Charge Selectivity of Proteinuria in Type I Diabetes Explored by Ig Subclass Clearance

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To investigate the role of protein charge in early diabetic proteinuria, the clearance of proteins differing in charge and/or size (anionic and cationic Igs, albumin) was evaluated in 98 insulin-dependent (type I) diabetic patients selected as a representative sample of the 418 patients attending our clinics. Of the patients, 12.9% were microalbuminuric and 4.8% were macroalbuminuric. Anionic and total IgG clearances were significantly increased in 30.6 and 12.2% of patients and were correlated with duration of disease. Anionic IgG4 clearances were increased in patients (9.2%) with normal IgG excretion, suggesting that charge-selectivity impairment is responsible for protein loss. Anionic Ig clearances were also higher in some patients (14.3%) with normal albumin clearance, probably as a result of different glomerular filtration and/or tubular reabsorption. The anionic-cationic IgG clearance ratio tended to increase in parallel with albumin clearance, but once above macroalbuminuric levels, it tended to fall again, indicating the concomitant presence of size-selectivity loss. The anionic IgG clearance and the anionic-cationic IgG ratio, in addition to albumin excretion, may be valuable in assessing early kidney protein charge-selectivity impairment and better characterizing normoalbuminuric patients and those in the preclinical stage of diabetic nephropathy. *Diabetes* 40:1685–90, 1991

Electrophysical interactions between the fixed anionic charges of the glomerular filtration barrier or the proximal tubule brush border and electrostatic charges of circulating macromolecules are important determinants in the regulation of protein excretion in both physiological and pathological conditions (1–8).

In diabetes, impairment of protein charge permselectivity has been invoked to explain the initial, prevalently anionic proteinuria in diabetic nephropathy (9,10) despite controversy about this point (11). A decrease in kidney fixed anionic charges is assumed to occur in diabetes mainly as a consequence of the decrease in sialic acid and heparan sulfate content (12–15). Furthermore, nonenzymic glycosylation may modify the net charge of both circulating and tissue proteins at all levels of the kidney filter (16–18). Because this decrease in fixed anionic charges is considered an early abnormality in the diabetic kidney occurring well before any clinical sign of the disease, importance has been attributed to the study of protein charge-selectivity loss in diabetes in the search for parameters to highlight the initial preclinical stages of diabetic nephropathy (19–21).

Various approaches have been used to explore protein charge-selectivity impairment in diabetes: parallel evaluation of IgG and albumin excretion (22,23); assessment of possible changes in the isoelectric point of different species of albumin or Igs excreted in the urine (20); relative excretion of glycosylated and nonglycosylated albumin, given that glycosylated albumin has a lower isoelectric point (9,24); and differential excretion of IgG subclasses with different ionic charges (21,25,26).

In this study, the working hypothesis was that two proteins similar in all main characteristics but differing in charge are expected to be found in the urine in proportions that differ from those in nondiabetic subjects when a protein charge-permselectivity impairment is present. Thus, clearance of IgG4, the anionic subclass of IgG, of which tiny amounts are present in the circulation, was compared to that of total IgG, >96% of which is made up of proteins of the same size and with the same main characteristics as IgG4 but are neutral or cationic (27). In

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this study, these parameters were used to gather data on the role of protein charge in the early stages of diabetic proteinuria and on the potentiality of this approach in a representative sample of insulin-dependent (type I) diabetic subjects from central Italy.

RESEARCH DESIGN AND METHODS

Albumin excretion rate was evaluated in 418 consecutive type I diabetic patients, i.e., all those who during the course of 1 yr attended the three outpatient diabetes clinics in Rome that participated in the study. Patient age (expressed as median and interquartile range [IR]) was 30 yr (IR 20–41 yr), duration of diabetes was 9 yr (IR 4–17 yr), and the male-female ratio was 212:206; the median albumin excretion rate was 6.25 μg/min (IR 3.3–13.6 μg/min). Of these, 98 patients were selected to represent the source population statistically, taking into account the albumin excretion rate, duration of disease, and age. In the 98 type I diabetic patients, median age, duration of disease, and albumin excretion rate were 32 yr (IR 22–46 yr), 10 yr (IR 5–19 yr), and 6.87 μg/min (IR 3.5–13.3 μg/min), respectively. In these patients, albumin, IgG, and IgG4 clearances were assayed, and their relative ratios were calculated. Protein clearances were used to take possible individual variations in protein blood concentrations into account and were expressed as $ml \cdot min^{-1} \cdot 10^{-3} \cdot 1.73\ m^{-2}$ body surface area. All urinary tests were performed on at least three different overnight collections, and the mean was taken as representing the individual patient's value.

One hundred one nondiabetic subjects from the same geographical area were included as control subjects (median age 31 yr, IR 19–43 yr). Albumin excretion rates were evaluated in all control subjects, whereas albumin, IgG, and IgG4 clearance rates were assessed in 22 of these subjects, taking age into account.

The albuminuria assay used was developed by us (28) and it is based on competitive binding between unknown urinary albumin to an anti-albumin Ig in solid phase. The IgG fraction of a rabbit anti-human albumin antiserum was left to coat highly adsorbent polystyrene microtiter tubes. Whereas nonspecific binding was overcome by saturation with a gelatin solution, increasing standard dilutions of albumin or the diluted urine (or serum) samples to be tested were left to incubate with an equal volume of radiolabeled albumin at room temperature for 1 h. The radioactive "cold/hot" albumin mixture was added to anti-albumin Ig-coated tubes that had been repeatedly washed and was left to incubate. After washing, radioactivity was assessed. The limit of detection of this assay is 25 ng albumin/ml with coefficients of variation (C.V.s) of 5.2 and 6.8% for intra-assay and for interassay evaluations, respectively.

The method for IgG4 detection was designed by us and has been reported elsewhere (29). Mouse monoclonal antibodies specific for the IgG4 subclass were bound to microtiter wells precoated with rabbit anti-mouse Ig antibody. IgG4 in standard preparations (or in the samples to be tested) was revealed with peroxidase-conjugated rabbit anti-human IgG. The procedure, carried out at 4°C, has a limit of sensitivity of 1 ng/ml IgG4. The intra-assay C.V. of positive samples was 4.3, and interassay C.V. was 8.9. A serum pool, obtained from the World Health Organization (serum no. 67/97), was used as standard.

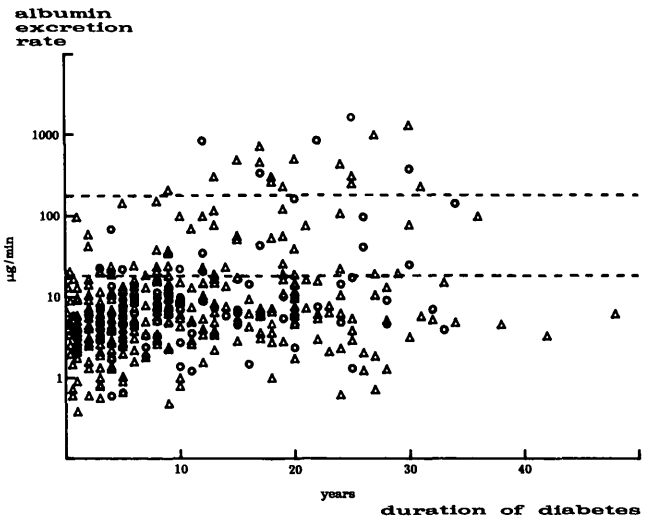

FIG. 1. Albumin excretion rates plotted on logarithmic scale in 418 insulin-dependent diabetic patients (*triangles* and *circles*) and in 98 patients (*circles*) selected for clearance studies versus duration of diabetes. *Dotted lines* indicate 20- and 200-μg/min limits of microalbuminuria and macroalbuminuria.

The method used to measure IgG was developed by our group and has previously been reported in detail (30). Briefly, the IgG fraction of a rabbit anti-human Ig antiserum in a basic calcium carbonate buffer is left to coat highly adsorbent polystyrene microtiter tubes. After the antibody-coated tubes have been saturated with gelatin, a 1:5 dilution of the urine (1:5000 of serum) to be tested and an equal volume of purified and radiolabeled human IgG are incubated and then added to the antibody-coated tubes. After incubation and repeated washings, radioactivity is counted. This method has a limit of sensitivity of 120 ng IgG. Intra-assay and interassay method precision for positive samples was 5.5 and 1.6, respectively.

We used linear regression analysis after logarithmic transformation of proteinuria values, χ^2 test, and Cox's test for trends in proportions to evaluate the results. Mean ± SD was used as the measurement of central value and dispersion for populations of data with a skewness <3, whereas median and IR were used when skewness was >3. Because there is no established limit of positivity for protein clearance values, the mean +3SD has been chosen.

RESULTS

Fifty-four of 418 patients (12.9%) showed albumin excretion rates ≥20 and <200 μg/min, whereas 20 patients (4.8%) presented values ≥200 μg/min (Fig. 1). In the 98 patients selected for this study, the percentages of microalbuminuric and macroalbuminuric patients were 13.2 and 5.1, respectively (Fig. 1). There was a significant trend for albumin excretion rates to rise with the

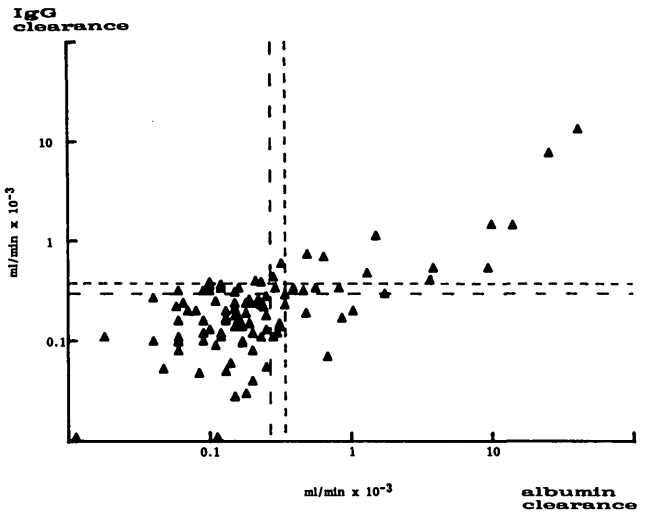

FIG. 2. IgG versus albumin clearance values in 98 insulin-dependent diabetic patients. Values have been log transformed, but for simplicity, their decimal corresponding values are indicated on *x* or *y* axis. *Dotted lines*, mean + 2SD and 3SD.

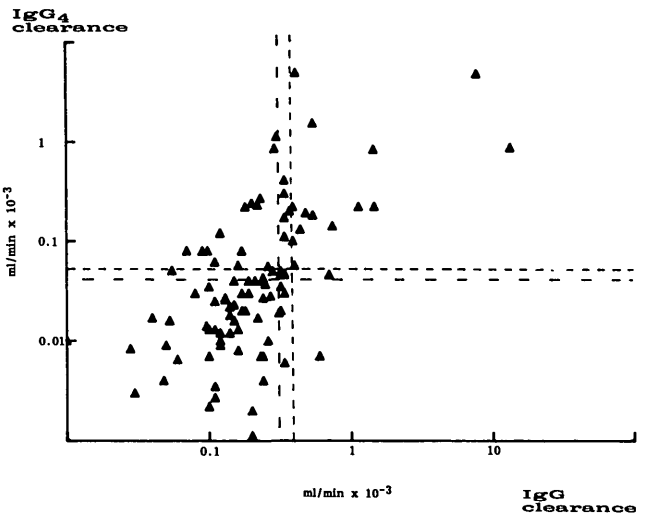

FIG. 3. IgG4 versus IgG clearance values in 98 insulin-dependent diabetic patients. Values have been log transformed, but for simplicity, their decimal corresponding values are indicated on *x* or *y* axis. *Dotted lines*, mean + 2SD and 3SD.

increasing duration of diabetes ($P < 0.0001$) and increasing age ($P < 0.005$) in both the larger and smaller groups, whereas no correlation was found with sex. The median albumin excretion rate in normal subjects was 4.36 μg/min (IR 2.58–6.59 μg/min).

The median albumin clearance value in the 98 type I diabetic patients was 0.19 ml · min^{-1} · 10^{-3} (IR 0.11–0.31 ml · min^{-1} · 10^{-3}). Twenty and four-tenths percent of patients were above the mean + 3SD of albumin clearance values found in the nondiabetic population (range 0.06–0.4 ml · min^{-1} · 10^{-3}, mean 0.13, SD 0.07). The correlation between albumin clearance, expressed as a logarithmic value, and duration of diabetes gave $r = 0.41$ ($P < 0.01$).

IgG clearance values showed a median of 0.2 ml · min^{-1} · 10^{-3}, (IR 0.11–0.32 ml · min^{-1} · 10^{-3}). Twelve and two-tenths percent of patients were above the mean + 3SD of IgG clearance values in the normal population (range 0.04–0.36 ml ·min^{-1} · 10^{-3}, mean 0.14, SD 0.08). After logarithmic transformation, the correlation of IgG clearance values with the duration of diabetes gave $r = 0.33$ ($P < 0.001$).

IgG4 clearance values were 0.028 ml · min^{-1} · 10^{-3}, (IR 0.01–0.08 ml · min^{-1} · 10^{-3}). Thirty and six-tenths percent of patients were above the mean + 3SD of normal IgG4 clearance values (range 0.003–0.05 ml · min^{-1} · 10^{-3}, mean 0.017, SD 0.012). The correlation of IgG4 values with the duration of diabetes was $r = 0.28$ after logarithmic transformation ($P < 0.01$).

After an overall analysis, IgG clearance showed a positive correlation with albumin clearance (log-transformed values, $r = 0.68$ $P < 0.0001$; Fig. 2). Taking into account the positivity limit of the mean + 3SD, 9.2% of patients with normal IgG clearance showed increased albumin clearance, whereas none presented an increase of IgG with normal albumin clearance.

The linear correlation between IgG4 and IgG clearances is illustrated in Fig. 3 (log-transformed values, $r = 0.59$, $P < 0.0001$). When patients were divided into normoalbuminuric, microalbuminuric, and macroalbuminuric, the correlation coefficients were 0.39 ($P < 0.001$), 0.08 (NS), and 0.3 (NS), respectively. With a positivity limit of mean + 3SD, 19.32% of patients with a normal IgG clearance showed an increased IgG4 clearance, whereas only 2% showed the opposite.

A linear correlation ($r = 0.68$, $P < 0.0001$) was found between IgG4 and albumin clearances (Fig. 4). Of normoalbuminuric patients, 14.3% showed increased IgG4 clearances, whereas 4.1% of patients with normal IgG4 clearance showed increased albumin clearance.

The ratio of IgG to albumin, taken as a selectivity index, is plotted against albumin clearance in Fig. 5. The correlation between the two variables appears to be parabolic

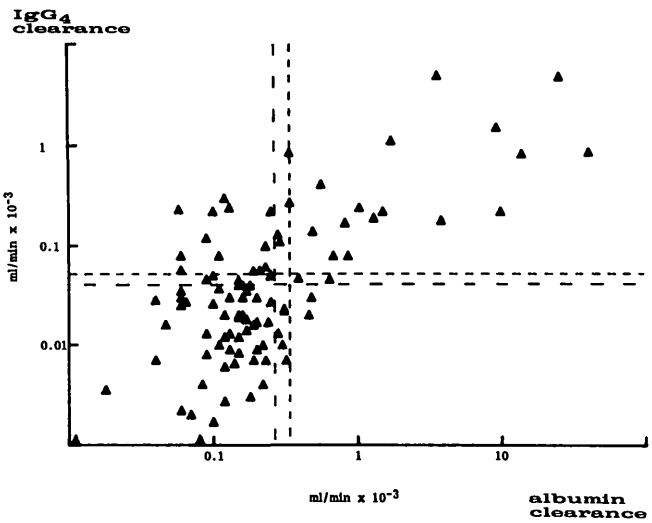

FIG. 4. IgG4 versus albumin clearance values in 98 insulin-dependent diabetic patients. Values have been log transformed, but for simplicity, their decimal corresponding values are indicated on *x* or *y* axis. *Dotted lines*, mean + 2SD and 3SD.

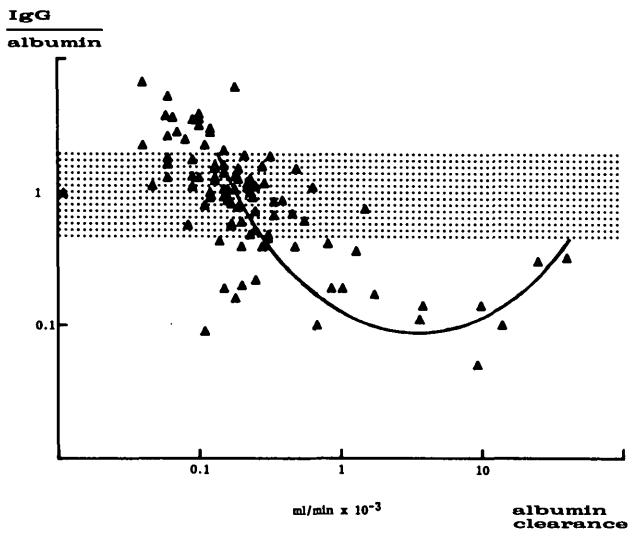

FIG. 5. Selectivity index, calculated with ratio of IgG and albumin clearances after log transformation, plotted against albumin clearance. *Shaded area*, range between 5th and 95th percentile of values in nondiabetic subjects; *curved line*, parabolic distribution of values (see text).

$$y = x^2(56 + 54\log 3)/198 - x(1 + \log 3)/2 + (45\log 3 - 155)/198$$

with the vertex

$$x = 99/2(1 + \log 3)/(56 + 54\log 3)$$

at an albumin clearance of 4.45 ml · min^{-1} · 10^{-3}. Thus, the ratio tends to decrease with increasing values of albumin clearance in patients with normo- or microalbuminuria but tends to increase again in macroalbuminuric patients.

When the selectivity index, obtained by calculating the ratio between anionic and total Ig, is plotted against the albumin clearance (Fig. 6) a different parabolic correlation is found

$$y = x^2(\log 2 - \log 3 - 1) + x + \log 3 - \log 2$$

with the vertex

$$x = -1/2(\log 2 - \log 3 - 1)$$

equivalent to 2.66 ml · min^{-1} · 10^{-3}. Consequently, the ratio tends to increase with higher values of albumin clearance in patients with normo- or microalbuminuria but decreases again in macroalbuminuric patients. Nineteen and three-tenths and 12.8% of patients, despite being normoalbuminuric, showed a ratio above or below the normal range of the IgG4-IgG ratio.

DISCUSSION

In diabetic nephropathy, it has been postulated that a charge-selectivity defect in protein excretion precedes and/or accompanies the size-selectivity loss (19,22,31). Various attempts have been made to find suitable tools to evaluate protein charge selectivity in diabetic patients (22–26). In this study, we opted to study the differential excretion of IgG subclasses with different electrostatic charges (27). Several studies in limited cohorts of diabetic patients have confirmed the potentiality of this approach and the validity of the hypothesis (21,24–26). Sensitive methods to detect the tiny amounts of urinary IgGs had to be developed (29,30), and clearances have been calculated to take into account possible individual variations in protein blood concentrations (29,32). Previous work in nondiabetic subjects has shown that the clearance of anionic IgGs is lower than that of neutral/cationic IgGs (32), thus confirming in humans the restricted filtration of anionic proteins seen in animal studies and that the anionic-cationic Ig ratio is not influenced by individual protein clearance changes in physiological conditions.

In this study in type I diabetic patients, both the clearance of anionic IgGs and the anionic-cationic IgGs ratio were clearly increased in several patients, suggesting a protein charge-permselectivity impairment. Several patients with an abnormal IgG4 clearance showed normal IgG clearance, thus highlighting the lack of parallelism between the excretion of proteins differing only in their charge. There was a high correlation between IgG4 and albumin clearances despite their differences in size but in accordance with their similarities in charge. Interestingly, several normoalbuminuric patients showed enhanced IgG4 clearances. The plotting of the anionic-cationic IgG ratio against albumin clearance values showed a parabolic correlation. In other words, this is evidence that, in many normoalbuminuric patients and most microalbuminuric ones, there is a disproportion in the relative excretion of proteins differing only in charge, the negative ones being the ones preferentially excreted; in the macroalbuminuric patients, the influence of charge decreases. Note that, among the normoalbuminuric patients, some presented a higher and others a lower anionic-cationic IgG ratio compared with nondiabetic values (Fig. 6, *left*).

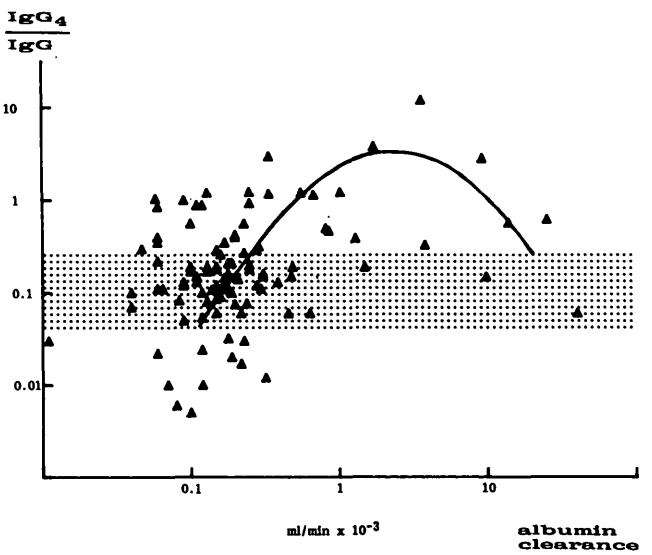

FIG. 6. Selectivity index, calculated with ratio of IgG4 and IgG clearances after log transformation, plotted against albumin clearance. *Shaded area*, range between 5th and 95th percentile of values in nondiabetic subjects; *curved line*, parabolic distribution of values (see text).

These findings reinforce and extend our preliminary studies in selected patients (21,26). They are in agreement with works from Deckert and associates where the importance of the ratio between the clearances of differently charged IgGs, in addition to albuminuria, in subgrouping and monitoring patients in the preclinical stages of diabetic nephropathy is stressed (24) and demonstrated in a small number of patients (25). Nakamura and Myers (20) in similarly directed research in proteinuric diabetic patients but with a different technical approach have shown data consistent with a charge selectivity of proteinuria. The parabolic correlation between a charge-selectivity index and the progression of diabetic nephropathy found in our study was suggested by Viberti et al. (22) analyzing their data on the IgG-albumin ratio.

When speculating on these findings, it should be remembered that both albumin and IgGs may pass through the glomerular filter in tiny amounts in normal conditions. Although albumin may filter through the ordinary glomerular pores, IgGs, because of their size, can only escape through the larger shunt pores of the alternative filtration pathway; like ordinary pores, the latter are thought to be negatively charged and to restrict the excretion of anionic proteins (20,33). At the tubular level, filtered proteins are to a large extent reabsorbed via a charge- and size-selective endocytotic mechanism: cationic and small proteins are preferentially absorbed (8,33,34). IgGs, different from albumin, can hardly be reabsorbed because of size (4,8), but this point is controversial (33–35). Indeed, in studies in humans, where proteins are investigated only before and after their passage through the kidney, it is difficult to differentiate the role of the glomerulus from that of the tubule in a presumed charge-permselectivity impairment.

It seems possible to identify different categories of patients according to the Ig ratio data (Fig. 6). In some patients with substantially intact kidney protein filtration mechanisms but with a slight increase in intraglomerular pressure, as occurs in the initial stages of diabetic nephropathy (22), a relative increase in the excretion of cationic proteins may be observed (Fig. 6, *lower left*) because the negative charges of the glomerular membrane exert a retropulsive force against anionic proteins; this agrees with previous reports (25).

In other patients with an initial reduction in fixed anionic charges, and consequently filtration restriction to circulating anionic proteins, we would expect to find a greater passage of anionic rather than cationic Igs, and thus an increased ratio. Even if excreted in small quantities, Igs are scarcely reabsorbed at the tubular level because of their size, whereas small quantities of filtered albumin may be handled by the high-capacity/low-affinity tubular reabsorption mechanisms (4,33,34). In this case, it is possible to observe an increased anionic-cationic Ig ratio coupled with normal albumin clearance (Fig. 6, *upper left*).

In those patients with a more severe reduction of fixed anionic charges of the kidney and thus with a manifest charge-selectivity impairment but without a substantial size-selectivity loss, we may find marked increases in albumin and anionic Ig clearances with no abnormalities in total Ig clearance (Fig. 6, *center*).

In patients in whom a loss of both charge and size selectivity in protein filtration is present, the clearance of albumin and anionic and cationic IgG is quantitatively increased: at this stage, the excretion of proteins is virtually independent of the protein charge, and the IgG ratio tends to return to normal values (Fig. 6, *right*).

In conclusion, our findings show that the parallel evaluation of albumin and anionic-cationic IgG ratio clearances gives insights into the size and charge permselectivity of proteins in type I diabetes. Disproportions in the anionic-cationic IgG ratio may be present even in patients with normal albumin clearance and seem to highlight initial abnormalities in the diabetic kidney. Thus, the assessment of anionic and cationic IgG clearance and of their ratio, in addition to the evaluation of albumin excretion, is proposed as an approach to evaluate the initial charge permselectivity abnormalities of the diabetic kidney and to better characterize and monitor diabetic patients without clinical nephropathy.

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