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ABSTRACT (245 words)

Autoantibodies against phospholipase A2 (PLA2R) and thrombospondin type 1 domain-containing 7A (THSD7A) are emerging as biomarkers to classify membranous nephropathy (MN) and to predict outcome or response to treatment. Anti-THSD7A autoantibodies are detected by western blot and indirect immunofluorescence test (IIFT). Here, we developed a sensitive ELISA optimized for quantitative detection of anti-THSD7A autoantibodies. Among 1012 biopsy-proven MN patients from 6 cohorts, 28 THSD7A-positive patients were identified by ELISA, indicating a prevalence of 2.8%. By screening additional patients, mostly referred because of PLA2R1-unrelated MN, we identified 21 more cases, establishing a cohort of 49 THSD7A-positive patients. Twenty-eight patients (57%) were male, and male patients were older than female patients (67 versus 49 years). Eight patients had a history of malignancy, but only 3 were diagnosed with malignancy within 2 years of MN diagnosis. We compared the results of ELISA, IIFT, western blot, and biopsy staining, and found a significant correlation between ELISA and IIFT titers. Anti-THSD7A autoantibodies were predominantly IgG4 in all patients. Eight patients were double positive for THSD7A and PLA2R1. Levels of anti-THSD7A autoantibodies were correlated with disease activity and with response to treatment. Patients with high titer at baseline had poor clinical outcome. In a subgroup of patients with serial titers, persistently elevated anti-THSD7A autoantibodies were observed in patients who did not respond to treatment or did not achieve remission. We conclude that the novel anti-THSD7A ELISA can be used to identify patients with THSD7A-associated MN and to monitor autoantibody titers during treatment.

INTRODUCTION

The primary form of membranous nephropathy (MN) is an autoimmune kidney disease in which circulating autoantibodies target podocyte autoantigens, leading to deposition of immune complexes in the glomerular capillary wall, podocyte injury and proteinuria [1-3]. Overall, MN affects more men than women (sex ratio 2:1), with a peak incidence at 50-55 years [4, 5]. Clinical outcome varies from spontaneous remission to persistent proteinuria and end stage renal disease in about 30% of cases.

In 2009, phospholipase A2 receptor 1 (PLA2R1) was identified as the major target autoantigen with circulating autoantibodies present in about 70% of MN patients [6]. In 2014, thrombospondin type 1 domain-containing 7A (THSD7A) was identified as a second autoantigen for another group of 2-5% MN patients [7]. Most cases of PLA2R1- and THSD7A-associated MN are mutually exclusive yet rare cases of dual positivity have been described [8-10].

Both PLA2R1 and THSD7A autoantigens are expressed in human podocytes and are membrane-bound proteins (180 and 250 kDa, respectively) with a long extracellular region comprising multiple but distinct domains with disulfide bonds [6, 7]. Anti-PLA2R1 and anti-THSD7A autoantibodies exclusively bind to conformational epitopes present in one or more respective domains and are predominantly of the IgG4 subclass [11-16]. Epitope spreading associated with disease worsening has been suggested for PLA2R1 [13, 14].

Although the pathogenic role of anti-PLA2R1 autoantibodies is still a matter of debate, multiple studies have shown that anti-PLA2R1 autoantibodies are specific and sensitive biomarkers for 50 to 80% of MN patients, depending on the studied cohorts [17, 18]. Anti-PLA2R1 autoantibodies are nowadays measured by robust biological assays such as IIFT [19] and ELISA [20, 21]. Furthermore,

PLA2R1 antigen accumulated in glomerular immune deposits is detected by standardized biopsy staining protocols [22-24]. These assays are now routinely used in clinical practice to identify and diagnose patients with PLA2R1-associated MN, predict clinical outcome and improve clinical management from conservative therapy to treatment with potent immunosuppressors [20, 25-36]. Other specific assays have also been described [37, 38].

Concerning THSD7A, patients' autoantibodies have recently been shown to be pathogenic in a mouse model [39, 40]. However, the detection of circulating autoantibodies is currently possible by WB [7] and IIFT [41], which provide only semi-quantitative titers. As for PLA2R1-related MN, standardized biopsy staining protocols that can detect THSD7A antigen in glomerular immune deposits have been reported [8, 9, 42, 43]. Developing more robust and rapid assays such as ELISA for the sensitive and quantitative measurement of autoantibody levels in THSD7A-associated MN patients would be helpful for both diagnosis and clinical follow-up.

In this study, we describe the set up of the first ELISA for the sensitive and quantitative detection of anti-THSD7A autoantibodies. We used the assay to screen a combined cohort of 1012 MN patients and identified 28 THSD7A-positive patients, indicating a prevalence of 2.8%. We also screened additional PLA2R1-negative patients and included in total 49 THSD7A-positive MN cases. We tested all cases by WB and IIFT, characterized the anti-THSD7A IgG subclasses and analyzed their reactivity for PLA2R1. We finally described the clinical characteristics of this population for age, gender, disease activity and possible links to etiology including malignancy. Our results show that this ELISA is rapid, sensitive and specific to measure anti-THSD7A autoantibodies and will be useful for better diagnosis and clinical follow-up of MN patients.

RESULTS

ELISA set-up

We prepared the full extracellular domain of human THSD7A in HEK293 cells (Figure S1) as soluble antigen and set up an ELISA that can specifically detect anti-THSD7A autoantibodies in serum from a subset of patients with MN but not from patients with other diseases or from healthy donors (Figure 1). Since many studies have shown that IgG4 is the predominant IgG subclass in MN patients [6, 7, 20, 21, 24, 42, 44, 45] and is more sensitive than total IgG to measure anti-PLA2R1 autoantibodies [6, 7, 13, 46, 47], we optimized the ELISA for detection of IgG4 anti-THSD7A autoantibodies. Using the above serum samples and receiver operating characteristics (ROC) curve analysis, we defined a cut-off value of 16 RU/mL above which serum samples are considered positive for anti-THSD7A (Figures 1 and S2A). We also established a standard curve for conversion of OD values into RU/mL (Figure S2B).

Identification of THSD7A-positive patients

Screening by ELISA of baseline serum from a total of 1012 biopsy-proven MN patients from 6 national and international cohorts [14, 20, 32, 33, 48-50] led to the identification of 28 THSD7A-positive patients, indicating an overall prevalence of 2.8% for THSD7A-associated MN (Table 1). Three of these patients were also positive for anti-PLA2R1 autoantibodies, indicating a prevalence of about 0.3% for double-positive MN patients (Table 1).

Additionally, our consortium screened THSD7A positivity in baseline serum or biopsy from other MN patients (mostly referred to our different nephrology centers because of PLA2R1-

unrelated MN over the last 4 years) by either of the four techniques available to identify THSD7A-associated MN, ie ELISA, IIFT, WB or enhanced THSD7A staining in biopsy [8, 9, 14, 33, 41]. This led to the inclusion of 21 additional THSD7A-positive patients, among which 5 were double positive. Collectively, our cohort thus included 49 THSD7A-positive MN patients (Figures 1 and S3, Tables 2 and S1).

Comparison of THSD7A positivity between ELISA, WB and IIFT and IgG subclasses

We then compared the positivity for THSD7A in baseline serum from the 49 patients by the 3 techniques: ELISA, WB and IIFT (Figure 2 and Table S1). In total, 43/49 patients were fully positive by ELISA (Figure 1 and Table S1, IgG4 detection), IIFT (Figure S4 and Table S1, total IgG detection) and WB (Figure S5 and Table S1, IgG4 detection). Among the 6 remaining patients, MN40 and MN41 were positive by IIFT when assayed at a 1:10 dilution with detection of total IgG (Figure S4) but negative by WB and ELISA when detected for IgG4 (Table S1). On the other hand, MN46 was positive by ELISA and WB but negative by IIFT (Figure 3). The last 3 patients (MN47, MN48 and MN49) were negative in serum by all 3 techniques but were positive based on THSD7A biopsy staining (Figures 3 and S6 and [9]).

Since ELISA titers ranged over several logs (Figures 2 and S3), we compared the different assays for 3 representative patients having low to high range anti-THSD7A titers and analyzed the correlation of titers measured by ELISA and IIFT for the whole cohort. Figure 2 shows that the 3 patients can be detected by ELISA at a 1:100 serum dilution (Figure 2A), by WB at the same dilution or lower (Figure 2B) and by IIFT, where appropriate dilution ranging from 1:10 to 1:1,000 gave a specific signal (Figure 2C). The autoantibody titers measured by ELISA and IIFT

correlated significantly (r=0.8592, p<0.0001, Figure 2D for IgG4 ELISA and Figure S3B for total IgG ELISA).

We also tested the positivity of the 49 patients by ELISA when detection of anti-THSD7A autoantibodies was performed with secondary antibodies specific for other IgG subclasses or total IgG. In our conditions, we detected IgG1, IgG2 and IgG3 anti-THSD7A autoantibodies in only 14, 18 and 20% of patients, respectively (Figure S3C). As for total IgG, 38 of the 49 patients (78%) were positive, with autoantibody titers spanning 3 log units and correlating significantly with IgG4 titers (r=0.925, p<0.0001, Figure S3D and E). More careful analysis showed that 37 of the 38 patients positive with total IgG were among the 44 patients positive for IgG4 (see above) while the last patient was negative for IgG4 (see below). Among the 7 patients negative for total IgG but positive for IgG4, six had low IgG4 anti-THSD7A titers and were negative for IgG1, IgG2 and IgG3 subclasses while the last one was positive for both IgG4 and IgG3. The higher number of patients detected with anti-IgG4 secondary antibodies is likely due to the better signal to noise ratio of these antibodies as compared to anti-total IgG (Figure S3), and the fact that anti-THSD7A autoantibodies are mostly IgG4 [7, 46, 47, 51, 52].

Among the 5 patients negative with IgG4 detection, 4 were also negative for total IgG, IgG1, IgG2 and IgG3. However, the last patient, MN40, illustrated a unique case in our cohort since it was negative for IgG4 but positive for total IgG. We carefully double-checked the positivity of this patient when using total IgG versus IgG4 and other IgG subclasses by ELISA, western blot and IIFT, and we confirmed that this patient was clearly positive for anti-THSD7A total IgG as well as IgG1, but not IgG2, IgG3 and IgG4 (Figures S3, S4 and S5).

MN patients double positive for THSD7A and PLA2R1

We carefully evaluated the 49 patients for their double positivity for THSD7A and PLA2R1 by detection of anti-THSD7A and anti-PLA2R1 autoantibodies in baseline serum by ELISA, WB and IIFT, and of THSD7A and PLA2R1 antigens in immune deposits (when biopsies were available). In total, 8 patients (MN42 to MN49) were found to be double positive (Figure 3). Four of these patients were previously reported from an American cohort (MN42 and MN45 [8]) and a Chinese cohort (MN47 and MN48 [9]) but none of them were compared for levels of anti-THSD7A and anti-PLA2R1 autoantibodies by quantitative ELISA. Analysis with baseline serum showed that double positive patients can have different titers of anti-THSD7A and anti-PLA2R1 autoantibodies (Figure 3A). Four patients had relatively higher titers of anti-THSD7A than anti-PLA2R1, one patient had low titers of both autoantibodies and the last three patients had no detectable levels of anti-THSD7A but high anti-PLA2R1 titers. The first five patients (MN42 to MN46) were clearly double positive in serum by ELISA and WB (Figure 3AB) but some of them appeared less positive by IIFT, which may be explained, at least in part, by the low titers measured by ELISA (Figure 3C). The last 3 patients (MN47, MN48 and MN49) were positive in serum by all 3 techniques (ELISA, WB and IIFT) for PLA2R1 but not for THSD7A (Figure 3ABC).

Among the 49 patients, renal biopsies were available for a total of 11 patients and were tested for THSD7A and PLA2R1 staining. This includes data previously published for MN42, MN45, MN47 and MN48 [8, 9]. In agreement with ELISA, 6 patients (MN 5, 6, 13, 26, 34 and 41) were positive for THSD7A but negative for PLA2R1 on biopsy (Figure S6). The 5 other patients had enhanced staining for both THSD7A and PLA2R1 ([8, 9] for MN42, MN45, MN47 and MN48 and Figure S6 for MN49[8, 9], data summarized in Figure 3D).

Taken together, we conclude that 8 patients are double positive for THSD7A and PLA2R1 (Figure 3E). Our analyses also illustrate the need of using all available techniques for detection of anti-THSD7A and anti-PLA2R1 in serum and antigen staining in biopsy to identify these rare cases of double positive MN patients.

Clinical characteristics of THSD7A-associated MN patients

Overall, the baseline clinical characteristics of the 49 THSD7A-associated MN patients (Tables 2 and S1) did not strongly differ from patients with PLA2R1-associated MN including those from the cohorts listed in Table 1 [23, 27, 33, 34, 41, 49, 53]. At baseline, the median age of the 49 anti-THSD7A-positive patients was 59.9 years. Median proteinuria and eGFR were 6.1 g/day [4.1-10.2] and 76.5 mL/min/1.73 m^2 [49.8-90.0], respectively. There was no significant correlation between anti-THSD7A titer and proteinuria (Figure S7).

Since we previously observed a high proportion of women in THSD7A-positive patients as compared to PLA2R1-positive patients [7], we investigated the influence of gender on the clinical parameters and anti-THSD7A titers in our cohort (Table 2). Among the 49 patients, 28 were males (57%) and 21 were females (43%), giving a gender ratio of 1.3:1 which contrasts with the 2:1 ratio typically observed in the general MN population (for instance it is 2.1:1 in our combined cohort of 1012 patients, Table 1) or the PLA2R1-associated MN specific subgroup [15]. Interestingly, females were significantly younger than males (48.8 versus 67.0 years, p=0.003). Data on menopausal age were lacking for our cohort. The average age at menopause of women with CKD is 51 years[54, 55], which suggest that a significant number of women developed MN before menopause (Table 2 and Figure 4A). However, the levels of anti-THSD7A titers did not

significantly differ between males and females, nor with age when analyzed by tertiles or when females were compared as two subgroups below and above 51 years (Figures 4B and S8A, Table S2). Titers separated by gender did not correlate with proteinuria (Figure S7). Proteinuria and serum albumin did not differ between males and females, irrespective of age (Table 2 and Figure S8B). Serum creatinine was higher and eGFR was lower in males, which might be partly age-related or due to more severe disease in males (Table 2).

It has been reported that THSD7A-associated MN may be linked to malignancy [41]. In our cohort, only 8 patients (16%) had a history of malignancy, including 6 males and 2 females (Tables 2 and S3). Clinical parameters were similar between these patients and others, except for serum albumin which was lower, possibly due to superimposed malnutrition [56]. Interestingly, all 8 patients with malignancy were significantly older, and only 3 of them were diagnosed for malignancy within 2 years of MN diagnosis (Tables S3 and S4). Titers of anti-THSD7A autoantibodies tended to be higher in patients with associated malignancy but the difference did not reach statistical significance (Table S3). Although the pattern of IgG subclass may differ between primary and secondary MN associated with malignancy [24, 45, 57], no differences in the relative levels of anti-THSD7A IgG subclasses were observed between patients with and without malignancy (Table S5).

We finally compared the clinical characteristics of patients with single positivity to THSD7A (n=41) versus double positivity to THSD7A and PLA2R1, which included 4 males and 4 females (n=8). No significant differences were observed for age, gender and baseline clinical values nor percentage of malignancy-associated MN between the two groups (Table S6).

Association of anti-THSD7A titer with disease activity

We compared anti-THSD7A titers at baseline and during follow-up to test the association between anti-THSD7A titers and disease activity, including response to treatment or spontaneous remission. Overall, we observed higher titers of anti-THSD7A autoantibodies in patients with active disease as compared to those in partial or complete remission (Figure 5A). We only had follow-up sera for 12 patients with a median follow-up of 17 months [7.0–31.3], but this number was sufficient to demonstrate that the anti-THSD7A titer is a relevant biomarker of the immunological autoimmune response and helps to monitor response to treatment (Figures 5B and 5C). At baseline, the 12 patients had nephrotic to sub-nephrotic range proteinuria (median 5.9 g/day [3.0–6.6]) and relatively high anti-THSD7A titers (median 206 RU/mL [68.8–472.8]). At the last follow-up serum sample available, patients who had reached spontaneous remission or remission after conservative or immunosuppressive treatment had non-nephrotic proteinuria levels (median 1 g/day [0.8–2.7]) and no detectable or strongly decreased anti-THSD7A titers [16–26.5 RU/mL]. In contrast, patients who did not reach remission, either untreated or resistant to immunosuppressive treatment, had nephrotic range proteinuria (median 10.8 g/day [6.5–14.0]) and persistently high anti-THSD7A titers (median 709 RU/mL [381.3–993.3].

The utility of anti-THSD7A titer to monitor disease activity and response to treatment was further illustrated by the clinical follow-up of patient MN13, a 4-year old girl treated twice with rituximab over 27 months (Figure S9). At baseline, the patient had high proteinuria (6.1 g/day) and anti-THSD7A titer (715 RU/mL). Treatment with rituximab led to a progressive decrease of anti-THSD7A titer which was followed by fluctuating but finally decreasing proteinuria down to the sub-nephrotic range at month 18. Between months 18 to 27, anti-THSD7A titer and proteinuria increased again, suggesting an ongoing relapse. Comparison of anti-THSD7A

autoantibody levels by ELISA, WB and IIFT showed that ELISA was the most accurate assay to detect subtle changes of anti-THSD7A levels, with IgG4 and IgG3 appearing as the most relevant IgG subclasses to monitor disease activity.

Anti-THSD7A titer and clinical outcome of THSD7A-associated MN patients

Among the 49 THSD7A–associated MN patients, we had clinical outcome and detectable anti-THSD7A autoantibodies at baseline for 36 patients during a median follow-up of 37 months (range 6.5–180 months) (Tables S1 and S7). During follow-up, 10 (28%) patients were untreated or treated with antiproteinuric therapy (angiotensin–converting enzyme inhibitors and/or angiotensin 2 receptor blockers and diuretics) while 24 (67%) patients received an additional immunosuppressive treatment (cyclosporine A, cyclophosphamide, rituximab or adrenocorticotropic hormone). Treatment was not available for 2 patients. Twelve (33%) patients remained in active disease among which two reached end stage kidney disease, while 24 (67%) reached complete (n=16) or partial (n=8) remission. Two patients (MN29 and MN33) experienced relapse after partial remission (one after spontaneous remission and the other after treatment with cyclosporine A). Among the 12 patients who remained in active disease, 10 (83%) patients had received an immunosuppressive treatment (Tables S1 and S7). Among the 24 patients who reached remission, 14 (58%) patients had received an immunosuppressive treatment (Tables S1 and S7).

We evaluated the association between anti-THSD7A titer at baseline and clinical outcome. Overall, baseline anti-THSD7A titer significantly differs between patients reaching remission or not during follow-up (134 [52; 955] versus 536 [250; 1671] RU/mL, respectively p=0.04)

(Figure 6A), while these two groups were comparable for age, sex ratio, proteinuria, albuminemia, eGFR and immunosuppressive treatment (Table S7).

We then divided the patients into tertiles based on anti-THSD7A titer and analyzed outcome. Patients in the lowest tertile (titers 23-122 RU/mL) tended to have a higher rate of remission (11/12, 92%) compared to patients in the middle (titers 134-566 RU/mL) and highest (titers 606-13920 RU/mL) tertiles (6/12, 50% and 7/12, 58%, respectively, p=0.07), while persistent proteinuria and renal failure tended to be more frequent in patients with high anti-THSD7A titer, but none of these trends reached statistical significance (not shown). Because of the small sample size, we combined the middle and highest tertiles and compared renal survival during the first three years after diagnosis to the lowest tertile. Patients from the lowest tertile had better renal survival with more remission compared to patients from the medium and highest tertiles (Figure 6B, p=0.006).

DISCUSSION

This study had two major aims: 1) the development of a robust ELISA allowing the sensitive and quantitative measurement of anti-THSD7A autoantibodies, and 2) the analysis of a relatively large retrospective cohort of THSD7A-positive patients from which we may identify clinical characteristics specific for THSD7A-associated MN.

We set up an ELISA to detect anti-THSD7A autoantibodies in serum with characteristics and performance similar to those previously reported for PLA2R1, using the purified full extracellular region of THSD7A as antigen for the solid-phase assay, validation of the ELISA with MN patients versus negative controls and detection for either total IgG or IgG4 secondary antibodies [20, 21]. Using this ELISA to screen a combined cohort of 1012 MN patients, we identified 28 patients, indicating a prevalence of 2.8%, in accordance with the prevalence range of 2-5% previously reported for Caucasian or Asian cohorts (data are summarized in Table S8) [7, 9, 41-43, 58, 59]. By screening additional patients mostly referred because of PLA2R1-unrelated MN, we included 21 more cases, providing a cohort of 49 patients with THSD7A-associated MN [7, 9, 41-43, 58, 59]. We validated the novel ELISA by comparing its sensitivity against the 49 MN patients with the original WB method [7] and the commercially available IIFT assay [41]. We found similar levels of sensitivity by ELISA and WB, and a significant correlation between anti-THSD7A titers measured by IIFT and ELISA. Among the 49 patients, 43 were positive by the 3 techniques: ELISA (IgG4 detection), WB (IgG4 detection) and IIFT (Total IgG detection). One was positive by ELISA, WB and IIFT (Total IgG detection). One was only positive by ELISA (IgG4 detection) and WB (IgG4 detection). One was only positive by IIFT (total IgG detection). The last 3 were negative in serum by all 3 techniques. These discrepancies may be explained by the different autoantibody detection systems and presentation of antigens in solid-phase ELISA,

WB and IIFT cell-based assay. We also found that the ELISA is more sensitive when detection of anti-THSD7A autoantibodies is made with anti-IgG4 as compared to anti-total IgG. Indeed, six patients could be detected only with anti-IgG4 while one patient could be detected only with anti-total IgG. It is thus preferable to use anti-IgG4 as a secondary antibody to detect the highest number of MN patients with THSD7A-associated disease and avoid false-negative cases, yet further screening with anti-total IgG may help to identify additional patients.

The 3 patients who had no circulating anti-THSD7A autoantibodies detected by ELISA, WB and IIFT were positive on biopsy with the presence of immune deposits containing the THSD7A antigen. Such discrepancies have been initially observed for PLA2R1-associated MN [22] and more recently for THSD7A-associated MN [41]. We conclude that the new ELISA is reliable and in accordance with available methods of detection such as WB and IIFT [7, 41].

Among the 49 patients, we identified 8 patients double positive for THSD7A and PLA2R1. No major clinical differences were observed between the single and double positive patients. Four double positive cases were novel and from Europe while two were already reported from an American cohort[8] and two from a Chinese cohort[9]. Three of the new cases were identified from the screening of 1012 MN patients, indicating an overall prevalence of about 0.3%. However, these 3 double positive cases actually represent about 10% of the 28 THSD7A-positive patients (identified from 1012 patients) but only about 0.4% of the 684 patients identified with PLA2R1-associated MN (Table 1). With the observed respective prevalence of about 70% and 3% for PLA2R1 and THSD7A in our combined cohort, and assuming that the respective production of anti-PLA2R1 and anti-THSD7A autoantibodies is a random event, one would expected much more double positive patients in the PLA2R1-positive group than in the negative one, suggesting a negative association between the two events. However, we could not determine from these

studies whether the presence of both anti-PLA2R1 and anti-THSD7A autoantibodies is co-incidental or associated one to another (for instance by inter-molecular epitope spreading [60]), and what autoantibody would precede the other one during the natural history of the disease.

Careful analysis of the 8 double positive patients led us to make two additional observations. First, the respective circulating levels of anti-THSD7A and anti-PLA2R1 could be very different between patients, with all scenarios observed, i.e. lower titer of anti-THSD7A than anti-PLA2R1 and vice-versa, or similar titers of both autoantibodies. Second, among the 5 patients with available serum and biopsy, two were fully positive, i.e. for both antigens in serum and biopsy (MN42 and MN45), while three (MN47, MN48 and MN49) were positive in both serum and biopsy for PLA2R1 but only positive for THSD7A in biopsy. This different pattern of positivity in serum versus biopsy is reminiscent of what was previously observed for MN patients with single positivity for PLA2R1 or THSD7A [22, 41].

In this study, we also present the clinical analysis of the largest cohort of patients with THSD7A-associated MN with quantitative analysis of anti-THSD7A titers for the different IgG subclasses. First, we observed that the titer of anti-THSD7A autoantibodies is heterogeneous and can vary by up to 3 orders of magnitude in both genders. Second, we showed that IgG4 is the predominant IgG subclass for anti-THSD7A autoantibodies in most patients, regardless of co-incidental diseases. Third, we observed no strong correlation between anti-THSD7A titer and levels of proteinuria, as previously observed for PLA2R1-associated MN [20]. Nonetheless, the anti-THSD7A titer appears to be a relevant biomarker to monitor disease activity during follow-up and treatment with immunosuppressors, as exemplified for the 12 patients with available follow-up. Fourth, we showed that patients with low anti-THSD7A titer at baseline had better clinical outcome, as previously observed for PLA2R1-associated MN [20, 25, 27, 28, 31, 35, 61]. In addition to

anti-PLA2R1 titer, PLA2R1 epitope spreading was recently identified as a prognosis biomarker to predict outcome in MN [14, 34]. Despite the recent identification of several epitopes in THSD7A [16], it remains to determine whether epitope spreading also occurs in patients with THSD7A-associated MN and may be a relevant biomarker of disease activity and clinical outcome.

The relatively large size of our cohort allowed us to stratify patients by gender and compare their epidemiological and clinical parameters. First, we observed a male:female ratio of 1.3:1 which contrasts with the 2:1 ratio typically reported when considering all MN cases or PLA2R1-associated MN [2-5, 62]. Second, we observed that females were younger than males at diagnosis, suggesting different etiologies. To confirm our findings, we compiled the available data on gender and age from all previous studies identifying cases of THSD7A-associated MN (Table S8) and observed that females were indeed more preponderant and younger in THSD7A-associated MN than PLA2R1-associated MN. Third, we observed that females had on average similar levels of anti-THSD7A autoantibodies, yet the levels seemed to vary with age. However, we could not evidence significant differences in disease activity, response to treatment and clinical outcome between males and females.

The underlying etiologies leading to PLA2R1- and THSD7A-associated MN are currently unknown [2, 3, 63]. We observed many associated diseases in our cohort of THSD7A-associated diseases (Table S1). Recent studies reported that 8 of 40 (20%) THSD7A-positive MN patients had an associated malignancy within a median time of 3 months from diagnosis of MN, suggesting that THSD7A-positive patients are at higher risk of having an underlying malignant disease and may be intensively screened for cancer as a possible etiology [41, 64]. In our cohort, we found that 8 of the 49 THSD7A-positive MN patients (16%) had a history of cancer, including one double positive patient. However, only 3 patients had cancer (gastric, colonic and prostatic)

within 2 years of MN diagnosis. Furthermore, we observed that 7 of the 8 patients were older than 65 years, with 6 males and 2 females. These observations are in line with the study by Hoxha et al where 5 of the 8 patients were older than 65 years, and with 5 males and 3 females [41]. We also observed that the 8 THSD7A-positive patients with associated cancer tended to have higher anti-THSD7A titers, but no significant differences in IgG subclasses. Since earlier studies reported that the prevalence of malignancy in MN patients is higher than in the general population, while it increases with age [65-68], our data converge to the point that most cases of THSD7A-associated MN with malignancy were likely co-incidental in our subgroup of 8 patients.

Another cause of MN may be associated with pregnancy and pre-eclampsia [69-72], including recent observations for THSD7A-associated MN [73, 74]. In line with this hypothesis, we observed a significant subgroup of young THSD7A-positive female patients (before menopause, Figure 4B) in our cohort but we could not evidence a relationship between MN and pregnancy or pre-eclampsia.

We also described 2 cases of pediatric and adolescent MN with no associated underlying disease. A 4-year-old girl who was treated with rituximab and reached partial remission, and a 17-year-old boy who had high anti-THSD7A titer at baseline and was resistant to rituximab.

In conclusion, we have reported a novel ELISA that is useful to diagnose MN patients for THSD7A-associated disease, to carefully monitor disease activity and response to treatment during follow-up, and predict clinical outcome. We also described new cases of patients with THSD7A-associated MN supporting the hypothesis that THSD7A-associated MN differs from PLA2R1-associated MN in the natural history and epidemiological features, such as gender, age

at onset and associated etiologies [15, 75]. Interestingly, this situation appears reminiscent to that observed for myasthenia gravis, a neuromuscular autoimmune disease with multiple autoantigens and different paths and etiologies towards the same disease entity [76].

METHODS

Patients

Since the prevalence of THSD7A-associated MN is low, we included patients from several national and international nephrology centers. Baseline serum samples of patients with biopsy-proven MN were collected within 6 months from renal biopsy. A total of 1012 patients originating from 6 independent retrospective cohorts and additional patients who were referred to us mostly for PLA2R1-unrelated MN were screened for anti-THSD7A autoantibodies. Estimated GFR (eGFR) was calculated by applying the Modification of Diet in Renal Disease 4 (MDRD4) formula. Disease activity was defined as no remission of nephrotic syndrome. Partial remission was defined as proteinuria lower than 3.5 g/24 h and at least 50% reduction from the time of inclusion in the study with normalization of the serum albumin concentration and stable serum creatinine. Complete remission of proteinuria was defined as proteinuria lower than 0.5 g/24 h, normal albuminemia and stable eGFR. Remissions were classified as spontaneous if they were occurring without the administration of immunosuppressive agents during follow-up. The negative control groups included serum samples from patients with other diseases such as lupus nephritis (n=9), membranoproliferative glomerulonephritis (n=9), ANCA vasculitis (n=3), IgA nephropathy (n=11), other renal diseases (n=18) and from healthy blood donors (n=52). The studies were approved by the relevant institutional review boards in the different countries and were conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from participants in all studies.

ELISA for the detection of anti-THSD7A and anti-PLA2R1 autoantibodies

Purified recombinant THSD7A protein (prepared as described in the supplementary appendix) or the full extracellular domain of human PLA2R1 prepared as described [21] were used as antigens to coat 96-well ELISA microplates (Thermo scientific) in 20 mM Tris pH 8.0 overnight at 4°C. Plates were blocked with SeramunBlock (Seramun Diagnostica) for 2 hours. Patients' serum samples diluted at 1:100 in 0.1% low-fat dry milk PBS were incubated for 2 hours. Plates were washed 3 times with PBS-Tween 0.02%. Bound human antibodies were detected with either anti-human HRP-conjugated IgG1, IgG2, IgG3, IgG4 or total IgG (Southern Biotech) diluted in SeramunStab ST (Seramun Diagnostica) at 1:5,000, 1:5,000, 1:20,000, 1:30,000 and 1:200,000, respectively. Secondary antibodies were all incubated for 1 hour. After washes, tetramethylbenzidine peroxidase substrate (TMB) was added and developed for 15 min. The reaction was stopped by adding 1.2 N HCl. All incubation steps were carried out at room temperature on a plate shaker. The optical density was read at 450 nm using a plate reader. A standard curve for IgG4 detection was made using a highly THSD7A-positive serum which was assigned a value of 99,000 RU/mL when not diluted. A standard curve consisting of 8 dilutions covering the range from 990 RU to 9.9 RU/mL was plotted using the GraphPad Prism software and applied to each plate to convert OD values into RU/mL (Figure S2B). Samples that were out of range were diluted at 1:500 and 1:1,000 and re-analyzed. The inter-assay variation was measured by incorporating a borderline positive serum sample on each plate. Results from 10 different plates showed a coefficient of inter-assay variation lower than 20% (not shown). Normal range for IgG1, IgG2, IgG3 and total IgG detection were defined using serum samples from disease controls and healthy donors (not shown).

WB and IIFT for the detection of circulating anti-THSD7A and anti-PLA2R1 autoantibodies and kidney biopsy staining for THSD7A and PLA2R1 antigens

Detailed procedures for WB [7] and biopsy staining [22] are provided in the supplementary appendix. We used the commercial cell–based IIFT kit from Euroimmun AG containing a mosaic biochip of formalin-fixed HEK293 cells overexpressing full-length human THSD7A or PLA2R1 or mock-transfected HEK293 as a negative control.

Statistical analysis

Baseline characteristics of the patients in the study were expressed as percentages for qualitative variables and medians and IQRs for quantitative variables. Non-parametric correlations between several parameters were calculated by Spearman's test. Quantitative variables were compared by Mann-Whitney U or one-way ANOVA tests and categorical variables were compared by a Pearson chi-squared test or a Fisher exact test. P values lower than <0.05 were considered as statistically significant. Statistics were performed using the GraphPad Prism version 6 software. Renal survival curves were calculated using Kaplan-Meyer estimates for survival distribution. The endpoint was the time where patients entered into remission (partial or complete) from baseline. Differences between groups based on tertiles of anti-THSD7A titer were analyzed with the log-rank test.

Supplementary figures and material

Supplementary information is available at Kidney International's website.

DISCLOSURE

Some co-authors are co-inventors on the patents "Diagnostics for membranous nephropathy" (LHB and GL), "Methods and kits for monitoring membranous nephropathy" (BSP and GL) and "Prognosis and monitoring of membranous nephropathy based on the analysis of PLA2R1 epitope profile and spreading" (BSP, GD and GL).

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TABLES

Table 1: THSD7A and PLA2R1 reactivity of patients' sera from the different MN cohorts screened in this study. The main clinical characteristics of each cohort can be found elsewhere: Nice [14], Saint-Etienne [49], Gemritux [32], Sweden [50], Italy [48] and Netherlands [77]. Values are shown as n (%).

Cohort	Patients			THSD7A Positive	THSD7A positive		THSD7A negative	
	All	Male	Female		PLA2R1 positive	PLA2R1 negative	PLA2R1 positive	PLA2R1 negative
Nice	275	183 (67)	92 (33)	8 (2.9)	1	7	150 (54.6)	117 (42.5)
Saint-Etienne	68	45 (66)	23 (34)	2 (2.9)	0	2	41 (60.3)	25 (36.8)
Gemritux	75	52 (69)	23 (31)	2 (2.7)	0	2	61 (81.3)	12 (16.0)
Sweden	25	16 (64)	9 (26)	1 (4.0)	0	1	16 (64.0)	8 (32.0)
Italy	251	175 (70)	76 (30)	6 (2.4)	2	4	182 (72.5)	63 (25.1)
Netherlands	318	219 (69)	99 (31)	9 (2.8)	0	9	234 (73.6)	75 (23.6)
Total (n) (%)	**1012**	**690 (68.2)**	**322 (31.8)**	**28 (2.8)**	**3 (0.3)**	**25 (2.5)**	**684 (67.6)**	**300 (29.6)**

Table 2: Epidemiological and clinical baseline characteristics of anti-THSD7A positive patients. The 49 patients were positive for THSD7A by at least one technique of detection: ELISA (44/49 positive patients with IgG4 detection and 1 additional patient with total IgG detection), IIFT (45/49 positive patients, total IgG detection), WB (44/49 positive patients, IgG4 detection) or biopsy staining (11/11 positive patients). Values are shown as n (%) or median [IQR]. eGFR was calculated using the MDRD4 formula.

Characteristics of THSD7A-positive patients	**All (n=49)**	**Male (n=28)**	**Female (n=21)**	**P value**
Gender M/F n (%)	28/21	28 (57)	21 (43)	
Age at diagnosis (year)	59.9 [48.5–75.0]	67.0 [54.3–75.0]	48.8 [36.5–64.5]	0.003
Proteinuria (g/day)	6.1 [4.1–10.2]	6.6 [5.3–11.2]	5.0 [3.0–7.7]	0.106
Serum creatinine (µmol/L)	89.0 [70.7–121.0]	112.0 [88.0–145.0]	70.7 [64.7–86.6]	0.0004
eGFR (CKD-EPI) (mL/min/1.73 m^2)	76.5 [49.8–90.0]	60.0 [39.8–80.0]	85.5 [64.5–91.8]	0.017
Serum albumin (g/L)	21.0 [16.5–25.3]	21.0 [15.3–25.8]	22.2 [17.5–25.3]	0.681
Anti-THSD7A titer (RU/mL)	278.0 [40.5–835.0]	256.0 [27.5–857.8]	302 [55.5–1035.0]	0.585
Cancer incidence n (%)	8 (16)	6 (21)	2 (10)	0.264

LEGEND TO FIGURES

Figure 1: Comparative analysis of IgG4 reactivity against THSD7A determined by ELISA. IgG4 anti-THSD7A reactivity was validated using THSD7A-positive MN patients (with respect to positivity by IIFT and WB) versus THSD7A-negative MN patients (PLA2R1-positive and DN patients) and other controls (other diseases and healthy blood donors, n=101). The data are expressed as OD_{450nm} and presented as median. The dotted line represents the cut-off (OD_{450nm} of 0.12 corresponding to 16 RU/mL) at a defined specificity of 97% with respect to IFT by ROC curve analysis (Figure S2). DN: double negative MN patients, IgA: IgA nephropathy, Lupus: Lupus nephritis, ANCA: anti-neutrophil cytoplasmic autoantibodies, MPGN: membranoproliferative glomerulonephritis, ORD: other renal diseases, HBD: healthy blood donors.

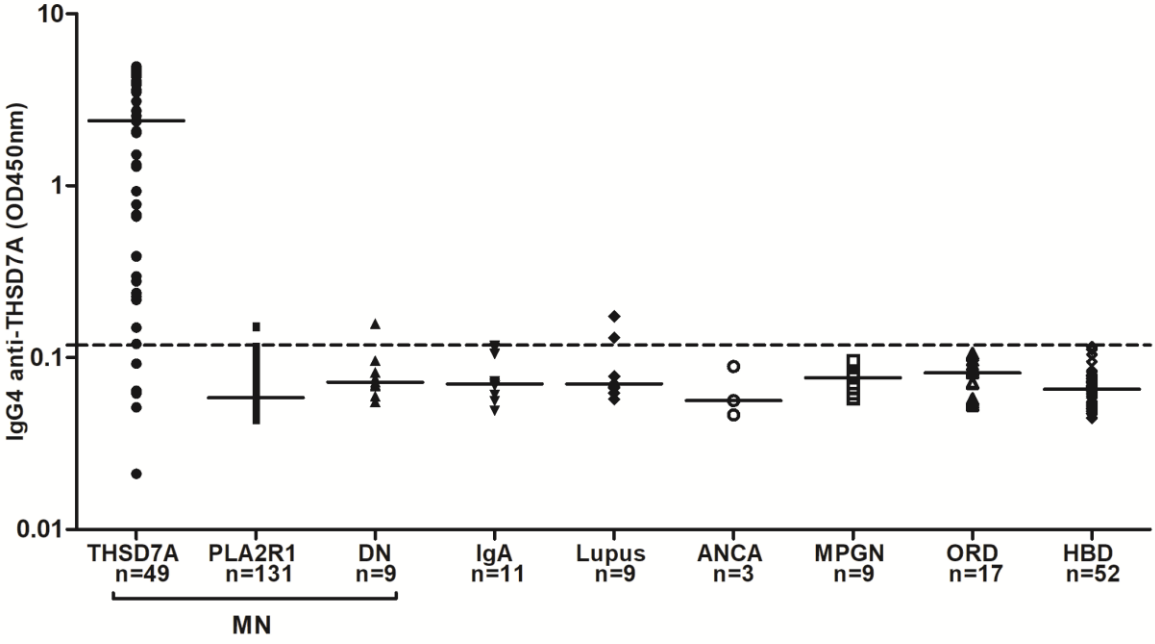
Figure 2: Comparison of anti-THSD7A detection in serum by ELISA, WB and IIFT. (A) ELISA reactivity for 3 MN patients with low to high range titers detected at a dilution of 1:100. MN5 was further diluted to determine the ELISA titer with accuracy; (B) WB reactivity of the same patients tested at optimal serum dilution against purified recombinant THSD7A and PLA2R1 (50 ng each). Blots were exposed for different times; (C) IIFT reactivity at optimal serum dilution. Scale bar 70 µm (D) Correlation between anti-THSD7A levels of MN patients (n=49) as measured by IIFT (detection for total IgG) and ELISA (detection for IgG4). The correlation is significant ($r=0.8592$, $p<0.0001$).

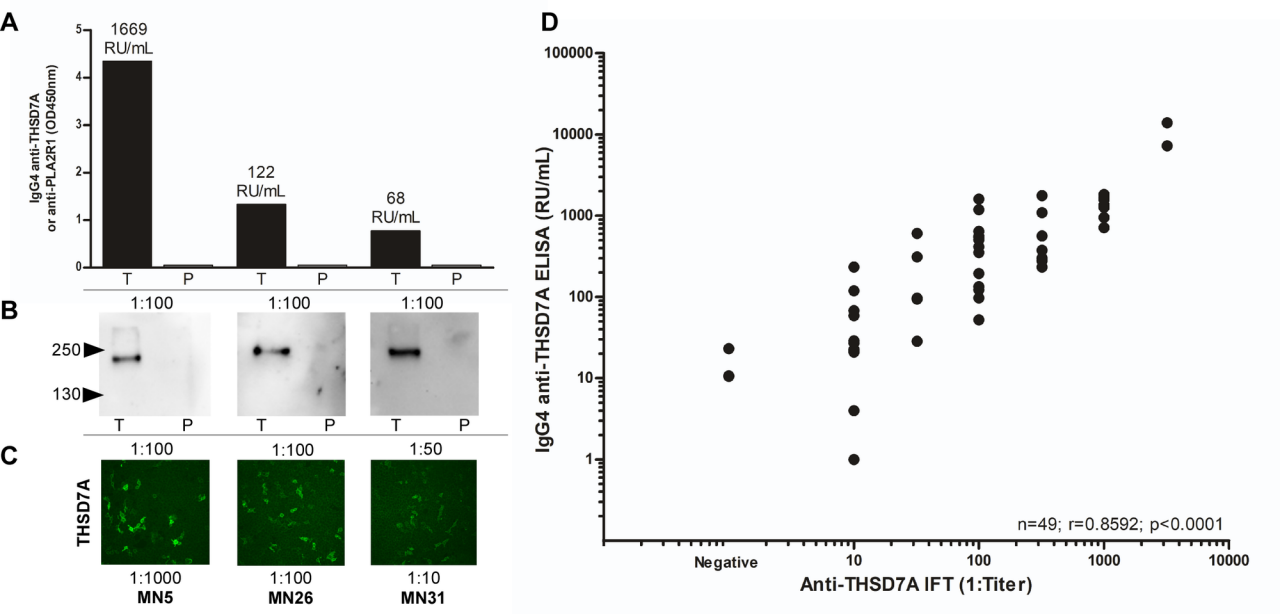
Figure 3: Detection of anti-THSD7A and anti-PLA2R1 autoantibodies in double positive MN patients. (A) Reactivity of sera from double positive MN patients (MN42 to MN49) and single positive MN controls (MNT+ and MNP+) against THSD7A and PLA2R1 antigens in ELISA. All sera were diluted at 1:100 except MN46 (1:25). (B) WB reactivity of sera from double positive MN patients (MN42 to MN49) against THSD7A and PLA2R1 antigens (50 ng each) loaded on SDS-PAGE (6%) under non-reducing conditions. All sera were used at a dilution of 1:25. Soluble forms of THSD7A and PLA2R1 have molecular masses of about 230 kDa and 170 kDa, respectively. (C) IIFT showing the reactivity of MN42 to MN49 against HEK293 cells transfected with THSD7A or PLA2R1 expression vectors. Sera were tested at a dilution of 1:10 or higher depending on titer. Scale bar 70 µm. (D) Summary of positivity by biopsy staining for THSD7A and PLA2R1 antigens (biopsies were available for 5 patients: MN49 staining is shown in figure S6 and for other patients the original data can be found elsewhere [8, 9]). (E) Summary of positivity for THSD7A and PLA2R1 autoantibody or antigen in serum or on biopsy respectively. NA: not available.

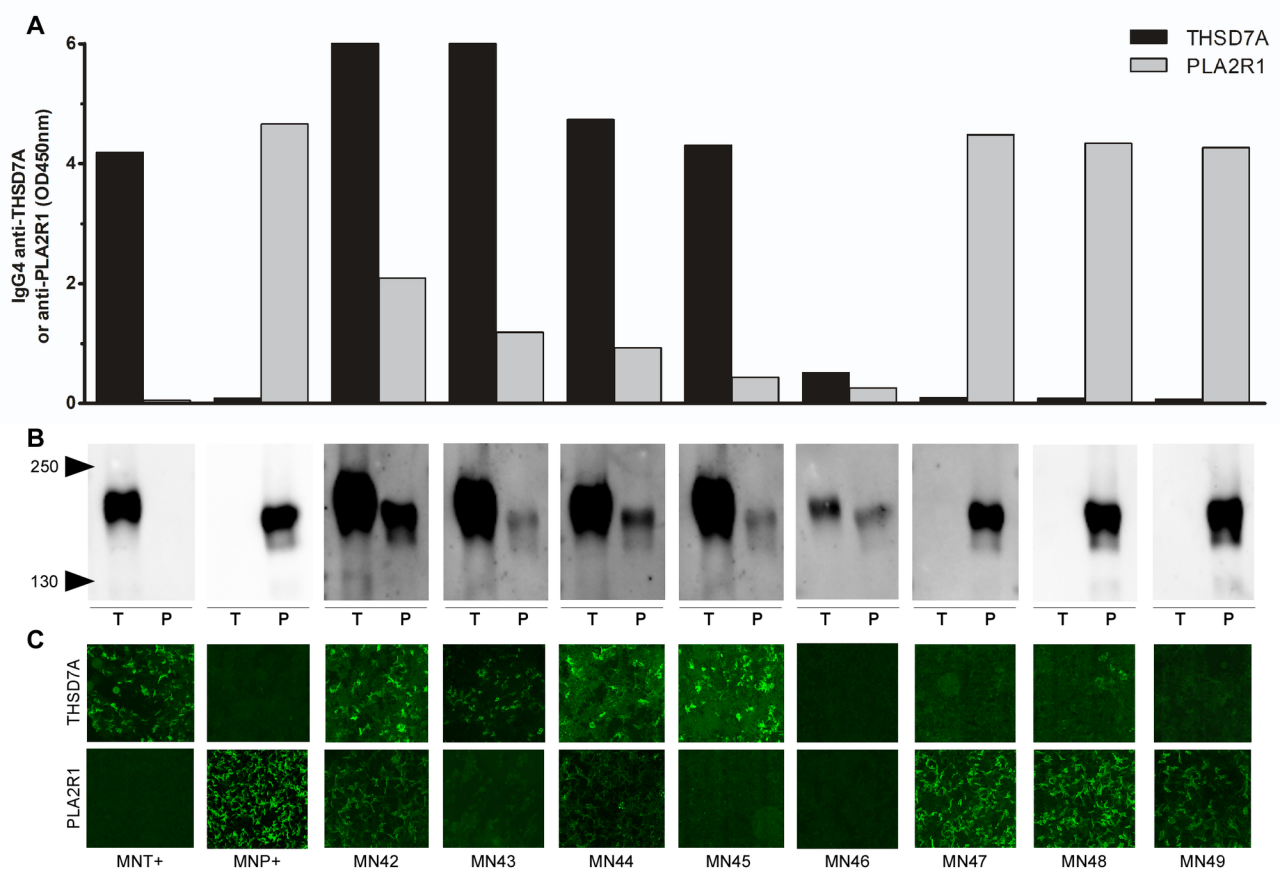
Figure 4: Distribution of age and anti-THSD7A titers among THSD7A-positive patients as a whole or by gender. (A) Age distribution of anti-THSD7A positive patients (n=49). The data are presented as median. Females were significantly younger than males using the Mann-Whitney U test ($p<0.003$). (B) Distribution of anti-THSD7A titers for THSD7A-positive patients, as measured with anti-IgG4 secondary antibodies. The dotted line represents the threshold value for IgG4 detection.

Figure 5: Relationships between anti-THSD7A titers and clinical status in THSD7A-positive patients. (A) Anti-THSD7A titers were measured from baseline and follow-up sera of anti-THSD7A patients with active disease (53 baseline and follow-up serum samples with a mean proteinuria of 7.4 g/day), partial remission (15 follow-up serum samples with a mean proteinuria of 1.9 g/day) or complete remission (5 follow-up serum samples with a mean proteinuria of 0.3 g/day). The anti-THSD7A titer was significantly lower in patients with complete remission compared to those in active disease. The difference in titer between patients in active disease vs partial remission did not reach significance. (B) Anti-THSD7A titers during follow-up of patients who received no or conservative treatment and (C) immunosuppressive treatment. Anti-THSD7A titers of patients who reached remission are shown with dotted lines. A, active disease; R, remission; LOCF, last observation carried forward.

Figure 6: Anti-THSD7A titer at baseline predicts clinical outcome. (A) Anti-THSD7A titers at baseline significantly differ between patients with remission versus active disease at last follow-up (n=36, p=0.04). (B) Renal survival at last follow-up. Renal event is defined by partial or complete remission within 3 years after anti-THSD7A ELISA measured at baseline. Patients with low titer of anti-THSD7A (titers 23-122 RU/mL, first tertile) had a higher incidence of renal events as compared to patients with medium and high titer (titers 134-13920, middle and high tertiles together) (n=36, p=0.006).


10
1
0.1
0.01
IgG4 anti-THSD7A (OD450nm)
THSD7A n=49
PLA2R1 n=131
DN n=9
IgA n=11
Lupus n=9
ANCA n=3
MPGN n=9
ORD n=17
HBD n=52
MN


A
1669 RU/mL
122 RU/mL
68 RU/mL
IgG4 anti-THSD7A or anti-PLA2R1 (OD450nm)
T P T P T P
1:100 1:100 1:100
B
250
130
T P T P T P
C
1:100 1:100 1:50
THSD7A
1:1000 1:100 1:10
MN5 MN26 MN31
D
IgG4 anti-THSD7A ELISA (RU/mL)
Negative
Anti-THSD7A IFT (1:Titer)
n=49; r=0.8592; p<0.0001

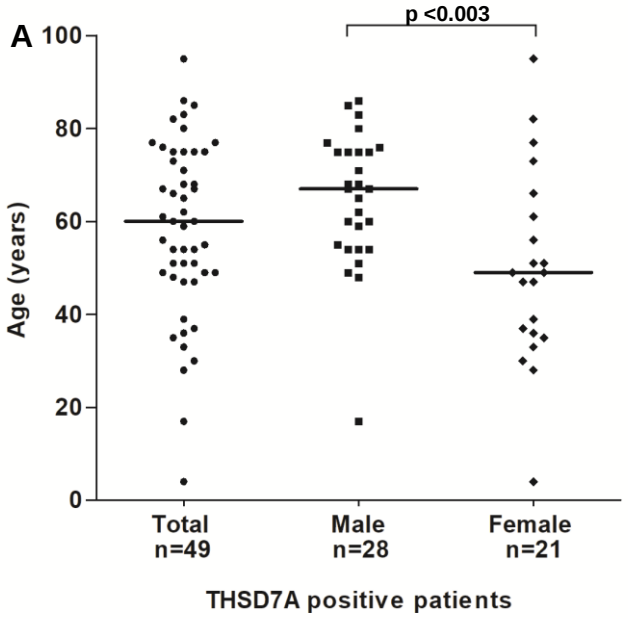


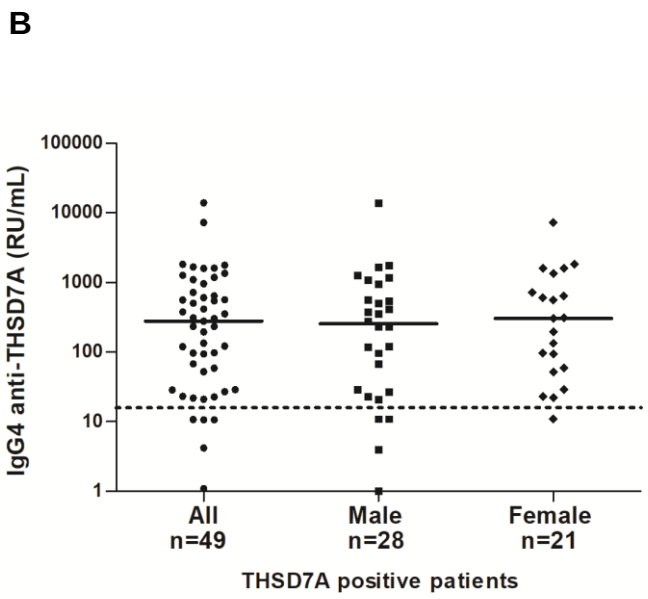
D

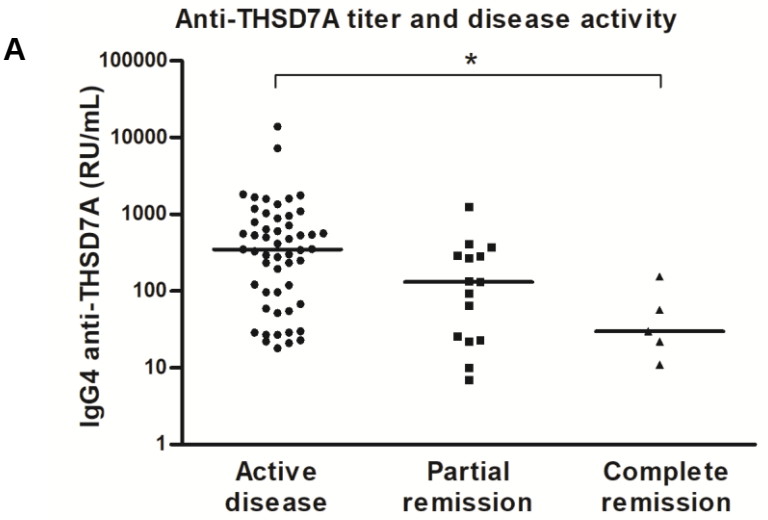
THSD7A antigen	+	NA	NA	+	NA	+	+	+
PLA2R1 antigen	+	NA	NA	+	NA	+	+	+
MN patient #	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**

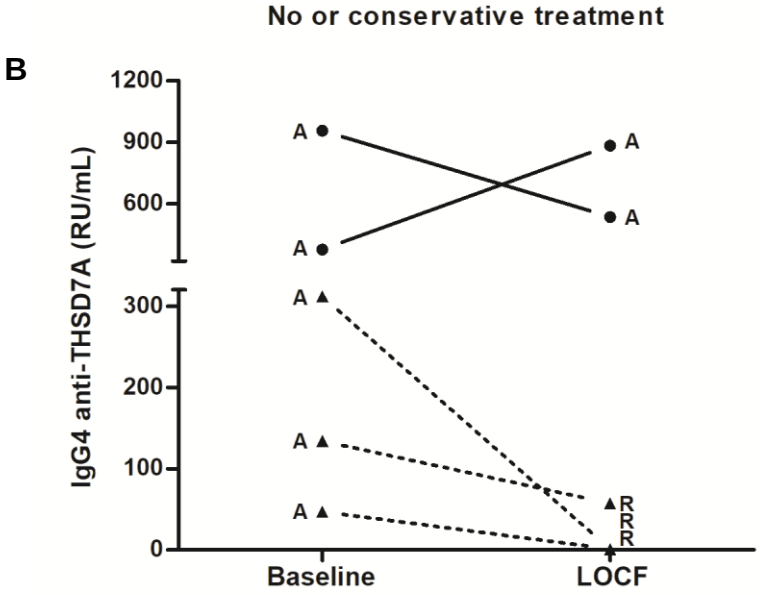
E

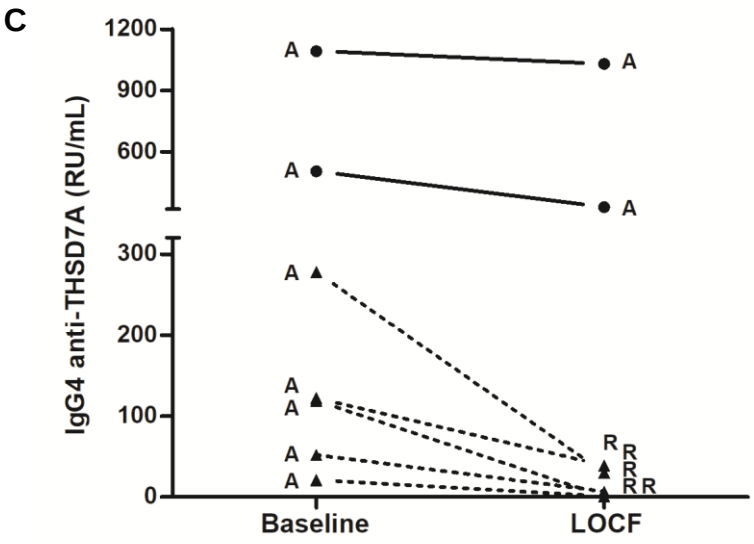
	T P	T P	T P	T P	T P	T P	T P	T P
Serum	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
Biopsy	+ +	NA NA	NA NA	+ +	NA NA	+ +	+ +	+ +
MN patient #	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**


A
p <0.003
100
80
60
40
20
0
Age (years)
Total
n=49
Male
n=28
Female
n=21
THSD7A positive patients

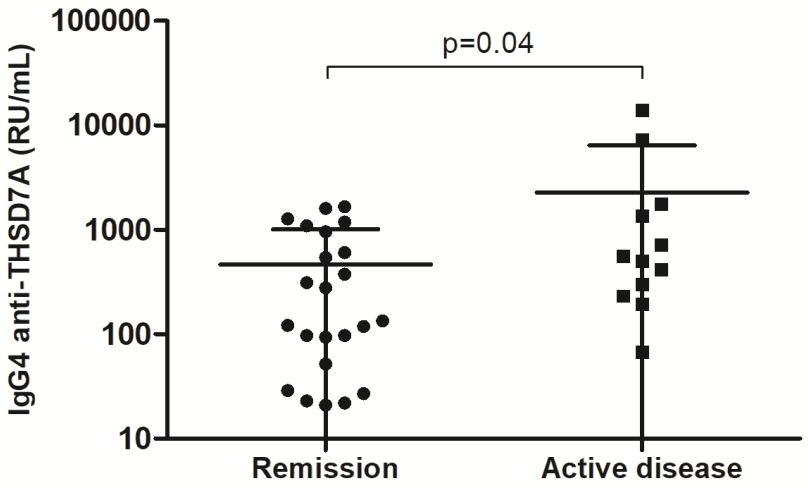

B
100000
10000
1000
100
10
1
IgG4 anti-THSD7A (RU/mL)
All
n=49
Male
n=28
Female
n=21
THSD7A positive patients


A
Anti-THSD7A titer and disease activity
IgG4 anti-THSD7A (RU/mL)
100000
10000
1000
100
10
1
*
Active disease
Partial remission
Complete remission

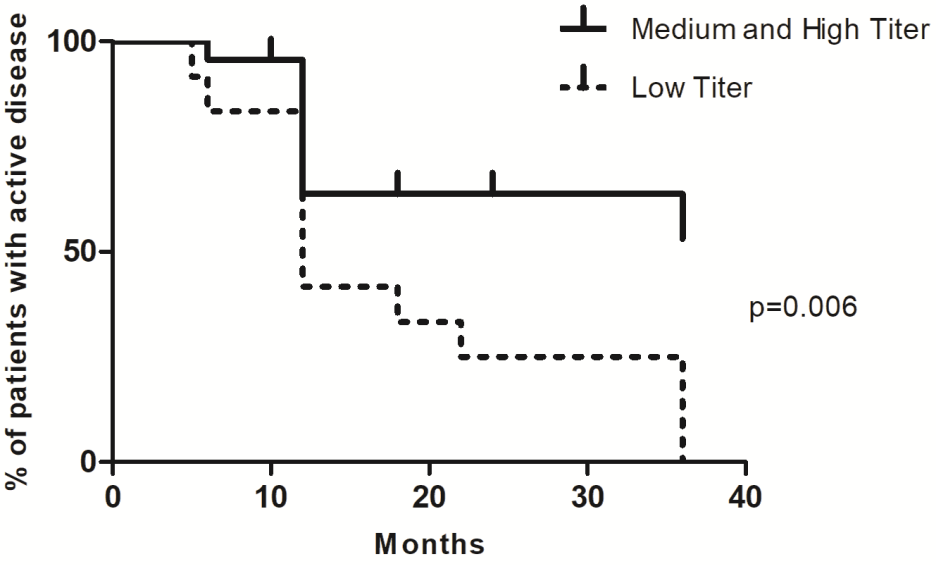

B
No or conservative treatment
IgG4 anti-THSD7A (RU/mL)
1200
900
600
300
200
100
0
A
R
Baseline
LOCF


C
Immunosuppressive treatment
IgG4 anti-THSD7A (RU/mL)
1200
900
600
300
200
100
0
A
R
Baseline
LOCF

A


IgG4 anti-THSD7A (RU/mL)
100000
10000
1000
100
10
p=0.04
Remission
Active disease

B


% of patients with active disease
100
50
0
0
10
20
30
40
Months
Medium and High Titer
Low Titer
p=0.006