Online Supplement

Anti-elastin and anti-collagen V antibodies:

Anti-elastin antibody levels were determined in duplicate on plasma samples of 463 subjects. To determine anti-elastin antibody levels, an enzyme-linked immunosorbent assay (ELISA) was used, according to a slightly modified protocol of Lee et al. Briefly, human lung elastin peptides (prepared by enzymatic hydrolysis of human lung elastin with human sputum elastase, QP45, Elastin Products Company, Owensville) were dissolved in PBS (Dulbecco's Phosphate buffer saline, Invitrogen, Merelbeke, Belgium) in a final concentration of 25 µg/ml and 60 µl was used to coat high protein binding flat bottom polystyrene microtiter plates (Immulon 4HBX, Fisher Scientific, Erembodegem, Belgium). Plates were incubated at 37 °C for 2 hours and subsequently washed with 0.05 % PBS-Tween solution (Tween 20, Sigma-Aldrich, Bornem, Belgium). Plates were then blocked by adding 200 µl of 1 % blocking buffer (Bovine serum Albumin or BSA, Sigma-Aldrich, Bornem, Belgium) and incubated at 37°C for 2 more hours. After washing, human plasma samples were diluted in sodium bicarbonate buffer (pH 9.6) and applied on the plates with a 2-fold serial dilution starting from 1:40 to 1:1280. Plates were incubated overnight at 4°C and washed with 0.05 % PBS-Tween solution before the application of a secundary antibody diluted in 1 % BSA at a concentration of 4 µg/ml (goat anti-human HRP conjugated IgG (Ab6759, Abcam, Cambridge, U.K.). After 2 hours of incubation at room temperature, several washing steps with PBS occurred after which 60 µl of TMB buffer (Invitrogen, Merelbeke, Belgium) was added for 15-30 minutes in the dark. The colorimetric reaction was stopped by adding 60 µl of stop solution (sodium hydroxide, Invitrogen, Merelbeke, Belgium). Optical density of individual wells was determined at 450 nm using standard microplate spectrophotometer. In our assay, a positive control was included on each plate. For this purpose, we selected a plasma

sample of a scleroderma patient with known positive titers of anti-elastin antibodies. The samples were calculated relative to the standard positive control and values were expressed as relative units: (OD sample/ OD positive control) x 100. Patients with relative units ≥ 100 were considered as positive. In the subgroup of 25 patients, anti-elastin antibody titers were repeated to determine variability over time. The average time between two samples was 2.61 years. Anti-collagen V antibody titers were also quantified by ELISA with following protocol changes: Human collagen V (C657, collagen V from human placenta, Sigma Aldrich, Bornem) was diluted at concentration of 25 µg/ml to coat high protein binding flat bottom polystyrene plates. Plasma samples were serially diluted and applied in concentrations starting from 1:10 to 1:640. The secondary antibody (goat anti-human HRP conjugated IgG) was used at a lower concentration (0.6 µg/ml) to minimize non-specific binding in our assay. We also included a positive control for anti-collagen V antibodies (scleroderma patient) on each plate to calculate relative units against positive controls.