Disease and immune diseases [2]. Hence, there is a possibility that Grp94 might also display antigenic activity in other immune diseases, such as RA and SLE [3, 4], and that anti-Grp94 antibodies can be taken as an early and sensitive indicator of the development and evolution of these diseases [4]. In support of this, the paper of Weber et al. [5] should add further evidence to previous experimental data, showing the increase of anti-Grp94 antibodies in patients with RA and SLE. Unfortunately, however, the paper suffers with several methodological errors that heavily affect validity of the results. One critical aspect relates to ELISA for measuring auto-antibodies in serum. The authors made determinations without previously validating the assay; i.e., they omitted to construct the calibration curve with both negative and true-positive samples (to establish the lower and higher limits of sensitivity), and determine intra- and inter-assay variability [6, 7].

Reproducibility of measures should also be tested using at least two dilutions of each serum sample in duplicate and the values, read on the linear portion of the curve, should be expressed as antibody titre (dilution factor of serum) or antibody concentration (μg/ml). Normalization by plasma proteins is preferable, since in patients with immune diseases (and also in normal subjects) protein concentration [especially that of immunoglobulin G (IgG)] cannot be assumed that is equal, and averaging antibody values without this correction might engender big errors.

It is not clear why the authors used western blotting (WB) to assess specificity of the immune reaction after having already made the same measure in ELISA. As a rule, WB is used first to identify both true-positive and negative samples that then serve for constructing the calibration curve in ELISA. The only difference between the two methods is the higher sensitivity and reliability of ELISA, which in addition, permits quantification of measurements. Since the same antigenic proteins are used in both methods (with the exception of a lower quantity of antigen in ELISA), the reason why the authors omitted Grp94 in experiments of WB to detect true-positive reactions (Fig. 2 in their paper) appears to contradict the principle of the method and negating validity of results obtained with Grp94 in ELISA (Table 1 in their paper).

A matter of concern is also the lack of adequate controls for excluding non-specific reactions of both serum samples and secondary anti-human IgG antibodies with the antigen. The authors used only BSA as control in half of the plate wells, BSA can be taken as the blank for positivity due to un-blocked sites in the plate, but the real blank is made with antigen incubated in the absence of primary antibodies (thus, rigorously speaking, with plasma proteins other than anti-chaperone antibodies; i.e., with control human serum albumin (HSA) and/or IgG at approximately the same concentration present in patients’ samples). In particular, the authors do not seem to know that Grp94 can also irreversibly bind IgG at sites other than the antigen-binding site [8], thus giving rise to significant false-positive reactions with IgG antibodies (of different species). We investigated this crucial property of Grp94 in depth and found that non-specific binding of Grp94 to IgG could only be prevented by thermal denaturation of

References

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Comment on: Antibodies to the endoplasmic reticulum-resident chaperones calnexin, Bip and Grp94 in patients with rheumatoid arthritis and systemic lupus erythematosus

Sini, The role of Grp94, the most represented endothelium-resident chaperone, in the pathogenesis of inflammatory/immune diseases and development of associated vascular complications has recently been demonstrated in experiments on plasma of type 1 diabetic patients [1], providing indirect experimental evidence of the immunogenicity of extracellular Grp94 [2]. Hence, there is a possibility...
Grp94 [9], whereas SDS at the concentrations used by authors was ineffective. The abnormally elevated dispersion of data even in healthy subjects, who instead should not show any positivity for autoantibodies, might thus originate from non-specific binding of non-immune IgG in any serum sample to antigen Grp94. The same troublesome aspect likely also occurs with Bip, since Bip is even more involved than Grp94 in binding IgG [10].

Another aspect that might contribute to the larger-than-expected variability of data (Table 1, Fig. 3 in the paper) is the lack of stratification of patients, especially for those analysed twice. It was of crucial importance to group patients for variables (such as the presence of other pathologies, type and duration of therapies, and plasma concentration of IgG(3), that can affect clinical evolution of disease, causing either a decrease or increase in the level of anti-Grp94 antibodies. Lack of information about relevant clinical and bio-humoral parameters on patients at the two separate visits makes the statistical analysis (Tables 2 and 3 in the paper) a purely mathematical exercise without any biological meaning. Changes in the mean values of both anti-Grp94 and anti-Bip antibodies in RA patients at Visit 2 compared with Visit 1 are evident in both Figs 3 and 4 in the paper (redundant), suggesting that some variables actually intervened in time in modifying the values of antibodies in the same patients. Strangely, however, the authors omitted to stress this difference, stating that the level of antibodies remained constant.

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Comment on: Antibodies to the endoplasmic reticulum-resident chaperones calnexin, Bip and Grp94 in patients with rheumatoid arthritis and systemic lupus erythematosus: reply
SIN, We appreciate the interest of Tramentozzi et al. in our work [1] and would like to respond to their comments. Tramentozzi et al. [2] questioned our ELISA system for detection of antibodies to endoplasmic reticulum (ER)-resident chaperones in serum of patients with RA and SLE. In the following, we explain that our ELISA system is highly reproducible and well established: first, negative and true-positive samples were clearly identified and verified by immunoblot. Secondly, inter-plate correlation was assessed both by carrying along one defined negative as well as one positive serum as reference on each plate. The ratio of these values allowed normalization of each serum for individual anti-ER chaperone antibody titres. Thirdly, sera were analysed in duplicate. Fourthly, an a priori test of several dilutions of sera was performed to determine the linear range and the optimal dilution factor (1:40) providing optical density (OD) values within the linear range. The dilution factor was kept constant to enable statistical evaluation. In this kind of investigation, values expressed as fold induction in comparison with healthy individuals are as meaningful as values expressed in antibody titres. However, for obvious reasons it is not feasible to exactly quantify the concentration of antigen-specific antibodies within polyclonal sera containing multiple antibody clones of various isotypes and immunoglobulin G (IgG) subclasses binding with different affinities and avidities,