Anti-CarP home-made ELISA technique

To detect IgG or IgA antibodies against carbamylated fetal calf serum (Anti-FCS and Anti-FCS-IgA, respectively) and IgG fibrinogen (Anti-Fib), an ELISA assay using both carbamylated FCS and Fib with non-modified FCS or Fib as antigens was developed. FCS and Fib were carbamylated by incubating a 4mg/ml concentration for FCS or 2mg/ml for Fib with 1M of KCNO (or with 1M of KCl for the controls) for 15 hours at 37ºC. After incubation, the samples were desalted by centrifugation (Amicon Ultra-0.5 centrifugal filter units, Merck). Carbamylation efficiency was assessed by amino acid analysis of the hydrolysed samples in a Biochrom 30 amino acid analyser (Biokrom, UK) using L-Norleucine as the internal standard. The conversion of Lys to homocitrulline was determined as the fraction of the total amount of amino acids.

Anti-FCS and Anti-Fib were determined by ELISA. All samples were assayed on separate plates (Nunc MaxiSorp, Thermo Fisher Scientific, Denmark) coated with FCS or Fib carbamylated and non-modified as antigens overnight at a concentration of 10 µg/mL of carbonate-bicarbonate buffer (0.1 M pH 9.6). Diluted serum samples (1:50 in PBS-1% BSA-0.05% Tween) were incubated overnight at 4ºC and IgG or IgA antibodies detected using goat anti-human IgG-AP or a rabbit anti-human serum IgA-AP (Jackson Immunoresearch Europe, UK) and SIGMAFAST p-Nitrophenyl phosphate as substrate.

Chimeric Fibrin/Filaggrin Homocitrullinated peptide (CFFHP): [HCit620,625] α-fibrin(617-631)-S306, S319 cyclo [Cys306,319, HCit312]filaggrin (304-324) and its non-homocitrullinated version were synthesized by solid-phase peptide synthesis as C-terminal carboxamides on a Novasyn TGR resin (Novabiochem Merck, Germany) following a 9-fluorenylmethoxycarbonyl (Fmoc) strategy with subsequent cyclization in solution by forming a disulfide bridge (1-2).

To determine IgG antibodies against CFFHP (Anti-CFFHP) home-made ELISA assays were performed. Firstly, CFFHP and non-homocitrullinated peptide as a control for homocitrulline specificity were coupled covalently to the microplates (Nunc Immobilizer)

diluted to 10µg/mL in 0.05 M carbonate/bicarbonate (pH 9.6) buffer. 100 µL of peptide solution was added to each microplate well and incubated overnight at 4ºC. Each plate contained control wells that included all reagents except the serum sample and the peptide in order to estimate the background reading. After incubation, the plates were blocked with 2% BSA in 0.05 M carbonate/bicarbonate (pH 9.6) buffer for 1 hour at room temperature. Sera were diluted 50-fold in RIA buffer (1% BSA, 350 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1% vol/vol Triton X-100, 0.5% wt/vol Na-deoxycholate, 0.1% SDS) supplemented with 10% foetal bovine serum; 100µL/well was added and incubated for 1.5 hours at room temperature. After washing 6 times with PBS/0.05% Tween-20, 100 µL/well of the anti-human secondary IgG antibody conjugated to peroxidase in RIA buffer was added and bound antibodies were detected using SIGMAFAST with o-phenylenediamine dihydrochloride as substrate.

Reactivity to non-homocitrullinated FCS, Fib and CFFHP peptide was subtracted from the reactivity to homocitrullinated FCS, Fib and CFFHP. A series of successive dilutions of a pool of sera from four positive patients was used as a reference standard in all plates and to convert optical density values to arbitrary units (AU). ROC curve analysis and regression analysis were conducted using the GraphPad Prism5 program and the cut-off values were determined with a specificity of 95% compared with a healthy population of blood donors (n=179). A positive cut-off value was defined as ≥ 173.5 AU/mL, ≥ 166,9 AU/mL, ≥ 146.5 AU/mL and ≥ 257.0 AU/ml for Anti-FCS, Anti-Fib, Anti-CFFHP and Anti-FCS-IgA respectively. The magnitude of the reactivity against FCS-CarP and Fib-CarP vs. native proteins rendered differences in OD values higher than 0.2 and 0.8, respectively; these differences were lower for the chimeric carbamylated peptide (>0.1). A test was only considered positive and specific for homocitrulline when the UA/mL values were higher than the respective cut-off and the OD difference between carbamylated (homocitrullinated) and native (non-homocitrullinated) antigens was at least 0.1, in

agreement with the methodology previously reported by van der Woude et al. for citrullinated peptide antigens (3).

References

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