

# Anti-Heat Shock Protein 27 Antibody Levels and Diabetes Complications in the EURODIAB Study

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**OBJECTIVE** — To assess whether serum anti-heat shock protein 27 (HSP27) antibody levels are associated with micro- and macrovascular complications of type 1 diabetes.

**RESEARCH DESIGN AND METHODS** — Anti-HSP27 IgG antibody levels were measured in 531 type 1 diabetic subjects recruited as part of the cross-sectional analysis of the EURODIAB Prospective Complications Study. Case subjects ( $n = 363$ ) were defined as individuals with one or more diabetes complications and control subjects ( $n = 168$ ) as individuals with no evidence of any diabetes complication.

**RESULTS** — Anti-HSP27 levels were comparable in case and control subjects (19.6 arbitrary units/ml [11.3–32.7] vs. 20.4 arbitrary units/ml [11.7–35.3], geometric mean [interquartile range]), and there was no correlation between HSP27 and anti-HSP27 levels ( $r = 0.01$ ,  $P = 0.81$ ). In logistic regression analysis, anti-HSP27 was not associated with the presence of complications, even after adjustment for main risk factors.

**CONCLUSIONS** — Anti-HSP27 antibody levels are not a marker of vascular complications in type 1 diabetes.

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Heat shock protein (HSP27) is a member of a family of proteins whose intracellular expression is increased to offset the deleterious effects of cellular stresses (1). HSP27 is also released into the circulation and can induce an autoimmune response with production of anti-HSP27 antibodies (2).

The immune response against HSPs has been implicated in the pathogenesis of atherosclerosis in the general population (3). In clinic-based cohorts, anti-HSP27 antibody levels were found to be associated with age and hypertension (4), although not consistently (5), and in-

creased in patients with acute coronary syndromes (4,6). However, no large epidemiological study has assessed anti-HSP27 levels in stable patients with established cardiovascular disease.

Type 1 diabetes is associated with a greatly increased risk of vascular complications, and we have recently reported that, in type 1 diabetic individuals, higher serum levels of HSP27 are independently associated with a threefold-increased risk of distal symmetrical polyneuropathy (DSP) (7). In the same study base, we have now assessed potential associations between anti-HSP27 antibodies and both

micro- and macrovascular complications of type 1 diabetes.

## RESEARCH DESIGN AND METHODS

The EURODIAB Prospective Complications Study is a follow-up of the EURODIAB IDDM Complications Study, designed to explore risk factors for diabetes complications in 3,250 randomly selected people with type 1 diabetes (8,9). A cross-sectional, nested case-control study was designed on the cohort recruited at follow-up (10). Case subjects were defined as individuals with cardiovascular disease, proliferative retinopathy, micro-/macroalbuminuria, or neuropathy. Control subjects were selected based on being completely free of complications. Only subjects with serum samples stored at  $-80^{\circ}\text{C}$  within 2 h from collection were included to reduce variability due to protein degradation. Applying these criteria, this yielded 363 case and 168 control subjects with full data on complications and samples available for analysis (7). The sample size provides a power of 95% ( $\alpha = 0.05$ ) to detect a difference in log anti-HSP27 of at least one-third of an SD between case and control subjects.

Anti-human HSP27 antibodies were measured using an in-house enzyme-linked immunosorbent assay. Microtiter plates were coated with 1  $\mu\text{g}$  rh-HSP27 (Stressgen, Milan, Italy). After blocking with 3% BSA, both standards and serum samples (diluted 1:500) were added in duplicate and incubated overnight at  $4^{\circ}\text{C}$ . After 2-h incubation with peroxidase-conjugated goat anti-human IgG (Sigma-Aldrich, Milan, Italy), the substrate 3,3',5,5'-tetramethylbenzidine dihydrochloride was added and the absorbance read at 450 nmol/l. Six serial dilutions of a control serum, highly positive for anti-HSP27 IgG antibodies, were assayed in every plate and used to generate a standard curve. The undiluted serum sample was assigned 125 arbitrary units per milliliter (AU/ml). The inter- and intra-assay coefficients of variation were 7.5 and 5.3%, respectively. Serum IgG levels were determined by immunoturbidometry (Dade Behring BN 100 Analyzer) with anti-IgG

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reagents and calibrators (Dade Behring). The coefficients of variation for both intra- and inter-assay were <4%.

Logistic regression analyses were used to estimate the odds ratios of anti-HSP27 for any complication (albumin excretion rate  $\geq 20$   $\mu\text{g}/\text{min}$ , retinopathy, neuropathy, and cardiovascular disease), independently of confounders and known risk factors. The likelihood ratio test was used to compare nested models examining the role of age, sex, diabetes duration, BMI, waist-to-hip ratio, A1C, blood pressure, lipids, albumin excretion rate, C-reactive protein, interleukin-6, tumor necrosis factor- $\alpha$ , homocysteine, Amadori albumin, soluble E-selectin, soluble vascular cell adhesion molecule, and smoking status. Variables were retained in the final model if they added significantly to the likelihood of models or to the estimated coefficients of predictors. In light of the hypothesis of a different role of anti-HSP27 antibodies in the pathogenesis of different complications, logistic regression models were also fitted separately for each complication. To assess the pattern of odds ratios across increasing levels of serum anti-HSP27 antibodies, levels were categorized into quartiles in control subjects. Both linear and U-shaped trends across quartiles were tested by entering a single ordinal term and a quadratic term into the models.

**RESULTS**— Anti-HSP27 levels were measurable in all 531 samples and showed a right skewed distribution. Values were similar in case and control subjects (19.6 AU/ml [11.3–32.7] vs. 20.4 AU/ml [11.7–35.3], geometric mean [interquartile range],  $P = 0.57$ ), even after adjustment for age and diabetes duration (20.0 vs. 20.5 AU/ml,  $P = 0.80$ ). Furthermore, no relation was found between HSP27 and anti-HSP27 serum levels ( $r = 0.01$ ,  $P = 0.81$ ). In the logistic regression analysis (Table 1), there was no significant association of anti-HSP27 antibodies with either the “any complication” category or with each complication separately, apart from cardiovascular disease (model 1 and 2). After adjustment for main risk factors, however, this association was no longer significant (model 3). No significant trend, either linear or U-shaped, across quartiles was present. Results were unmodified after adjustment for IgG levels.

**CONCLUSIONS**— We have recently reported that in type 1 diabetic patients,

**Table 1—Odds ratios for diabetes complications by anti-HSP27 values in the nested case-control study within the EURODIAB study**

	Odds ratio (95% CI)*	Odds ratio (95% CI)†	Odds ratio (95% CI)‡
All complications			
Log anti-HSP27	1.07 (0.84–1.36)	1.00 (0.76–1.30)	1.12 (0.80–1.58)
Anti-HSP27 (AU/ml)			
<11.33	1.00	1.00	1.00
11.33–21.27	1.20 (0.72–2.02)	1.05 (0.59–1.89)	1.56 (0.75–3.28)
21.28–32.68	0.87 (0.51–1.48)	0.57 (0.31–1.05)	0.59 (0.28–1.28)
>32.68	1.25 (0.75–2.09)	1.24 (0.69–2.23)	1.65 (0.80–3.43)
<i>P</i> for linear trend	0.65	0.87	0.56
DSP			
Log anti-HSP27	1.10 (0.84–1.43)	0.97 (0.71–1.33)	0.94 (0.62–1.41)
Anti-HSP27 (AU/ml)			
<11.33	1.00	1.00	1.00
11.33–21.27	1.26 (0.71–2.24)	0.98 (0.49–1.94)	1.76 (0.70–4.40)
21.28–32.68	0.93 (0.51–1.70)	0.56 (0.27–1.15)	0.45 (0.18–1.21)
>32.68	1.26 (0.71–2.24)	1.16 (0.59–2.30)	1.17 (0.48–2.87)
<i>P</i> for linear trend	0.66	0.98	0.63
Micro-/macroalbuminuria			
Log anti-HSP27	1.02 (0.78–1.34)	0.98 (0.71–1.35)	0.96 (0.60–1.54)
Anti-HSP27 (AU/ml)			
<11.33	1.00	1.00	1.00
11.33–21.27	1.16 (0.66–2.05)	1.13 (0.58–2.23)	2.25 (0.80–6.34)
21.28–32.68	0.90 (0.50–1.63)	0.66 (0.33–1.34)	0.59 (0.20–1.66)
>32.68	1.08 (0.61–1.92)	1.30 (0.65–2.59)	1.30 (0.47–3.62)
<i>P</i> for linear trend	0.99	0.82	0.79
Retinopathy			
Log anti-HSP27	1.08 (0.83–1.38)	1.03 (0.76–1.39)	1.11 (0.74–1.67)
Anti-HSP27 (AU/ml)			
<11.33	1.00	1.00	1.00
11.33–21.27	1.28 (0.75–2.18)	1.37 (0.71–2.63)	2.28 (0.93–5.60)
21.28–32.68	0.97 (0.56–1.68)	0.66 (0.33–1.30)	0.69 (0.28–1.70)
>32.68	1.25 (0.73–2.13)	1.50 (0.78–2.90)	2.09 (0.84–5.19)
<i>P</i> for linear trend	0.66	0.60	0.49
Cardiovascular disease			
Log anti-HSP27	1.47 (1.09–1.99)	1.33 (0.93–1.90)	1.33 (0.85–2.07)
Anti-HSP27 (AU/ml)			
<11.33	1.00	1.00	1.00
11.33–21.27	1.72 (0.88–3.40)	1.56 (0.70–3.43)	1.72 (0.64–4.62)
21.28–32.68	1.50 (0.75–2.99)	0.76 (0.33–1.77)	0.69 (0.24–1.90)
>32.68	2.41 (1.25–4.64)	2.43 (1.11–5.43)	2.35 (0.90–6.10)
<i>P</i> for linear trend	0.02	0.09	0.21

\*Unadjusted. †Adjusted for age and diabetes duration. ‡Adjusted for age, diabetes duration, hypertension, A1C, smoking, and log tumor necrosis factor- $\alpha$ .

serum levels of HSP27 are an independent marker of DSP (7). In contrast, in the present study, performed on the same study base, we found that anti-HSP27 levels were similar in type 1 diabetic patients with or without micro- and macrovascular complications, including DSP.

Although serum HSP27 levels are enhanced in type 1 diabetic patients with DSP (7) and anti-HSP27 antibodies induce neuronal apoptosis in vitro (11), both the absence of relation between

HSP27 and anti-HSP27 levels and the similarity in anti-HSP27 antibody values among patients with or without DSP do not support the hypothesis of a role of anti-HSP27 antibodies in the pathogenesis of the neuronal damage in type 1 diabetes.

An immune response against HSPs has been implicated in the pathogenesis of atherosclerosis (3). This is the first large epidemiological study assessing circulating anti-HSP27 levels in type 1 diabetic

subjects with cardiovascular complications. Strengths of our study are the use of a large representative sample of people with type 1 diabetes and the ability to account for confounding by other risk factors and complications. There are limitations, such as the cross-sectional study design and the reduced power of the analyses due to lower number of control subjects compared with case subjects. However, altogether, our data do not support the hypothesis that anti-HSP27 antibody levels are a suitable marker for micro-/macrovascular complications in type 1 diabetes.

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