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Generation of catalytic antibodies is an intrinsic property of an individual's immune system: a study on a large cohort of renal transplant patients

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The present work is dedicated to her.

Authors' contribution

Designed the work: AM, IP, OT, DNR, SVK, CL, SLD

Performed the research: AM, IP, CD, MS, BW, SPL, DB, AMe

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Abstract

Renal transplant is the treatment of choice for patients with terminal end-stage renal disease. We have previously identified low levels of catalytic IgG as a potential prognosis marker for chronic allograft rejection. The origin and physiopathological relevance of catalytic antibodies is not well understood owing to the fact that catalytic antibodies have been studied in relatively small cohorts of patients with rare diseases and/or without systematic follow-up. In the present study, we have followed the evolution of the levels of catalytic IgG in a large cohort of renal transplant patients over a 2-year period. Our results demonstrate that, prior to transplant, patients with renal failure present with heterogeneous levels of IgG hydrolyzing the generic PFR-MCA substrate. PFR-MCA hydrolysis was greater for patients' IgG than for a therapeutic preparation of pooled IgG from healthy donors. Renal transplant was marked by a drastic decrease in levels of catalytic IgG over three months followed by a steady increase during the next 21 months. Patients who displayed high levels of catalytic IgG pre-transplant recovered high levels of catalytic antibodies 2 years post-transplant. Interestingly, IgG-mediated hydrolysis of a model protein substrate, pro-coagulant factor VIII, did not correlate with that of PFR-MCA prior transplantation, while it did 12 months post-transplant. Taken together, our results suggest that the level of circulating catalytic IgG under pathological conditions is an intrinsic property of each individual's immune system, and that recovery of pre-transplant levels of catalytic IgG is accompanied by changes in the repertoire of target antigens.

Keywords

kidney transplant; catalytic antibodies; factor VIII

Introduction

Catalytic antibodies are immunoglobulins with enzyme-like properties. The advent of hybridoma technology fuelled a remarkable progress and in the past 26 years catalytic antibodies with more than 100 tailor-made specificities have been made (1). From the late 80's, scientists have also investigated the pathophysiological association of catalytic antibodies in several pathological conditions. Thus, IgG with catalytic activity against vasoactive intestinal peptide, thyroglobulin, myelin basic protein, DNA/RNA and coagulation factor VIII, have been reported in pathological conditions including asthma (2), Hashimoto's thyroiditis (3), multiple sclerosis (4), systemic lupus erythmatosus (5), and hemophilia A (6), respectively. Recent findings however suggest the presence of naturally occurring catalytic antibodies in physiology. The naturally occurring catalytic antibodies display promiscuity in antigen/substrate specificity and are believed to act in defence mechanism against viral/bacterial pathogens. In fact, immunoglobulins of the IgA and IgM type isolated from healthy individuals are shown to possess nuclease and/or protease activity against bacterial or viral antigens (7–9). In this context, catalytic antibodies have been

proposed to participate in maintaining immune homeostasis and clearing of biological wastes. Whether catalytic antibodies are a feedback control mechanism aimed at re-establishing immune homeostasis under pathological conditions still remains elusive. In this line, our earlier investigations provide some hints. We have observed that high levels of circulating catalytic IgG correlate with a favourable outcome in some diseases. Patients with high IgG-mediated catalytic activity had a better survival rate in sepsis and a tendency towards better survival was observed in the case of patients with acquired hemophilia, that possess factor IX-activating antibodies in plasma (10, 11). Moreover, in patients undergoing renal transplant, better graft survival correlated with the presence of high levels of IgG-mediated catalytic activity. High IgG-mediated catalytic activity as early as at 3 months was predictive of absence of chronic allograft rejection (CAN) 2 years post-transplant (12).

Despite the efforts invested to date, our understanding of the physiopathological relevance of catalytic antibodies in human health remains poor. We know that patients with different diseases generally exhibit heterogeneous levels of catalytic antibodies. We also know that the levels of catalytic antibodies may evolve with time, although not necessarily in a manner that correlates with disease progression. However, it is not clear whether high levels of catalytic antibodies in some individuals are an intrinsic property of their immune system or are associated with peculiar disease conditions. In the present study, we followed a large cohort of patients with renal transplant for a period of 2 years, with regular and systematic blood sampling prior to transplantation as well as 3, 12 and 24 months later. Interestingly, we observed that high or low IgG catalytic activity is an intrinsic property of an individual’s immune system that fluctuates within the course of disease and is dependent upon treatment regimes. Conversely, antigen specificity of catalytic antibodies emerges gradually during the course of disease.

Patients and methods

Study population

From October 2008 to August 2009, we prospectively collected plasma from 100 consecutive patients 3 months following renal transplant at the Renal Transplantation Department of the Necker Hospital (Paris, France). Patients were followed-up and we also collected plasma at 12 months (92 patients) and 24 months (73 patients) post-transplant. Frozen pre-transplant plasma samples were retrieved retrospectively in the case of 59/100 patients. Clinical characteristics of the patients are depicted in Table 1. Written informed consents were obtained from each patient according to the Declaration of Helsinki. The blood samples were taken during the normal follow-up of the patient and since the study did not require additional blood sampling, an approval from an ethics committee was not required under French law according to the article L.1121-1 of the public health code. The article states that: the research organized and performed on human beings in the development of biological knowledge and medical research are permitted under the conditions laid down in this book and are hereinafter referred to by the term "biomedical research". The article further states that it does not imply under conditions: "For research in which all actions are performed and products used in the usual way, without any additional or unusual diagnostic procedure or surveillance."

Plasma collection

Blood was collected in citrate vacutainer tubes (BD biosciences), and centrifuged at 1500 rpm for 10 min at 20°C. Plasma was stored in aliquots at −20°C until use.

Purification of IgG

IgG were isolated from plasma by affinity-chromatography on protein G-Sepharose (Amersham Pharmacia Biotech). In brief, plasma was incubated with protein G-Sepharose overnight at 4°C, eluted using 0.2M glycine-HCl pH 2.8, dialyzed against PBS-0.02% NaN_3 overnight at 4°C, and concentrated using Amicon (Millipore). A therapeutic preparation of pooled normal human IgG (intravenous Ig (IVIg); Sandoglobulin) was used as a source of control IgG. Size-exclusion chromatography of patients' IgG and IVIg was performed on a Superose-12 column (GE Healthcare Europe) equilibrated with urea-containing buffer (50 mM Tris pH 7.7, 8 M urea and 0.02% NaN_3), at a flow rate of 0.5 ml/min to exclude potentially contaminating proteases. IgG-containing fractions were then pooled and dialyzed against PBS-0.02% NaN_3 for 2 days with four changes in buffer at 4°C, followed by dialysis against catalytic buffer containing 5 mM $CaCl_2$ (pH 7.7) for 1 day with two changes in buffer at 4°C. The purity of IgG preparations was confirmed by SDS-PAGE and immunoblotting under non-reducing conditions. IgG was quantified by Bradford assay.

IgG-mediated hydrolysis of PFR-MCA

IgG (66.67 nM) were mixed with 100 μM PFR-MCA (Peptide Institute, Inc.) in 40 μl of catalytic buffer containing 5 mM $CaCl_2$ (pH 7.7) in white 96-well U-bottom plates (Thermo Scientific) and incubated in the dark for 24 h at 37°C. Hydrolysis of the PFR-MCA substrate was determined by the fluorescence of the leaving group (aminomethylcoumarin; λem 465 nm, λex 360 nm) using a spectrofluorometer (GENios; Tecan Trading). Fluorescence values were compared with a standard curve of free MCA and the corresponding quantities of released MCA were computed. At each time point, background release of MCA, measured in wells containing the substrate alone, was subtracted from the value observed in the presence of the IgG. Data are expressed as the quantity of released MCA computed at time zero subtracted from the quantity of released MCA computed at a given time point per amount of time per amount of IgG.

Biotinylation of FVIII

Recombinant human factor VIII (FVIII, Kogenate FS, BayerPharma, Lille, France) was reconstituted in distilled water to a final concentration of 600 μg/ml, desalted by dialyzing against borate buffer (100 mM borate (pH 7.0), 150 mM NaCl, and 5 mM $CaCl_2$). Sulfo-NHS-LC-biotin (440 μl at 25 μg/ml) was allowed to react with 600 μg of FVIII with gentle agitation in the dark for 2 h at 4°C. Biotinylated FVIII was dialyzed against catalytic buffer containing 5 mM $CaCl_2$ for 3 h at 4°C, aliquoted, and stored at −20°C until use.

Hydrolysis of biotinylated FVIII

Biotinylated FVIII (185 nM) was incubated in 40 μl of catalytic buffer containing 5 mM $CaCl_2$ with the purified patients' IgG (10 μg/ml, 66.67 nM) in the dark for 24 h at 37°C. Samples were mixed with Laemmli's buffer without 2-ME (1:1, v/v) and 25 μl of each

sample was subjected to 10% SDS-PAGE. Protein fragments were then transferred onto nitrocellulose membranes (Schleicher & Schuell's Microscience). Following overnight blocking in TBS containing 0.2% Tween 20 at 4°C, membranes were incubated with streptavidin-coupled alkaline phosphatase (Southern Biotech) diluted 1:3000 in blocking buffer, for 60 min at room temperature. After washing in TBS containing 0.1% tween-20 and TBS, labeled FVIII was revealed using the BCIP/NBT kit (Kirkegaard & Perry Laboratories). Blots were scanned using a scanner (EPSON Perfection V10) and rates of hydrolysis were calculated by densitometric analysis (NIH ImageJ software).

Statistics

The statistical comparisons of groups of patients were performed using the non- parametric Mann-Whitney U test, with two-tailed P values, unless indicated.

Results

IgG from pre-transplant patients show heterogeneous levels of catalytic activity, which is not related to particular underlying pathology

We collected plasma from 100 consecutive renal-transplant patients after 3 months of transplant and followed-up to obtain plasma at 12 (92 patients) and 24 months (73 patients) post-transplant. Pre-transplant plasma samples from 59 patients were retrieved retrospectively. Overall, 27 patients were lost during the 24 months of the study period. The cohort included as many men as women, with a mean age of 48.3±1.5 years (mean±SEM; range: 21 to 83, Table 1 and supplementary Table 1). Causes for end-stage renal dysfunction included diabetes (4% of the patients), vasculopathy (8%), glomerulopathy (24%), uropathy (23%), interstitial nephropathy (15%) or were not known (26%). Fifteen and 2 patients had one or two previous transplants, respectively.

IgG was purified and tested for hydrolysis of the peptide PFR-MCA, a surrogate substrate for catalytic antibodies with serine protease-like activity (13). The absence of contamination of the IgG samples by adventitious proteases was ensured by the use of a double-step purification procedure that involves a step of purification based on affinity and a step of purification based on protein size under denaturing conditions. Incubation of patients' IgG with PFR-MCA resulted in hydrolysis of the peptide and release of the fluorescent MCA moiety. The released fluorescence allowed for the calculation of rates of hydrolysis.

Pooled IgG from healthy individuals (IVIg) demonstrated a marginal hydrolysis of PFR-MCA with an activity of 0.65±0.03 fmol/min/pmol (mean±SEM for 29 repeats). Irrespective of the time-point considered, IgG from renal-transplanted patients demonstrated significantly higher hydrolysis rates of PFR-MCA than IVIg (Figure 1A). There was no significant difference in the levels of PFR-MCA-hydrolyzing IgG at any time point with respect to sex, age, transplantation rank, cause for end-stage renal failure and time of dialyses prior to transplant (data not shown).

The levels of PFR-MCA hydrolyzing IgG were extremely heterogeneous prior to transplantation, with a mean activity of 6.6±0.9 fmol/min/pmol (mean±SEM; coefficient of variation: 1.04, Figure 1A). To investigate whether high or low levels of PFR-MCA

hydrolyzing IgG were associated with a particular disease condition, we compared the hydrolysis rates between patients with different causes for end-stage renal failure. IgG from patients with uropathy displayed the lowest mean rate of PFR-MCA hydrolysis, that was statistically different from that of IgG from patients with interstitial nephropathy (3.7±0.7 vs 7.9±1.2 fmol/min/pmol, P=0.003, Figure 1B). Of note, several underlying pathologies had only a low number of cases, thus hampering powerful statistical comparison.

IgG-mediated catalytic activity varies overtime in renal-transplant patients

A longitudinal follow-up of the levels of PFR-MCA-hydrolyzing IgG was performed to determine the evolution of IgG-mediated catalytic activity during the course of the disease. The differences in the rates of IgG-mediated catalytic activity between each group were evaluated using the two-tailed Mann-Whitney test. As compared to the rates of IgG-mediated catalytic activity pre-transplant, the rates decreased sharply 3 months post-transplant (6.6±0.9 vs 2.4±0.2 fmol/min/pmol, P<0.0001, Figure 1A). However, an increase in the rates of hydrolysis of PFR-MCA was observed at 12 months post-transplant as compared to 3 months (3.2±0.3 vs 2.4±0.2 fmol/min/pmol, P=0.015). The rates of hydrolysis further increased significantly at 24 months (5.1±0.6 fmol/min/pmol) in comparison to 3 months (P<0.0001) and 12 months (P=0.004). There was no difference in the rates of IgG-mediated catalytic activity between patients prior to transplant and 24 months later (6.6±0.9 vs 5.1±0.6 fmol/min/pmol), indicating that the pre-existing levels of PFR-MCA-hydrolyzing IgG had been recovered in the due course of time. Linear regression analysis between the groups of patients pre-transplant and 24 months post-transplant, showed a significantly positive correlation in the rates of IgG-mediated PFR-MCA hydrolysis (P<0.001, R^2=0.23, Figure 1C). No correlation in IgG-mediated catalytic activity was observed between the groups of patients at other time points.

To confirm that patients with elevated levels of catalytic antibodies before transplant recovered elevated levels two years later, we divided the patients into quartiles based on the rates of IgG-mediated PFR-MCA hydrolysis measured in pre-transplant samples. At each time point, the IgG-mediated catalytic activity of the upper quartile was compared with the rates of catalytic activity of the cumulated lower three quartiles (Figure 1D). The rate of IgG-mediated catalytic activity in the upper quartile of patients was significantly high both pre-transplant (12.03±1.6 vs 2.7±0.2 fmol/min/pmol, P<0.0001) and 24 months post-transplant (6.8±1.2 vs 4.6±0.7, fmol/min/pmol, P=0.0004). Clinical characteristics of patients from the top quartile, who account for the overall initial decrease and subsequent increase of catalytic antibody levels, did not differ from that of the remaining patients (Table 2). Further, there was no statistically significant association between the changes in catalytic antibody levels with time and the numbers of HLA mismatch or occurrence of HLA-reactive antibodies. Of note, levels of IgM-mediated PFR-MCA hydrolysis in 12-month post-transplant plasma did not differ between 5 patients randomly chosen from the upper quartile and 5 patients randomly chosen from the rest of the patients (Supplementary Figure 1).

IgG-mediated FVIII and PFR-MCA hydrolysis correlate at 12 months but not prior to transplant

IgG from patients' plasma prior to transplant, and 3 and 12 months post-transplant were tested for their ability to hydrolyze human recombinant FVIII. Patients' IgG were incubated with biotinylated FVIII and profiles of FVIII hydrolysis were revealed by Western blotting (Fig 2A). The rates of IgG-mediated FVIII hydrolysis were calculated by densitometric analysis after subtracting the amount of FVIII hydrolysed spontaneously in the absence of IgG. Patients' IgG demonstrated heterogeneous profiles of FVIII hydrolysis (Fig 2A). Thus, the rates of hydrolysis of FVIII among pre-transplant patients were highly heterogeneous. As observed for PFR-MCA hydrolysis, the rate of IgG-mediated FVIII hydrolysis decreased significantly 3 months post-transplant (157±9.8 vs 43.8±10.2 μmol/min/mol, $P<0.0001$, Fig 2B), and recovered after 12 months (113.7±17.6 μmol/min/mol). Pooled IgG from healthy individuals demonstrated a marginal hydrolysis of FVIII with an activity of 11.1±1.2 μmol/min/mol (mean±SEM for 22 repeats).

While the mean rate of hydrolysis of PFR-MCA and FVIII by patients' IgG was relatively high prior to transplant, no correlation was observed between IgG-mediated PFR-MCA hydrolysis and IgG-mediated FVIII hydrolysis ($P=0.17$, $R^2=0.03$, Figure 2C). The longitudinal follow-up however demonstrated a correlation tendency at three months ($P=0.053$, $R^2=0.01$), which became significant 12 months post transplantation ($P<0.0001$, $R^2=0.4$, Figure 2D). For information, rates of IgG-mediated hydrolysis of FVIII and PFR-MCA are indicated in supplementary Table 2 for comparison between patients with different diseases.

Discussion

Our results demonstrate that levels of PFR-MCA-hydrolyzing IgG were extremely heterogeneous among patients prior to transplant (T0 in Figure 1A) and were systematically greater than that measured for pooled IgG from healthy donors. These observations raise the question of the origin of the B-cell clones producing catalytic IgG and of the nature of the triggering signals/inflammatory environment implicated in their positive selection. Interestingly, among the different patients included in our cohort, patients with uropathy displayed the lowest levels of catalytic IgG. In contrast to the other underlying conditions that may lead to renal graft (i.e., diabetes, vasculopathy, glomerulopathy and interstitial nephropathy), uropathy is a congenital defect that presumably does not results from a global alteration of inflammatory, immune or cardiovascular processes. Accordingly, patients with uropathy do not generally relapse following kidney transplant. We have demonstrated in the past an association between the increased prevalence of catalytic IgG and positive outcomes in human disorders including acquired hemophilia, septic shock and renal transplant (12). Further, catalytic antibodies naturally occur in body fluids, such as blood, saliva or milk (7–9, 14), and have been proposed to participate in maintenance of immune homeostasis (15). Taken together, these observations suggest that the production of catalytic antibodies is part of physiological process that is exacerbated under inflammatory conditions. It is tempting to speculate that it represents a feed-back mechanism triggered by a dysregulated immuno-inflammatory status.

The 24 month-long follow-up analysis of catalytic antibodies in transplanted patients revealed a drastic decrease in levels of PFR-MCA-hydrolyzing IgG 3 months post-transplant. It is plausible that the decrease in IgG-mediated catalytic activity post-transplantation is linked to the associated treatments. Indeed, all the patients received different combinations of steroids, ciclosporin, tacrolimus and/or mycophenolate mofetil. Moreover, about 90% of the patients received adjunct immuno-suppressive therapy under the form of Basiliximab, anti-lymphocyte rabbit serum, Rituximab, plasmapheresis and/or IVIg. A specific assessment of the effects of steroids on the levels of catalytic IgG was not possible, since all patients were administered with at least one form of steroid. Of note, in a previous analysis using the same patients cohort (16), we showed that the decrease in levels of catalytic IgG was greater in the case of patients who were treated with repeated doses of IVIg post-transplant. We had proposed that the further reduction of catalytic IgG in IVIg-treated patients could result either from a dilution of the patients' IgG by the administered IVIg or from a direct immunomodulatory effect of IVIg on the patients' immune system (16). Importantly, the levels of catalytic IgG were assessed at fixed concentrations of total IgG. Thus, reduction in levels of catalytic antibodies reflect variations in the populations of catalytic antibodies in the total IgG pool, and are independent from disease/treatment-related fluctuations of total IgG levels. The reasons accounting for a specific and preferential immunosuppression towards catalytic IgG (and/or B-cell clones producing catalytic antibodies) remain unexplained. We may however speculate that the reduction in IgG catalytic activity at 3 months is due to the change in inflammatory status caused by the use of immunosuppressive drugs and that the subsequent revival of IgG catalytic activity at 12–24 months is associated with a concomitant significantly lower dosage of some of the drugs (Supplementary Figure 2). An alternative hypothesis pertaining to this effect is that the immune response against the transplanted kidney may bias the antibody repertoire away from catalytic activity, as has been documented earlier in the case of a suppressed production of the natural PFR-MCA directed catalytic antibodies following immunization with irrelevant antigens (17).

Our data indicates consistency in the levels of PFR-MCA hydrolyzing IgG in the plasma of patients before transplantation and 24 months later. We observed a significant correlation in the rate of IgG-mediated PFR-MCA hydrolysis between pre-transplant samples and the corresponding 24 month-follow-up post-transplant samples. Furthermore, patients who displayed high IgG-mediated catalytic activity before transplant recovered significantly high catalytic activity at 12 and 24 months post-transplant. Altogether, the data show that the suppression imposed on catalytic antibody levels by kidney replacement surgery and/or by the associated anti-inflammatory and immunosuppressive treatments, disappears with time. The organism replenishes the depleted pool of catalytic antibodies at levels similar to that existing prior to transplantation. The results thus suggest that the selection (and activation) of catalytic IgG-producing B cells is an intrinsic property of each individual's immune system.

A deregulated activation of the coagulation cascade due to the uncontrolled activation of the endothelium of the transplanted kidney may result in total loss of kidney function due to increased graft fibrosis (18). Factor VIII plays a central role within the coagulation cascade where it promotes the amplification loop of thrombin generation. Our previous observations

in chronic allograft nephropathy and severe sepsis document that the hydrolytic activity of catalytic IgG is directed against coagulation factor VIII and/or factor IX (10). We thus complemented our follow-up of IgG with catalytic activity towards a generic substrate for serine proteases (i.e., PFR-MCA) with the study of factor VIII-hydrolyzing IgG. As in the case of PFR-MCA, IgG-mediated factor VIII hydrolysis was significantly greater in patients before kidney transplant than in pooled normal IgG, experienced a drastic decrease within 3 months post-transplant and increased to almost initial levels by 12 months post-transplant. However, while levels of factor VIII-hydrolyzing and PFR-MCA-hydrolyzing IgG did not correlate when measured in pre-transplant samples, they exhibited a strong correlation 12 months post-transplantation. The later correlation is in agreement with our previous analysis of catalytic IgG in patients with autoimmune anti-factor VIII IgG (19). Of note, we had documented in the latter work the reciprocal inhibition of the hydrolysis of PFR-MCA by factor VIII and of the hydrolysis of factor VIII by PFR-MCA (19, 20). The finding of a correlation between the two substrates in post-transplant samples, but not in pre-transplant samples is an interesting lead for further studies on the transplant-driven evolution of catalytic antibody repertoires. Whether the differences are related to the ‘simplicity’ of cleavage of a single amide bond in the case of PFR-MCA as compared to the complexity of the hydrolytic processes required for IgG-mediated factor VIII proteolysis (20), remains to be determined. In the future, screening large arrays of random peptides displayed on phages, or investigating for the presence of DNA-hydrolyzing antibodies, using IgG from renal transplant patients, may be informative in this respect.

While the present data does not allow to conclude on the plasticity or stability of the repertoire of catalytic antibodies under physiological conditions, or in an “un-manipulated” diseased organism (because the patients are treated with immunosuppressive drugs), the data clearly plead for an intrinsic capacity of the patients’ immune system to express catalytic antibodies at higher than physiological levels. In other words, our data suggest that, when fluctuations on the levels of catalytic antibodies are imposed on the organism (by “extrinsic factors”, i.e., drugs), then the organism replenishes the depleted pool of catalytic antibodies. Evolution of levels of circulating catalytic antibodies may thus be envisaged as a complex process: under physiological conditions, B cells secreting catalytic antibodies are controlled and their expression is repressed to minimal levels; under some pathological conditions, most probably inflammatory or autoimmune, the B-cell clones are activated in an individual-dependent manner – some patients have the intrinsic capacity to mount a strong “catalytic immune response” (without presuming of its role, significance, efficiency), while other patients mount only marginal catalytic immune responses; the levels of circulating catalytic IgG are then stable, and are re-established if eliminated by transient immuno-suppressive treatments – which may be due to the chronic stimulation of the “catalytic immune system” by a yet unidentified disease-related trigger, or to the fact that the repressor mechanisms that were at play under physiological conditions have been perturbed and cannot be re-established.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PFR-MCA	proline-phenylalanine-arginine-methylcoumarinamide
FVIII	factor VIII
CAN	chronic allograft nephropathy
IVIg	intravenous immunoglobulins

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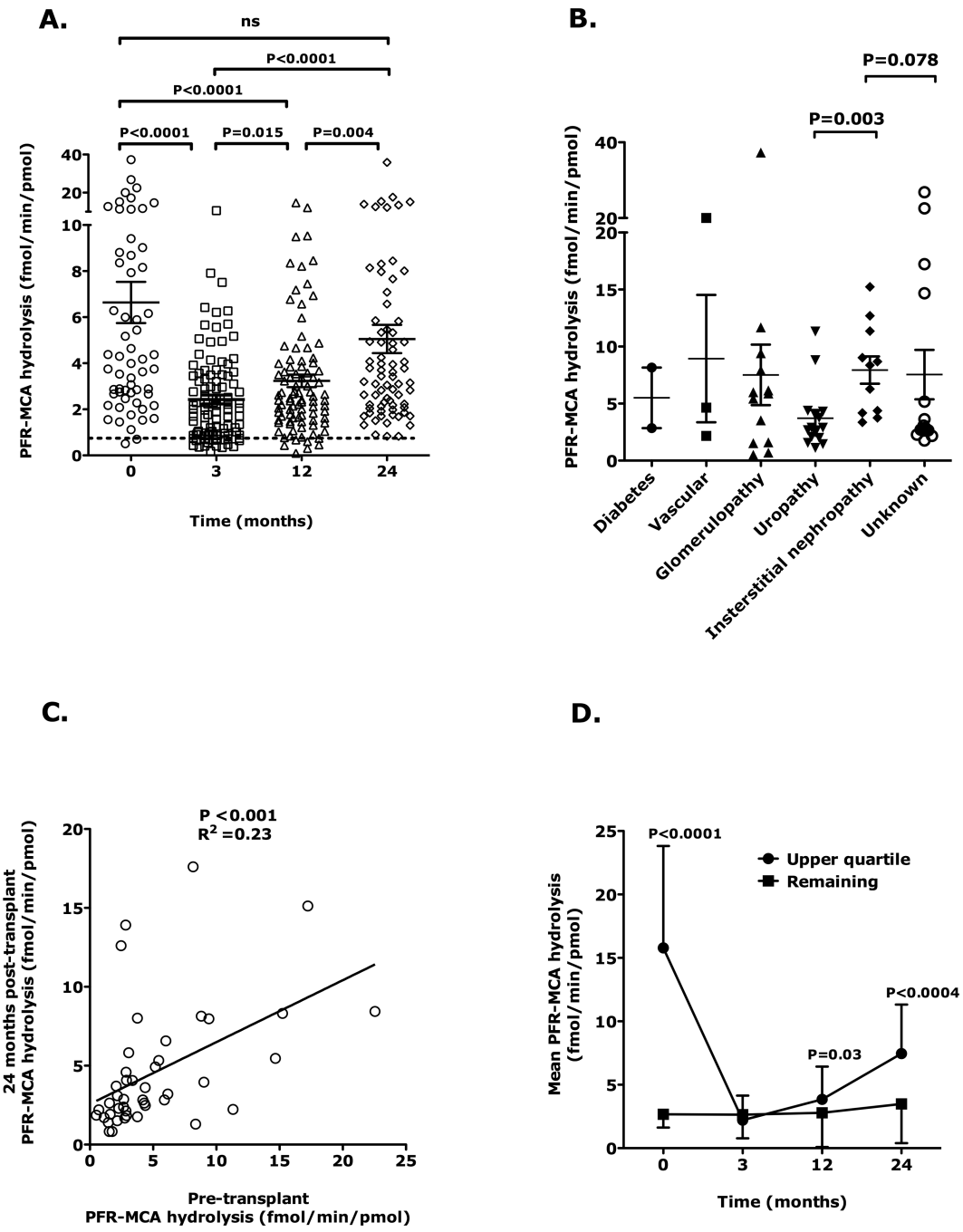

Figure 1. Longitudinal analysis of catalytic IgG in patients undergoing renal transplant

Panel A. Evolution of IgG-mediated catalytic activity in patients with renal transplant. IgG were purified from the plasma of patients collected prior to kidney transplant (n=59), as well as 3 (n=100), 12 (n=92) and 24 (n=73) months following transplantation. Purified IgG (66.67 nM) were incubated with the PFR-MCA substrate (100 μM) at 37°C for 24 hr. PFR-MCA hydrolysis was quantified by measuring the fluorescence of the leaving fluorescent MCA moiety, and is expressed in fmol of hydrolyzed substrate as a function of time per pmol of IgG. The hydrolysis of PFR-MCA by pooled IgG from healthy donors is depicted

by a dotted line. Hydrolysis was compared between groups using the two-tailed Mann Whitney U test. **Panel B.** Catalytic IgG in patients with different causes for end-stage renal failure. The study cohort included 2 patients with diabetes, 3 with vasculopathy, 13 with glomerulopathy, 15 with uropathy, 11 with interstitial nephropathy and 15 with unknown cause for end-stage renal failure. Statistical significances were assessed using the two-tailed Mann Whitney U test. **Panel C.** Correlation between IgG-mediated PFR-MCA hydrolysis prior to transplantation and 24 months later. The rates of IgG-mediated PFR-MCA hydrolysis measured prior to renal transplant and after 24 months were positively correlated as analyzed by linear regression. **Panel D**. Longitudinal follow-up of the patients displaying high levels of catalytic IgG. The pre-transplant patients were divided into quartiles and the mean rates of PFR-MCA hydrolysis of the upper quartile was compared with that of the pooled remaining quartiles. Hydrolysis was compared between groups using the two-tailed Mann Whitney U test.

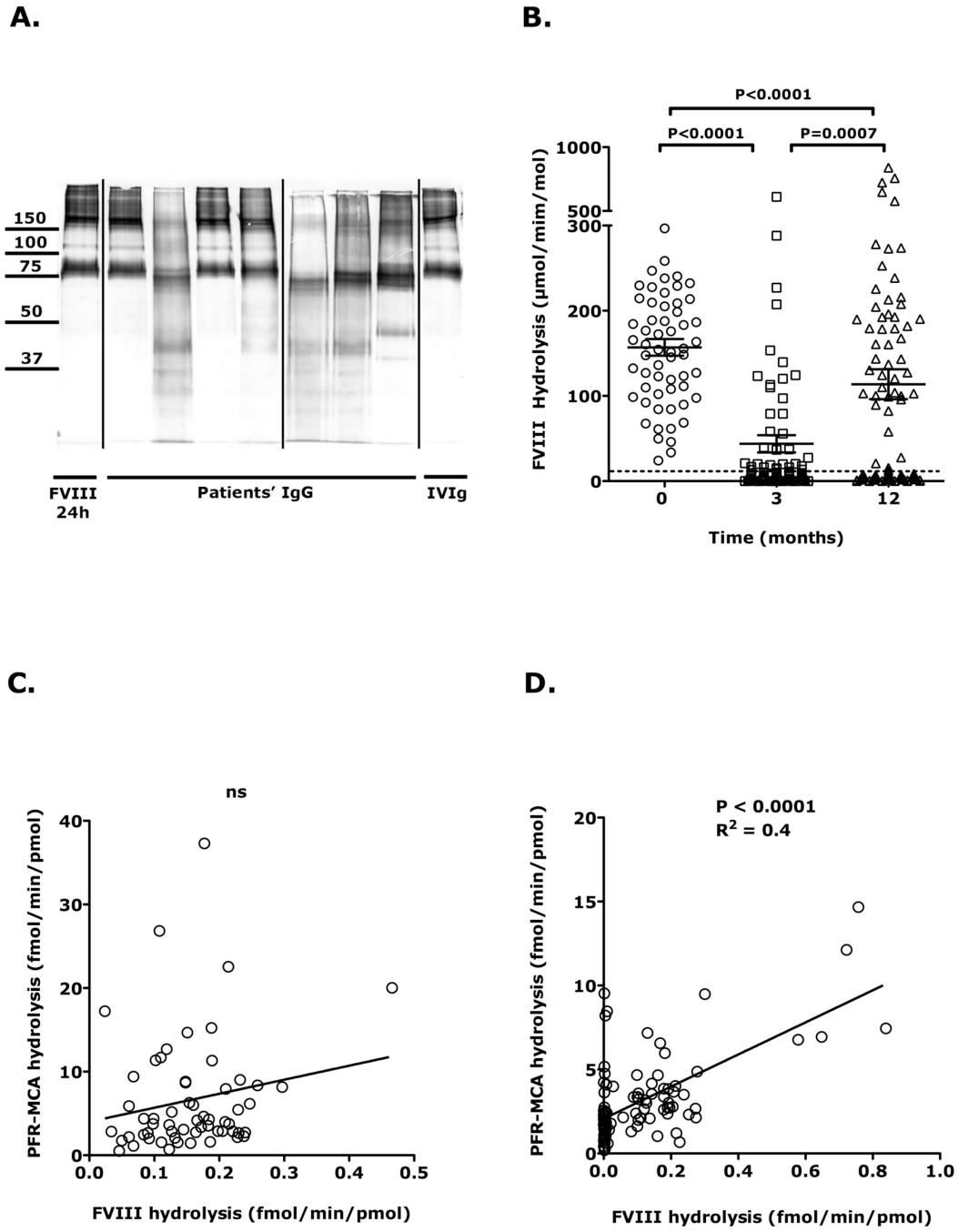

Figure 2. Hydrolysis of FVIII by IgG from renal-transplant patients

Panel A. IgG-mediated FVIII hydrolysis. Biotinylated recombinant human FVIII (185 nM) was incubated alone (lane 1) or in the presence of IgG (66.67 nM) from 7 randomly selected renal-transplanted patients (lanes 2–8) for 24 hr at 37°C. Pooled normal IgG from healthy donors (IVIg) was used as control IgG. FVIII was subjected to 10% SDS-PAGE and transferred onto a nitrocellulose membrane before revelation of biotinylated fragments. **Panel B.** Longitudinal follow-up of FVIII-hydrolyzing IgG. Rates of IgG-mediated FVIII hydrolysis were obtained by densitometric analysis of the blots (Panel A). Results are

expressed as μmol of hydrolyzed FVIII per min per mol of IgG. Statistical differences where assessed using the two-tailed Mann Whitney U test. **Panels C and D.** Correlation of rates of PFR-MCA hydrolysis and FVIII hydrolysis by IgG prior transplant (Panel C; ns: not significant) and 12 months later (Panel D).

Table 1

Patients' characteristics at inclusion

	Nb (%) or median [range]
Age (years)	**100**
	48 [21–83]
Sex	**100**
Females	50
Males	50
Cause for end-stage renal failure	**100**
Diabetes	4
Vascular diseases	8
Glomerulopathy	24
Uropathy	23
Interstitial nephropathy	15
Unknown	26
HLA reactive antibodies	**68**
HLA1	18 (27)
ND	32
HLA2	29 (43)
ND	32
Both HLA1 and HLA2	10 (15)
ND	34
HLA mismatch[†]	**98**
0	5
1	10
2	22
3	12
4	32
5	16
6	1
Previous kidney transplant	**100**
None	82
1	15
2	2
ND	1

ND: not documented

[†]HLA mismatch: Recipient and donor HLA A, B and DR antigens were defined and the number of mismatches of the pair (0 to 6) is indicated.

Table 2

Drug usage among patients with high and low IgG-mediated PFR-MCA hydrolysis

	Upper quartile (15 patients*)				Lower quartiles (44 patients)			
Drugs administered	**Pre-transplant**	**0–3 months**	**3–12 months**	**12–24 months**	**Pre-transplant**	**0–3 months**	**3–12 months**	**12–24 months**
Ciclosporin	2 (13.3)†	2 (13.3)	1 (6.7)	1 (6.7)	6 (13.6)	8 (18.2)	5 (11.4)	1 (2.3)
Tacrolimus	14 (93.3)	12 (80)	10 (66.7)	1 (6.7)	34 (77.3)	35 (79.5)	32 (72.7)	7 (16)
Mycophenolate mofetil	14 (93.3)	15 (100)	15 (100)	2 (13.3)	44 (100)	44 (100)	41 (93.2)	8 (18.2)
Azathioprine	0	0	1 (6.7)	0	0	1 (2.3)	2 (4.5)	0
Corticosteroid	15 (100)	15 (100)	14 (93.3)	1 (6.7)	43 (97.7)	43 (97.7)	41 (93.2)	7 (16)
Other immune suppressants	13 (86.7)	1 (6.7)	4 (26.7)	0	37 (84)	1 (2.3)	5 (11.4)	0

*Of the 100 patients included 3 months following renal transplant, samples from 59 patients were retrieved retrospectively. Among these 59 patients, 15 belong to the upper quartile group and 44 to the remaining quartiles.

†Number (percentage)