

# Plasma C3d Levels and Ischemic Heart Disease in Type II Diabetes

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**OBJECTIVE** — To test the hypothesis that the complement system may be activated in patients with type II diabetes and CAD.

**RESEARCH DESIGN AND METHODS** — The plasma C3d concentration was measured in 106 type II diabetic patients and 25 nondiabetic control subjects. The patient group was subdivided according to AER, and the groups were adjusted for age, sex, and known duration of diabetes. For the assignment to a given subgroup, normoalbuminuria was defined as AER <15  $\mu\text{m}/\text{min}$ , microalbuminuria as AER 16–250  $\mu\text{g}/\text{min}$ , and macroalbuminuria as AER >250  $\mu\text{g}/\text{min}$ . The presence or absence of coronary disease was assessed through clinical examination, ECG, and coronary angiography. An RIA system was used for measurement of urinary albumin levels, and the plasma C3d concentrations were measured by ELISA.

**RESULTS** — Within each of the AER-defined subgroups, the plasma C3d levels were significantly higher in patients with IHD than in those without. Thus, in the normoalbuminuric group, plasma C3d levels were 16.3 AU/ml (95% CI 13.9–19) in patients with IHD vs. 11.6 AU/ml (95% CI 10.5–12.7) in those without ( $P < 0.001$ ). The corresponding data for the microalbuminuric and macroalbuminuric groups were 21.8 (95% CI 18.1–26.3) vs. 13.6 (95% CI 12.3–15.1) and 31.6 (95% CI 24.9–40) vs. 17.5 (13.6–22.6) AU/ml ( $P < 0.01$ ), respectively. Patients with IHD also had significantly higher plasma C3d levels than normal control subjects, regardless of AER subgroup. A multiple logistic regression analysis demonstrated an association between the plasma C3d concentration and IHD and AER.

**CONCLUSIONS** — Activation of the complement system may play a role in the development of macrovascular disease in type II diabetes.

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IHD, ISCHEMIC HEART DISEASE; TYPE II DIABETES, NON-INSULIN-DEPENDENT DIABETES MELLITUS; TYPE I DIABETES, INSULIN-DEPENDENT DIABETES MELLITUS; CAD, CORONARY ARTERY DISEASE; AER, ALBUMIN EXCRETION RATE; RIA, RADIOIMMUNOASSAY; ELISA, ENZYME-LINKED IMMUNOSORBENT ASSAY; CI, CONFIDENCE INTERVAL; AU, ARBITRARY UNIT; DAF, DECAY ACCELERATING FACTOR; TG, TRIGLYCERIDE; BP, BLOOD PRESSURE; DBP, DIASTOLIC BLOOD PRESSURE; WHO, WORLD HEALTH ORGANIZATION; SBP, SYSTOLIC BLOOD PRESSURE; BMI, BODY MASS INDEX; LDL, LOW-DENSITY LIPOPROTEIN; HS, HEPARIN SULFATE.

In activation of the complement system in atherosclerosis has been demonstrated by the presence of C5b-9 complexes and of the complement regulatory glycoprotein DAF in atherosclerotic lesions (1–3). In a clinical context, high plasma level of products of the activation of the complement cascade have been detected in patients with acute myocardial infarction and with unstable angina (4).

Type II diabetes is a major risk factor for CAD, particularly so in patients with increased AER (5). Abnormal profiles of several components of the complement system have been reported in patients with type II diabetes and vascular complications (6). In a study of 256 type II diabetic patients, high plasma C3d levels were detected in 26% of cases (7). In type I diabetes, a significant correlation has been found to exist between AER and plasma SC5b-9 levels (8).

In this study we measured the plasma concentrations of C3d (a split product arising on activation of C3) in a selected group of 106 type II diabetic patients, then assessed their correlation with AER level and the presence or absence of IHD.

## RESEARCH DESIGN AND METHODS

The study group consisted of 106 type II diabetic patients selected from among those regularly attending (2–3 times/yr) our diabetes outpatient clinic. We used the following criteria to determine if patients had type II diabetes: no previous ketoacidosis episodes; absence of ketonuria; use of insulin only because of secondary failure or to improve control, and in any case after  $\geq 5$  yr duration of the disease; and diagnosis of diabetes >30 yr of age. Patients with overt heart failure, hepatic disease, or a history of renal or urinary tract disease were excluded. Table 1 shows pertinent clinical data on these patients.

AER was assessed at each regular visit to the outpatient clinic. Timed overnight urine samples were collected on

Table 1—Characteristics of study patients grouped by AER

	AER GROUPS		
	NORMOALBUMINURIC	MICROALBUMINURIC	PROTEINURIC
N	50	33	23
SEX (M/F)	20/30	14/19	11/12
AGE (YR)	62 ± 6	62 ± 5	65 ± 5
KNOWN DURATION OF DIABETES (YR)	10 ± 4	12 ± 6	13 ± 4
BMI (KG/M <sup>2</sup> )	28.1 ± 3.9	28.7 ± 4.5	29.1 ± 5
HbA <sub>1c</sub> (%)	9.2 ± 1.7	9.6 ± 1.8	9.1 ± 2.1
RETINOPATHY N/B/P*	34/15/1	16/12/5	2/13/8
SMOKERS/NONSMOKERS	17/33	10/23	7/16

Data are means ± SD or ratios.

\*N/B/P, normal/background/proliferative

three different days, and the patients were classed as normo-, micro-, or macroalbuminuric according to their AER levels (cut-off points are the means of three consecutive determinations). Normoalbuminuria was defined as AER ≤ 15 µg/min, microalbuminuria as AER 15–250 µg/min, and macroalbuminuria as AER ≥ 250 µg/min. We selected 33 microalbuminuric patients (age range 51–70 yr) for the study; 50 normoalbuminuric and 23 macroalbuminuric patients also were selected after adjustment for age, sex, and known duration of the disease. In addition, 26 age- and sex-matched normal individuals served as the control group.

The serum creatinine levels were measured using the Jaffe reaction in an autoanalyzer. The HbA<sub>1c</sub> concentration was measured by chromatography (Boehringer Mannheim, Mannheim, Germany). Commercially available enzymatic kits (Boehringer Mannheim) were used to measure total cholesterol and TG. Urinary albumin concentration was determined by RIA (Pharmacia Albumin RIA 100, Uppsala, Sweden). We used ELISA to measure plasma C3d, as described previously (9), using a rabbit anti-human C3d antibody (Dakopatts, Copenhagen, Denmark). Results were expressed in AU/ml.

The presence or absence of IHD was established by clinical examination,

ECG, and coronary angiography in symptomatic patients with normal ECGs (12 patients). At the time of the examination all patients were clinically stable. BP was measured twice, after 10 min of supine rest, using the disappearance of the Korotkoff sounds (phase V) for establishing the dBp. Arterial hypertension was diagnosed according to the WHO criteria (sBP >160 mmHg and/or dBp >95 mmHg) or when antihypertensive/diuretic therapy had been prescribed. Retinal status was assessed by ophthalmoscopy and vitreous fluorophotometry in 36 patients (10).

AER and plasma C3d, creatinine, and TG were logarithmically transformed (log<sub>10</sub>) for further processing. Differences between groups were evaluated using Student's *t* test for unpaired observations. Differences in the C3d plasma levels in each subgroup (expressed as geometric means and 95% CI) and other continuous data were analyzed using Scheffe's test for multiple comparisons. Correlations were calculated as the correlation coefficient, *r*. Multiple regression analyses were performed using the R-SIGMA program 1.991 (Horus Hardware, Madrid, Spain). *P* < 0.05 was considered statistically significant.

**RESULTS**— In addition to the matching parameters, the albuminuric groups had comparable BMI, smoking habits,

and HbA<sub>1c</sub> levels. Hypertension and retinopathy, as expected, were more prevalent in the micro- and macroalbuminuric groups. Serum creatinine levels were highest in the macroalbuminuric group.

Within each AER group, patients with and without IHD were compared as to prevalence of hypertension, plasma TC and TG, creatinine, C3d, and AER. Plasma TC and TG, serum creatinine, AER, and prevalence of hypertension did not differ within groups. The plasma C3d levels, however, were significantly higher in the IHD patients (compared with those without IHD) within each AER group. In the 11 patients with IHD in the normoalbuminuric group, the plasma C3d level was (geometric mean and 95% CI) 16.3 (13.9–19) vs. 11.6 (10.5–12.7) AU/ml in the 39 patients without IHD (*P* < 0.001). The corresponding figures for the other two groups were: microalbuminuria, 13 patients with IHD, 21.8 (18.1–26.3) vs. 13.6 (12.3–15.1) in 20 patients without IHD (*P* < 0.01); macroalbuminuria, 12 patients with IHD, 31.6 (24.9–40) vs. 17.5 (13.6–22.6) AU/ml in 11 patients without IHD (*P* < 0.01). (Table 2).

Patients with IHD had higher plasma C3d levels than the control group, regardless of their AER group assignment (Fig. 1). Among the patients without IHD, only those with macroalbuminuria had plasma C3d levels significantly higher than those of the control group (Fig. 2).

We further examined the influence of various factors—AER, HbA<sub>1c</sub>, serum creatinine, TC, TG, prevalence of hypertension, and IHD—on the plasma C3d levels. The analysis disclosed a strong association between C3d and the presence of IHD. This effect was at least in part independent of AER (Tables 3 and 4).

**CONCLUSIONS**— In type II diabetes, an association between an abnormal AER and coronary heart disease is well established, and our findings are in agreement with this. Some studies have suggested

Table 2—Risk factors and C3d plasma values in AER study patients grouped by the absence or presence of IHD

	AER GROUPS					
	NORMOALBUMINURIC		MICROALBUMINURIC		MACROALBUMINURIC	
	ABSENCE	PRESENCE	ABSENCE	PRESENCE	ABSENCE	PRESENCE
N	39	11	20	13	11	12
TOTAL CHOLESTEROL (MM)	6.25 ± 1.12	6.3 ± 1.16	6.3 ± 1.02	6.4 ± 1.03	6.28 ± 1.6	6.1 ± 1.5
TOTAL TG (MM)	2.58 (2.41–2.76)	2.62 (2.33–2.94)	2.71 (2.53–2.90)	2.61 (2.37–2.87)	1.95 (1.57–2.41)	1.94 (1.46–2.57)
AER (μG/ML)	3.9 (2.76–3.92)	3.36 (2.35–4.8)	33.4 (23.5–47.5)	57.8 (32.7–102.3)	1131.1 (826.2–1548.1)	1008.8 (698.7–1456.5)
CREATININE (μM)	84.86 (79.56–90.16)	88.4 (81.32–97.24)	88.4 (87.51–96.35)	89.28 (79.56–101.66)	143.2 (118.45–173.26)	176.8 (146.74–214.8)
C3d (AU/ML)	11.6 (10.5–12.7)	16.3 (14–19)*	13.6 (12.3–15.1)	21.8 (18.1–26.3)*	17.5 (13.6–22.6)	31.6 (24.9–40)*

Data are geometric means (95% CIs), except total cholesterol, which are means ± SD.

\*P < 0.01, absence vs. presence in each group.

that changes in the plasma lipid profile contribute to the increased cardiovascular mortality observed with increasing albuminuria in both types of diabetes (11,12). These abnormalities, however, have not been consistently observed in all diabetic patients (13,14). Evidence of complement system activation has been demonstrated in arterial atherosclerotic lesions by in vitro immunohistochemical methods (1–3). Studies of in vivo complement activation in patients with IHD are scarce. In one study (4), patients with acute myocardial infarction or unstable angina showed significantly increased plasma levels of C3d, C4d, and SC5b-9. Patients with clinically stable CAD also showed increased levels of these products of complement activation, but the differences were not statistically significant. High plasma C3d concentrations were demonstrated in 26% of cases in a series of 256 patients with type II diabetes, but no clinical correlations were given (7). In another study, abnormal profiles of several components of the complement system were found more frequently in type II diabetic patients with vascular complications (6). In type I diabetes, the plasma concentrations of SC5b-9 have been shown to correlate with the overnight AER (8).

Our study also found a significant overall correlation between AER and plasma C3d levels. A more important observation, however, is that patients with IHD had significantly higher plasma C3d levels than those without, at matching levels of AER. Only in the microal-

buminuric group does the mean AER tend to be higher in the IHD subgroup, but the difference was not statistically significant. Moreover, all IHD subgroups, regardless of AER level, showed plasma C3d levels significantly higher than those of the normal control sub-

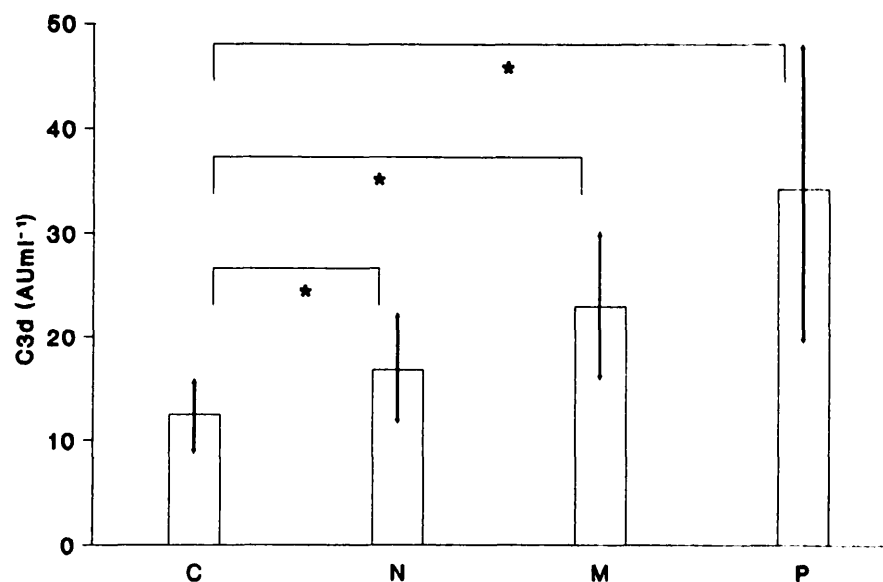
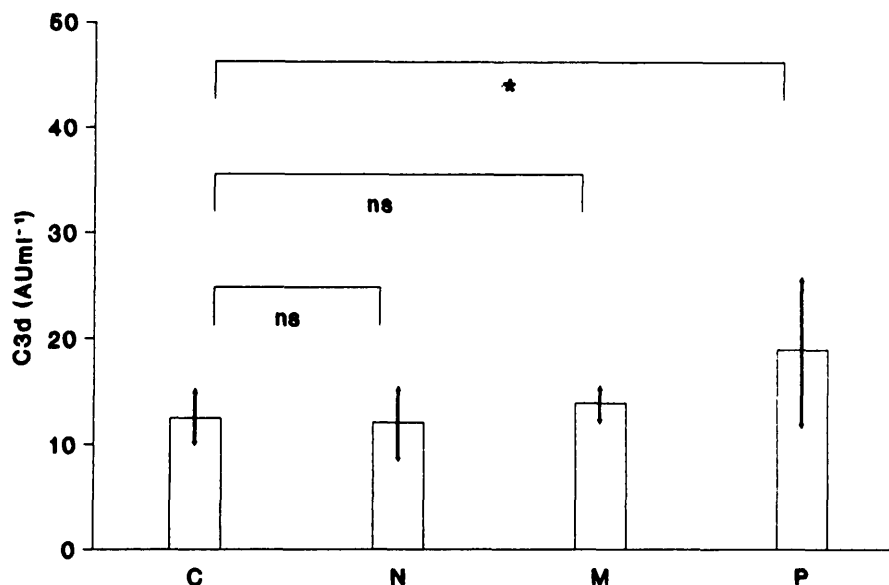


Figure 1—Plasma C3d levels in patients with IHD. (C), normal control subjects; (N), normoalbuminuric patients; (M), microalbuminuric patients; (P), patients with proteinuria. Plasma C3d levels in N and M patients were not significantly different from that of C subjects. \*P < 0.01.



**Figure 2**—Plasma C3d levels in patients without IHD. (C), normal control subjects; (N), normoalbuminuric patients; (M), microalbuminuric patients; (P), patients with proteinuria. Plasma C3d Levels in N, M, and P patients were significantly higher than that of C subjects. \*P < 0.05.

jects. Among the patients without IHD, only the macroalbuminuric group had plasma C3d concentrations significantly higher than those of the normal control group.

The activation of the complement system either by the classical (immune complex-mediated) and/or by the alternative (antibodies not required) pathways lead to changes in the proteins constituting the system, which then interact sequentially generating or inducing mediators of inflammation. The quantification of several stable cleaved fragments, such as C3d, may be used for the clinical assessment of the activation of the system

(9). The assembly of the final components of the system's sequence results in the formation of the membrane attack complex (MAC, C5b-9), which is able to damage cell surface membranes (15).

The mechanisms by which the complement system may be activated in patients with type II diabetes and CAD are unknown. Increased plasma levels of circulating immune complexes and of SC5b-9 have been found to be associated, although in many cases not significantly (8). The nonenzymatic glycosylation of all kinds of proteins, including

lipoproteins, is enhanced in diabetes mellitus, and one effect of glycosylation is to render these proteins immunogenic (16). Glycosylated LDL has been shown to bind to advanced glycosylated end products present in the endothelium of the arterial wall (17). Autoantibodies that react with glycosylated LDL have been demonstrated in the sera of type II diabetic patients (18). Thus, immune complexes might be formed in situ in the arterial wall.

Nonimmunological mechanisms may operate through the reduced amounts of sialic acid and mucopolysaccharides found in diabetic basal membranes (19). One study showed that human atherosclerotic lesions contain a lipid that is a dose-dependent activator of the alternative pathway of the complement system (20). Split products of the complement components are chemotactic for monocytes and can induce the synthesis of many biologically active molecules, including arachidonic acid derivatives, reactive oxygen forms, proteolytic enzymes, interleukins, etc. (21). Platelets and endothelial cells also can be affected. An increased procoagulant activity has been demonstrated in blood platelets exposed to activated complement components (22). Finally, loss of membrane-associated HS and antithrombotic property has been observed in cultured endothelial cells under the effect of terminal complement activation (23).

Our results suggest a role for

**Table 3**—Correlation of logC3d with various factors in type II diabetes

	r	P
LOGAER	0.58	<0.05
LOGCREATININE	0.48	<0.05
LOGTG	-0.14	NS
TOTAL CHOLESTEROL	0.06	NS
HbA <sub>1c</sub>	0.09	NS

**Table 4**—Effect of IHD on logC3d estimated by multiple regression analysis

FACTOR	EFFECT	P
CONSTANT	1.017 ± 0.026	
LOGCREATININE	0.173 ± 0.117	0.143 (NS)
LOGAER	0.070 ± 0.017	0.0001
IHD*	0.183 ± 0.029	0.00000001
HTA†	0.013 ± 0.028	0.640 (NS)

Data are means ± SE.  
\*IHD, absent vs. present (0 vs. 1)  
†HTA, absent vs. present (0 vs. 1)

complement activation in patients with type II diabetes and coronary heart disease. We believe the possibility of complement activation in other vascular complications of diabetes merits further examination. Deposits of several components of the complement system have been demonstrated in proliferative retinopathy (24), and in our study the plasma C3d levels were significantly higher than normal in macroalbuminuric patients without clinical evidence of IHD.

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