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# UNIVERSITÀ DEGLI STUDI DI TORINO

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

\*Davina Burt Ph.D., \*Graziella Bruno M.D., †Nish Chaturvedi MRCP, ‡Casper Schalkwijk Ph.D, ‡Coen D Stehouwer Ph.D., §Daniel R Witte Ph.D., ||John H Fuller FRCP, \*Silvia Pinach M.Sc., \*Paolo Cavallo Perin M.D., \*Gabriella Gruden Ph.D.

\*Department of Internal Medicine, University of Turin, Italy; †National Heart and Lung Institute, Imperial College, London, U.K.; ‡Department of Internal Medicine, Maastricht University Medical Centre Maastricht, the Netherlands; §Epidemiology Research Group, Steno Diabetes Center, Gentofte, Denmark; ||Department of Epidemiology and Public-Health, Royal Free and University College London, Medical School, London, U.K.

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### **Corresponding author:**

Dr. Davina Burt, PhD  
Diabetic Nephropathy Laboratory  
Department of Internal Medicine  
University of Turin  
C/so Dogliotti 14, 10126, Turin, Italy  
Tel: 0039-011-663-6035  
Fax: 0039-011-633-4751  
e-mail: [davina.burt@unito.it](mailto:davina.burt@unito.it)

## **Abstract**

**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 those with one or more diabetic complications and control subjects (n=168) as those with no evidence of any diabetic complication.

**Results.** Anti-HSP27 levels were comparable in cases and control subjects [19.6 AU/ml (11.3-32.7) vs. 20.4 AU/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.

## **Introduction**

Heat shock protein (HSP) 27 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), though not consistently (5), and increased in patients with acute coronary syndromes (4,6). However, no large epidemiological study has assessed anti-HSP27 antibody 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 3-fold 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/macrovascular complications of type 1 diabetes.

## **Research design and Methods**

The EURODIAB Prospective Complications Study is a follow-up of the EURODIAB Type 1 Diabetes Complications Study, designed to explore risk factors for diabetic complications in 3250 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 those with cardiovascular disease (CVD), 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° C within two hours 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-antiHSP27 of at least one third of a standard deviation between cases and control subjects.

Anti-human HSP27 antibodies were measured using an in-house ELISA. Microtitre plates were coated with 1 $\mu$ g of 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°C. After 2-hour 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 nM. 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-assay and intra-assay CVs were 7.5% and 5.3%, respectively. Serum IgG levels were determined by immunoturbidometry (Dade Behring® BN 100 Analyzer) with anti-IgG reagents and calibrators (Dade Behring®). The CVs for both intra- and inter-assay were below 4%.

Logistic regression analyses were used to estimate the odds ratios (ORs) of anti-HSP27 for any complication [albumin excretion rate (AER)  $\geq 20\mu$ g/min, retinopathy, neuropathy, CVD], 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, WHR, HbA<sub>1c</sub>, blood pressure, lipids, AER, C-reactive protein, interleukin-6, tumor necrosis factor (TNF)- $\alpha$ , 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 pattern of ORs across increasing levels of serum anti-HSP27 antibodies, levels were categorized into quartiles in controls. 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 cases 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 AU/ml 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 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 CVD (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 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 (6) 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 certain limitations, such as the cross-sectional study-design and the reduced power of the analyses due to lower number of controls as compared to cases. 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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### **Disclosure**

The authors have no relevant conflict of interest to disclose.

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

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

\* unadjusted

\*\* adjusted for age and diabetes duration;

\*\*\* adjusted for age, diabetes duration, hypertension, HbA<sub>1c</sub>, smoking, log-TNF $\alpha$