriguing point to be addressed is the apparent overlap between the complement system and adipose tissue biology. Currently, no data exist regarding the Acylation-Stimulating Protein (ASP) pathway (see above) in South Asians. As ASP deficiency is associated with reduced fat mass and increased

energy expenditure [63], the ASP pathway might be an interesting target in preventing the deleterious effects of central adiposity.
Second, as CTGF might be an important mediator of renal and vascular fibrosis, blocking its actions might open a new therapeutic avenue with respect to prevention of cardiovascular disease, as preliminary data suggest [64,65] Third, as complement activation is involved in the progression of proteinuric renal disease and properdin acts as a focal point, specifically blocking the interaction between properdin and the tubular brush border – without blocking the entire complement cascade- might retard progression of renal damage.
Of course, our studies raise new questions. The contribution of MBL to cardiovascular and renal damage should be further clarified. Is MBL an innocent bystander or a real mediator of progressive renal damage and if so, what is the pathophysiologic pathway involved? What is the relative contribution of low MBL levels to the process of atherosclerosis? It has been shown that high MBL levels after myocardial infarction are associated with an increased rate of re-infarction [66], suggesting that high MBL levels might prevent cardiovascular events, but are deleterious once an event occurs.
Proteinuria is an important predictor of progression of renal disease, and in our study urinary albumin/creatinine ratio was indeed associated with progressive renal failure. However, we were surprised that subjects with progressive renal disease had varying though overall mild degrees of baseline proteinuria (urinary albumin/ creatinine ratio ranging from 2.56 to 662 mg/mmol). Even in daily clinical practice, many subjects with chronic renal failure (including many diabetic subjects!) are seen with relatively little proteinuria. Although many studies address the role of complement-induced renal damage in proteinuric renal disease (see above), the role of complement in the progression of non-proteinuric renal disease is largely an undeveloped area.
South Asians have a 40-fold increased risk of reaching end-stage diabetic renal disease [67]. However, once on dialysis, they show an increased survival compared to Caucasians [68-70], while central obesity continues to be associated with decreased survival, even on dialysis [71]. Apparently yet undefined, initially harmful factors might lose their harmful potential or rather become protective in a dialysis setting. Interestingly, whereas high MBL levels are associated with progressive renal failure, in hemodialysis patients high MBL levels were shown to be associated with improved rather than worse survival [72]. As alternative pathway complement activation on the membrane of the artificial kidney is a well-known phenomenon, the complement system may

influence morbidity and mortality on dialysis [73]. Therefore, the role of complement in South Asians on dialysis might be an interesting future focus of investigation.

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Nederlandse samenvatting
Curriculum Vitae
Nawoord

Nederlandse samenvatting

Hindoestanen (Engels: South Asians) zijn oorspronkelijk afkomstig van het Indiase subcontinent. In de 19e eeuw zijn velen van daaruit naar Suriname verscheept om te werken op de plantages. Na de onafhankelijkheid van Suriname in 1975 en tijdens de politiek instabiele periode daarna zijn veel Hindoestaanse Surinamers naar Nederland geëmigreerd, waar zij zich vestigden in en rond de grote steden. In Den Haag en omgeving telt de Surinaams-Hindoestaanse gemeenschap rond 50.000 personen.

Personen van Hindoestaanse komaf hebben een verhoogd vóórkomen van type 2 diabetes mellitus (suikerziekte). Type 2 diabetes mellitus is geassocieerd met centrale adipositas (teveel buikvet), en ook dit komt bij personen van Hindoestaanse komaf vaak voor. Diabetes mellitus is geassocieerd met schade aan hart, bloedvaten en nieren leidend tot hartinfarct, beroerte en nierinsufficientie (onvoldoende nierfunctie), zelfs tot aan dialyse-noodzaak toe. Ook deze complicaties komen bij Hindoestanen vaker voor dan men zou verwachten. De precieze oorzaak hiervan is niet bekend.

Bij de ontstaanswijze van complicaties aan hart, bloedvaten en nieren staat een ontstekingsproces in bloedvaten en/of nieren centraal. Een teveel aan buikvet is geassocieerd met toegenomen ontstekingsactiviteit. Bij een ontstekingsproces zijn allerlei afweercellen en eiwitsystemen betrokken. Een van die eiwitsystemen is het complementsysteem. Dit systeem bestaat uit ongeveer 30 verschillende eiwitten die elkaar trapsgewijs activeren. De eiwitten van het complementsysteem brengen schade toe aan bacteriën, zorgen dat deze gemakkelijker door afweercellen opgeruimd kunnen worden, en bevorderen tevens de ontstekingsreactie. Het complementsysteem kan via 3 routes worden geactiveerd, en elke route heeft zijn eigen “gangmaker”-eiwit. Bij de lectine-route is dat Mannose-bindend lectine (MBL), dat onder andere suikerstructuren op bacteriën herkent. De hoogte van de MBL waarde in het bloed wordt voornamelijk genetisch bepaald. De twee andere routes worden op gang gebracht door immuuncomplexen (de klassieke route) of doordat spontaan optredende complement-activatie wordt gestimuleerd (alternatieve route). Dit proefschrift onderzoekt de mogelijke rol van het complementsysteem bij hart-, vaat- en niercomplicaties in Surinaams Hindoestaanse type 2 diabeten.

De personen die aan dit onderzoek hebben meegewerkt zijn afkomstig uit een eerder uitgevoerde studie, de zogenaamde HINDINEF studie, die in de jaren 1998-2000 465 Surinaamse Hindoestanen “in kaart heeft gebracht”. Het huidige

onderzoek betreft een vervolgonderzoek onder alle Surinaamse Hindoestanen die destijds diabetes mellitus hadden (168 personen). Na een gemiddelde periode van 7,66 jaar werd nagegaan in hoeverre zij complicaties hebben ontwikkeld aan hart, bloedvaten en nieren. Met de gegevens die uit het eerdere HINDINEF onderzoek waren verkregen is vervolgens gezocht naar factoren (en dan met name eiwitten van het complementsysteem) die geassocieerd zijn met het optreden van deze complicaties.

In **hoofdstuk 2** vinden we dat Surinaamse Hindoestanen hogere bloedwaarden hebben van complementeiwit C3, het centrale eiwit in het complementsysteem, vergeleken met blanke Caucasiers. Daarnaast hebben Surinaamse Hindoestanen ook hogere bloedwaarden van complementeiwit SC5b-9, het eindproduct van het complementsysteem. Dit zou erop kunnen wijzen dat het complementsysteem bij Surinaamse Hindoestanen actiever is dan bij Caucasiers. Echter, de hoogte van zowel C3 als SC5b-9 waren niet voorspellend voor het optreden van hart-en vaatcomplicaties. Bovendien waren C3 en SC5b-9 waarden niet aan elkaar gecorreleerd. Een hoger C3 lijkt dan ook niet noodzakelijkerwijs te leiden tot toegenomen complementactivatie. C3 waarden zijn wel gecorreleerd met maten voor de vetmassa (tailleomtrek, heupomtrek, overgewicht). Inderdaad is aangetoond dat vetcellen C3 kunnen produceren. Dit C3 dient als voorloper voor Acylatie Stimulerend Proteine (ASP). ASP heeft als functie om vetzuren – die na de maaltijd in het bloed komen – op te nemen in vetcellen. Verhoogde C3 waarden bij Surinaamse Hindoestanen zijn dus wellicht een uiting van toegenomen vetmassa.

In **hoofdstuk 3** vinden we dat het genotype (erfelijk materiaal) dat zorgt voor lage MBL waarden in het bloed wél geassocieerd is met het optreden van hart- en vaat complicaties. Behalve bij het bestrijden van bacteriën is MBL ook betrokken bij het opruimen van vetdeeltjes die schadelijk zijn voor bloedvaten en tot atherosclerose (in de volksmond “aderverkalking” genoemd) leiden. Bij lage MBL waarden verloopt deze opruimreacties onvoldoende, en dit zou mogelijk de verklaring kunnen zijn waarom een laag MBL-genotype geassocieerd is met hart-en vaatziekten. Ook zou een laag MBL gehalte een verhoogde vatbaarheid kunnen betekenen voor infecties met Chlamydia die in verband zijn gebracht met het optreden van atherosclerose. Opvallend genoeg zijn MBL waarden zelf niet voorspellend voor hart-en vaatziekten. Een eenmalige meting van MBL (zoals in onze studie) is wellicht onvoldoende nauwkeurig om de totale hoeveelheid MBL waaraan het lichaam in de loop van jaren wordt blootgesteld te weerspiegelen.

In tegenstelling tot hart-en vaatziekten was complementeiwit SC5b-9 wél geassocieerd met het optreden van nierfunctie verslechtering. Ook hoge MBL waarden waren geassocieerd met nierfunctieverslechtering. Dit zou kunnen duiden op betrokkenheid van het complementsysteem bij nierfunctie verslechtering, hoewel het exacte mechanisme niet is opgehelderd. Wellicht speelt toegenomen bindweefselvorming (fibrose) in de nier een rol. Er is wat MBL betreft dus een paradoxale situatie: een erfelijke aanleg voor lage MBL waarden is geassocieerd met hart-en vaartziekten, terwijl hoge waarden geassocieerd zijn met progressieve nierschade.
Hoofdstuk 4 en 5 gaan verder in op de rol van het complementsysteem bij progressieve nierschade. Wanneer nieren beschadigd raken lekt er dikwijls eiwit in de urine (proteïnurie). Onder deze eiwitten bevinden zich ook complementeiwitten, en die kunnen van binnen uit de nier verder beschadigen. Proefdierstudies laten zien dat wanneer het complementsysteem geremd wordt er minder nierschade optreedt. Het was al langer bekend dat met name de alternatieve route van complement activatie (zie boven) hierbij betrokken is, en dat complementeiwit C3 kan binden aan niercellen. Hoe deze binding tot stand komt was echter niet bekend. In **hoofdstuk 4** laten wij zien dat een ander complementeiwit, properdine genaamd, specifiek bindt aan niercellen. Deze binding van properdine, wat hier dus de functie van "gangmaker-eiwit" vervult, is essentieel voor het optreden van complement activatie. In **hoofdstuk 5** tonen wij aan dat properdine inderdaad in de urine gevonden kan worden van patiënten (in dit geval geen Hindoestanen maar 70 willekeurige patiënten van de polikliniek nierziekten in het LUMC) die een duidelijk eiwitlek hebben. Properdine in de urine is vervolgens weer geassocieerd met SC5b-9 in de urine. Dit duidt erop dat properdine een belangrijke bijdrage levert bij de door het complement systeem toegebrachte nierschade. Wanneer het molecuul geïdentificeerd kan worden waaraan properdine bindt is het in de toekomst wellicht mogelijk om proteïnuriegemedieerde nierschade (deels) te voorkomen.

Zoals boven reeds vermeld hebben Hindoestanen een aanleg voor het ontwikkelen van teveel buikvet (centrale adipositas). De verhouding tussen buikomtrek en taille-omtrek (Engels: waist-to-hip ratio (WHR)) is een maat voor centrale adipositas. In hoofdstuk 6 beschrijven wij dat de WHR bij Surinaams-Hindoestaanse type 2 diabeten de belangrijkste voorspeller is van hart-en vaatziekten. Personen met de hoogste WHR hadden een 5 x zo hoge kans op hart-en vaatziekten dan personen met de laagste WHR. Hoewel deze bevinding

nog niet bewijst dat vermindering van buikvet automatisch zal leiden tot minder hart- en vaatziekten, is het wel aannemelijk dat, specifiek in deze bevolkingsgroep, een gezondere leefstijl beschermend werkt voor hart- en vaatziekten.
In **hoofdstuk 7** beschrijven wij de resultaten van vaatfunctieonderzoek (IMT en PWV, zie verder) bij 54 Surinaams-Hindoestaanse type 2 diabeten. Tevens werden bloedwaarden gemeten van Connective Tissue Growth Factor (CTGF). Dit is een eiwit dat betrokken is bij bindweefselvorming (fibrose). Bij IMT meting (Intima-Media dikte) wordt met behulp van een echo-apparaat de binnenbekleding van de halsslagader gemeten, en de dikte hiervan is een maat voor atherosclerose ("vaatverkalking"). Bij een PWV meting (Pulse Wave Velocity) wordt de snelheid gemeten waarmee een drukgolf zich via de bloedvaten voortplant. Hoe stijver het bloedvat, hoe sneller de drukgolf zich voortplant (hoge PWV). Stijve bloedvaten leiden tot een grotere belasting voor het hart, en er komen steeds meer aanwijzingen dat stijve bloedvaten het risico op hart-en vaatziekten voorspellen. Wij vonden dat CTGF waarden gecorreleerd zijn aan zowel IMT (maat voor vaatverkalking), PWV (maat voor vaatstijfheid) als de mate van eiwit-lek in de urine (maat voor nierschade). Ook de tailleomtrek (als maat voor hoeveelheid buikvet) was geassocieerd met vaatstijfheid.
Hoewel de vraag of en op welke manier CTGF bijdraagt aan vaat- en nierschade nog niet is opgehelderd, lijkt CTGF een veelbelovend aangrijpingspunt om vaat- en niercomplicaties bij diabetes tegen te gaan.

Vanuit dit proefschrift kunnen enkele verbanden worden gelegd naar de medische praktijk. Aangezien centrale obesitas een belangrijke voorspeller is voor hart-en vaatziekten in Surinaams-Hindoestaanse type 2 diabeten, lijkt gewichtsreductie een primair aangrijpingspunt bij het voorkómen van hart-en vaatziekte, hoewel de effectiviteit ervan natuurlijk nog moet worden bewezen. Fascinerend is de kennelijke overlap tussen het complementsysteem en de vetweefsel stofwisseling. Er zijn geen gegevens met betrekking tot ASP in Surinaamse Hindoestanen. Proefdierstudies tonen aan dat een tekort aan ASP leidt tot gewichtsreductie en toegenomen energieverbruik. ASP zou daarom een interessant aangrijpingspunt kunnen zijn in het voorkómen van de schadelijke effecten van centrale adipositas.
De studies uit dit proefschrift roepen natuurlijk ook nieuwe vragen op. De bijdrage van MBL aan hart-en vaatziekte en nierschade dient verder opgehelderd te worden. Met name is nog onduidelijk of MBL zelf echt schadelijk is of slechts een

epifenomeen (dwz een andere –onbekende- factor leidt zowel tot toegenomen nierschade als toegenomen MBL waarden)

Eiwitlekkage in de urine is een belangrijke voorspeller voor progressieve nierfunctieverslechtering. Het verbaasde ons echter dat de personen die een verslechtering van nierfunctie hadden helemaal niet zoveel eiwit in de urine lekten. Ook in de dagelijkse praktijk worden veel patiënten gezien met progressieve nierfunctieverslechtering maar slechts een gering eiwitlek. Hoewel de rol van het complementsysteem bij eiwitlekkage steeds duidelijker wordt is er hoegenaamd niets bekend over de rol van het complementsysteem wanneer er nauwelijks eiwitlek is.
Surinaamse Hindoestanen met type 2 diabetes hebben een bijna 40 keer zo hoge kans om door diabetes aan de dialyse te geraken. Echter, eenmaal aan de dialyse hebben zij een betere overleving dan Caucasische lotgenoten. De oorzaak hiervan is niet duidelijk. Er zijn blijkbaar - tot op heden onbekende- factoren die hun schadelijkheid verliezen of juist beschermend werken in dialysepatiënten. Opmerkelijk genoeg heeft ook MBL zo'n "dubbel gezicht": hoge MBL waarden zijn geassocieerd met achteruitgang van nierfunctie, maar bij dialysepatiënten zijn lage MBL waarden juist gekoppeld aan slechtere overleving. Complement activatie op het oppervlak van de kunstnier is een welbekend fenomeen, en het complementsysteem zou de overleving aan de dialyse kunnen beïnvloeden. Het zou daarom interessant zijn om de rol van het complementsysteem bij Surinaams Hindoestaanse dialysepatiënten te bestuderen.

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 8 mei 1974 in Rijnsburg. Na het eindexamen VWO aan het dr. W.A Visser 't Hooft lyceum in Leiden werd in 1992 gestart met de studie Geneeskunde aan de Rijksuniversiteit Leiden. Na het behalen van het artsexamen in 1999 werkte hij als arts-assistent Interne Geneeskunde in het toenmalige Leyenburg (thans HAGA) Ziekenhuis in Den Haag. In 2001 werd hier met de opleiding tot internist gestart (opleiders dr. J.C.M. van der Vijver, dr. R.H. Kauffmann) en vanaf 2004 werd de opleiding voortgezet in het Leids Universitair Medisch Centrum (opleiders prof. A.E Meinders, prof. J.J. Romijn). In 2005 startte hij met de opleiding tot nefroloog (prof. A.J. Rabelink) en werd gestart met dit proefschrift onder leiding van prof. A.J. Rabelink, prof. M.R. Daha en dr. S.P. Berger. In 2006 en 2008 volgde respectievelijk registratie als internist en internist-nefroloog. Vanaf mei 2009 is hij werkzaam als internist-nefroloog in ziekenhuis Gelderse Vallei in Ede.

Nawoord

In den beginne hield ik mijzelf voor geen promotietraject te ondernemen, aangezien ik mezelf primair als clinicus en niet als wetenschapper beschouw. Gelukkig kwam ik er op tijd achter dat het één het andere niet hoeft uit te sluiten en kan ik terugkijkend zeggen dat het goed was.
Het moest gaan over de relatie tussen het complement-systeem en diabetes-gerelateerde complicaties. Het HINDINEF-cohort leek hiervoor bij uitstek geschikt en interessant: dat waren type 2 diabeten met een hoog cardiovasculair risicoprofiel.
Hoewel Moh mij waarschijnlijk graag iets vaker in het lab had gezien, kwam ik er al snel achter dat labwerk zich moeilijk met klinische taken laat combineren. Ik ben daarom zeer dankbaar voor de hulp die velen mij hebben gegeven, zowel in het lab als daarbuiten. Met name dank ik Reinier van der Geest, Nicole Schlagwein en Danielle van Gijlswijk-Janssen voor hun laboratoriumwerk, Jos op 't Roodt, Martin Spaans, Sabrina Hendriksen, Sonja van Berkel en Annemieke Sloeserwij voor het verrichten van de vaatfunctie-metingen. Voorts de secretaressen van de afdeling nierziekten voor administratieve ondersteuning, Romana Nasroe voor het opsporen en uitnodigen van de studiepersonen, Wim Poldermans, Herman Lemkes en Hanno Pijl voor het verrichten en beoordelen van fundusfoto's (hoewel de data hiervan nog wachten op analyse). Ook dank aan Tri Nguyen, Amelie Dendooven en Roel Goldschmeding voor de prettige samenwerking op het CTGF-artikel. En uiteraard Marko Mallat, die altijd bereid was om statistisch significante hulp te verlenen.
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De meeste offers worden achter de schermen en in stilte gebracht. Dat geldt ook voor dit proefschrift. Lieve Silvia: ik ben je intens dankbaar dat je me de gelegenheid hebt gegeven om dit avontuur aan te gaan en -belangrijker- het tot een (goed ?) eind te brengen. Als geen ander weet jij me in balans te houden. Bij jou ben ik thuis!