



HAL
open science

Prediagnostic Appearance of THSD7A Autoantibodies in Membranous Nephropathy

Peter D Burbelo, Stephen W Olson, Jason M Keller, Megha Joshi, Daniella M Schwartz, Yung-Jen Chuang, Gérard Lambeau, Laurence H Beck, Meryl Waldman

► **To cite this version:**

Peter D Burbelo, Stephen W Olson, Jason M Keller, Megha Joshi, Daniella M Schwartz, et al.. Prediagnostic Appearance of THSD7A Autoantibodies in Membranous Nephropathy. *Kidney360*, 2022, pp.10.34067/KID.0005112022. 10.34067/KID.0005112022 . hal-03896524

HAL Id: hal-03896524

<https://hal.science/hal-03896524v1>

Submitted on 13 Dec 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

How to Cite this article: Peter Burbelo, Stephen Olson, Jason Keller, Megha Joshi, Daniella Schwartz, Yung-Jen Chuang, Gérard Lambeau, Laurence Beck, and Meryl Waldman, Prediagnostic Appearance of THSD7A Autoantibodies in Membranous Nephropathy, *Kidney360*, Publish Ahead of Print, 2022, 10.34067/KID.0005112022

Article Type: Original Investigation

Prediagnostic Appearance of THSD7A Autoantibodies in Membranous Nephropathy

DOI: 10.34067/KID.0005112022

Peter Burbelo, Stephen Olson, Jason Keller, Megha Joshi, Daniella Schwartz, Yung-Jen Chuang, Gérard Lambeau, Laurence Beck, and Meryl Waldman

Key Points:

*The entire extracellular domain of THSD7A in the LIPS immunoassay was required to detect autoantibodies with high sensitivity in MN.

*In THSD7A seropositive MN patients, changes in antibody levels precedes a change in clinical status.

*THSD7A-antibodies were detected in some MN cases considered to be “secondary” to autoimmunity or cancer.

Abstract:

Background: Pathogenic autoantibodies against thrombospondin type-1 domain 7A (THSD7A) are present in approximately 3% of patients with membranous nephropathy (MN). Compared to PLA2R antibodies, less is known about THSD7A autoantibodies (AB) due to the relative rarity and lack of a commercially available quantitative immunoassay. Methods: Here we describe the development and validation of a highly quantitative, luciferase immunoprecipitation systems (LIPS) assay for detecting THSD7A AB and used it to study dominant THSD7A epitopes, disease associations and monitoring disease activity. The Department of Defense Serum Repository (DODSR) was then used to analyze THSD7A AB in 371 longitudinal serum samples collected before clinical diagnosis of MN from 110 PLA2R-negative MN subjects. Results: LIPS analysis demonstrated that a near full-length THSD7A (amino acids 1-1656) detected robust autoantibody levels in all known seropositive MN patients with 100% sensitivity and specificity compared to ELISA and/or Western blotting. The majority of THSD7A seropositive subjects in our pilot cohort had evidence of coexisting autoimmunity or cancer. Moreover, three THSD7A seropositive cases undergoing immunosuppressive therapy showed longitudinal autoantibody levels that tracked clinical status. Additional epitope analysis of two smaller protein THSD7A fragments spanning amino acids 1-416 and 1-671 demonstrated lower sensitivity of 32% and 44%, respectively. In the DODSR cohort, THSD7A seropositivity was detected in 4.5% of PLA2R negative MN cases. In one “primary” and in one secondary MN-associated with cancer, THSD7A AB were detectable less than one month prior to biopsy-proven diagnosis. In addition, 3 cases of lupus membranous nephropathy had detectable THSD7A AB years before hypoalbuminemia and biopsy-proven diagnosis. Conclusions: While further studies are needed to explore the significance of THSD7A AB in lupus membranous nephropathy, this study describes a novel, highly sensitive LIPS immunoassay for detecting THSD7A AB and adds to the existing literature on THSD7A-associated MN.

Disclosures: P. Burbelo reports the following: Patents or Royalties: NIH, HHS. L. Beck reports the following: Consultancy: Alexion, Novartis, Visterra, Ionis; Research Funding: Pfizer; Honoraria: UpToDate, Inc.; Patents or Royalties: I am a co-inventor on and receive royalties related to the US patent “Diagnostics for Membranous Nephropathy”; and Advisory or Leadership Role: Kidney Medicine editorial board. J. Keller reports the following: Employer: BioNTech SE. G. Lambeau reports the following: Patents or Royalties: Euroimmun DE. S. Olson reports the following: Employer: Novartis employee (4/2021). D. Schwartz reports the following: Other Interests or Relationships: I have an agreement to consult through Guidepoint Consulting on intracellular signal transduction and cytokines. I have not consulted through this company or other companies in the last 12 months. The remaining authors have nothing to disclose.

Funding: HHS | National Institutes of Health (NIH): Peter D. Burbelo, Meryl Waldman, Z99 DE999999; HHS | NIH | Office of Extramural Research, National Institutes of Health (OER): Laurence H. Beck, DK097053; Agence Nationale de la Recherche (ANR): Gérard Lambeau, ANR-20-CE14-0024-01 to G.L.; Fondation de la Recherche Medicale; DEQ20180339193

Author Contributions: Peter Burbelo: Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review and editing Stephen Olson: Conceptualization; Data curation; Supervision; Writing - review and editing Jason Keller: Methodology; Writing - review and editing Megha Joshi: Investigation; Resources Daniella Schwartz: Resources; Writing - review and editing Yung-Jen Chuang: Resources; Writing - review and editing Gérard Lambeau: Investigation; Writing - review and editing Laurence Beck: Investigation; Writing - review and editing Meryl Waldman: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Project administration; Supervision; Writing - original draft; Writing - review and editing

Data Sharing Statement: All data is included in the manuscript and/or supporting information.

Clinical Trials Registration: Yes

Registration Number: NCT00977977

Registration Date: 9/16/2009

The information on this cover page is based on the most recent submission data from the authors. It may vary from the final published article. Any fields remaining blank are not applicable for this manuscript.

Prediagnostic Appearance of THSD7A Autoantibodies in Membranous Nephropathy

Peter D. Burbelo^{1*}, Stephen W. Olson², Jason M. Keller¹, Megha Joshi², Daniella M. Schwartz³, Yung-Jen Chuang⁴, Gérard Lambeau⁵, Laurence H. Beck Jr⁶ and Meryl Waldman^{7*}

¹National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, 20892

²Nephrology Department, Walter Reed National Military Medical Center, Bethesda, Maryland, MD 20889.

³University of Pittsburgh Medical Center, Division of Rheumatology and Clinical Immunology, Pittsburgh, PA 15213.

⁴School of Medicine, National Tsing Hua University, Hsinchu, Taiwan, R.O.C.

⁵Université Côte d'Azur, Centre National de la Recherche Scientifique, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne Sophia Antipolis, France.

⁶Department of Medicine, Section of Nephrology, Boston University School of Medicine, Boston, MA, 02118.

⁷Kidney Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892.

*Corresponding Authors: email: Burbelop@nidcr.nih.gov and Waldmanm@niddk.nih.gov

Key points:

- The entire extracellular domain of THSD7A in the LIPS immunoassay was required to detect autoantibodies with high sensitivity in MN.
- In THSD7A seropositive MN patients, changes in antibody levels precedes a change in clinical status.
- Seropositive THSD7A antibodies were detected in some MN cases considered to be “secondary” to autoimmunity or cancer.

Abstract

Background: Pathogenic autoantibodies against thrombospondin type-1 domain 7A (THSD7A) are present in approximately 3% of patients with membranous nephropathy (MN). Compared to PLA2R antibodies, less is known about THSD7A autoantibodies (AB) due to the relative rarity and lack of a commercially, available quantitative immunoassay.

Methods: Here we describe the development and validation of a highly quantitative, luciferase immunoprecipitation systems (LIPS) assay for detecting THSD7A AB and used it to study dominant THSD7A epitopes, disease associations and monitoring disease activity. The Department of Defense Serum Repository (DODSR) was then used to analyze THSD7A AB in 371 longitudinal serum samples collected before clinical diagnosis of MN from 110 PLA2R-negative MN subjects.

Results: LIPS analysis demonstrated that a near full-length THSD7A (amino acids 1–1656) detected robust autoantibody levels in all known seropositive MN patients with 100% sensitivity and specificity compared to ELISA and/or Western blotting. The majority of THSD7A seropositive subjects in our pilot cohort had evidence of coexisting autoimmunity or cancer. Moreover, three THSD7A seropositive cases undergoing immunosuppressive therapy showed longitudinal autoantibody levels that tracked clinical status. Additional epitope analysis of two smaller protein THSD7A fragments spanning amino acids 1–416 and 1–671 demonstrated lower sensitivity of 32% and 44%, respectively. In the DODSR cohort, THSD7A seropositivity was detected in 4.5% of PLA2R negative MN cases. In one “primary” and in one secondary MN-associated with cancer, THSD7A AB were detectable less than one month prior to biopsy-proven

diagnosis. In addition, 3 cases of lupus membranous nephropathy had detectable THSD7A AB years before hypoalbuminemia and biopsy-proven diagnosis.

Conclusions: While further studies are needed to explore the significance of THSD7A AB in lupus membranous nephropathy, this study describes a novel, highly sensitive LIPS immunoassay for detecting THSD7A AB and adds to the existing literature on THSD7A-associated MN.

Introduction

Membranous nephropathy (MN) is an autoimmune disease affecting the kidneys and a leading cause of adult nephrotic syndrome (1). In MN, autoantibodies directed against extracellular proteins expressed by podocytes form electron dense immune deposits in the kidney (2). Before the discovery of MN antigens, MN was classified as “primary” or “idiopathic” when an etiology could not be identified and “secondary” when another condition (e.g., systemic autoimmune disease, malignancy, infection) was identified as the cause. The discovery of numerous target autoantigens has transformed how we classify patients with MN (3). The first major antigenic target, M-type phospholipase A₂ receptor (PLA2R), to which 70-80% of patients with “primary” MN develop autoantibodies, was discovered in 2009 (4). Autoantibodies against a second podocyte protein, thrombospondin type-1 domain-containing 7A (THSD7A), was identified in 2014 (5) and are present in 2–10% MN patients (6, 7). Due to widely available PLA2R immunoassays, autoantibody titers and trajectory have been well described before and at MN diagnosis, as well as after treatment and relapse and now inform diagnosis, management, and prognosis in PLA2R-associated MN (8). In contrast, fewer data exist for clinical applications of THSD7A AB testing. This is due to a combination of low seroprevalence and lack of a widely available commercial assays, in part due to the relatively large size of the THSD7A protein required for autoantibody screening. Current THSD7A AB assays including Western blot and the semi-quantitative indirect immunofluorescence assay, have limitations (5, 9, 10). A quantitative ELISA for detecting THSD7A AB is currently not commercially available and in limited use (6).

We previously developed highly sensitive and specific LIPS assays, based on recombinant luciferase-tagged antigen, to detect PLA2R AB (11, 12). In the current study, a LIPS immunoassay was developed for quantitative measurements of THSD7A AB and used to describe the trajectory and dynamics of autoantibody levels before MN diagnosis in pre-clinical phase, at diagnosis, after treatment, and before relapse.

Material and Methods

Clinical Trial Registration Number: NCT00977977; Registration Date: 9/16/2009

Patients and Samples

The study screened 502 patient sera for THSD7A AB from three different cohorts (**Figure. 1**). A pilot cohort consisted of de-identified patient samples collected at the National Institutes of Health, Bethesda, MD, under protocols approved by the IRB of the National Institute of Diabetes and Digestive and Kidney Diseases. This cohort established the optimal THSD7A AB assay to maximize assay sensitivity and specificity. The pilot cohort included a total of 96 samples, from 34 untreated PLA2R seropositive and 35 PLA2R seronegative MN subjects and 27 disease controls with other glomerular diseases. THSD7A AB status was assigned for each sample before unblinding. After unblinding, samples identified as THSD7A seropositive by LIPS were confirmed by Western Blot for THSD7A AB and/or by THSD7A tissue staining of renal biopsy. LIPS was also used for quantification of serial autoantibody levels in THSD7A seropositive subjects in the pilot cohort undergoing immunosuppressive treatment.

A validation cohort consisted of samples provided by L. B. (Boston University) was used to assess the diagnostic performance of the assay and contained 35 blinded serum samples from MN subjects previously classified by Western blot as THSD7A seropositive (n=25) or seronegative (n=10). Antibody levels based on LIPS in 24 THSD7A seropositive samples were also compared to the IgG4 anti-THSD7A ELISA (6).

The third cohort was from the Department of Defense Serum Repository (DoDSR) (protocol #0042, Walter Reed National Military Medical Center). We previously reported the emergence

of prediagnostic PLA2R antibody in longitudinal, DODSR biobanked, serum samples collected before MN diagnosis (12). For the present study, samples previously identified as PLA2R AB negative were analyzed for THSD7A autoantibodies prior to the diagnosis of MN based on renal biopsy. In total, 371 longitudinal samples from 75 cases classified as “primary” membranous nephropathy and 35 with “secondary” membranous nephropathy were evaluated in a single blinded manner. For the DODSR cohort, the terms “primary” and “secondary” were used because sample collection and classification in this database predates identification of MN antigens. Seropositive and seronegative status was assigned for each sample before unblinding. The measurements of serum albumin measurements were also evaluated as previously described (12).

THSD7A Luciferase Fusion Proteins for LIPS Autoantibody Testing

To study THSD7A AB, a near full-length protein (1656/1657 amino acids) along with two protein fragments were developed as *Gaussia* luciferase fusion proteins for LIPS. For plasmid construction, a clone containing the entire human THSD7A cDNA clone (13) was used as template for PCR and the cDNA was subcloned as an N-terminal fusion protein with *Gaussia* luciferase. Two THSD7A fragments encompassing amino acid residues 1–416 and 1–671 were also generated in a similar fashion. While a general protocol describing LIPS testing is available (14), a detailed LIPS methodology used to study THSD7A antibodies is provided in Supplemental Information 1. A small number of additional LIPS immunoassays for measuring autoantibodies against known lupus and other autoimmune targets, including Ro52, Ro60, RNP-A, Smith-D3, GAD65, and interferon-alpha, were tested in selected patients and have been described previously (15).

Statistics

GraphPad Prism software (San Diego, CA) was used to analyze and plot autoantibody measurement data. Cut-off values for determining THSD7A seropositivity was based on optimal separation using receiver operator characteristics (ROC) analysis. The heatmap was constructed based on Z-scores of THSD7A seropositive antibody levels based on the THSD7A seronegative controls as described (15).

Results

Detection of THSD7A AB by LIPS in a Pilot Cohort

A near full-length THSD7A chimeric fusion protein with *Gaussia* luciferase was used in LIPS to evaluate autoantibodies in a pilot cohort. After decoding, the control group (n=27) all had low THSD7A AB levels with an average value of approximately 10,000 LU (**Figure 2**). Similarly low autoantibody levels were found in 34 (out of 34) known PLA2R seropositive and 30 (out of 35) PLA2R seronegative MN subjects (**Figure 2**). Five PLA2R seronegative subjects showed higher seropositive THSD7A AB levels (**Figure 2**). Three of these samples showed approximate values of 215,000 LU, 220,000 LU and 313,000 LU, one had modest autoantibody levels (125,000 LU) and one had a lower value of 35,000 LU. Western blot and/or tissue staining of the renal biopsy from the five subjects confirmed THSD7A seropositivity. Using ROC analysis, a tentative cut-off value of 30,000 LU was chosen and yielded 100% sensitivity and specificity for detecting all five subjects.

Clinical data for the five THSD7A seropositive subjects revealed three females and two males with a mean age at presentation of 42 ± 8 years (**Table 1**). Mean proteinuria at diagnosis was 11.6 ± 4 grams and eGFR was 82 ± 30 ml/min/1.73m². Subject 1 had onset of nephrotic syndrome during the third trimester of pregnancy and underwent renal biopsy after delivery. Subject 4 was diagnosed with MN shortly after delivering a baby complicated by eclampsia, but the timing of renal disease onset was unknown. In addition, three patients had coexisting autoimmune or inflammatory disorders: lupus, heterozygous loss-of-function *TNFAIP3* gene mutation causing A20 haploinsufficiency (HA20) and refractory pustular psoriasis.

All five THSD7A seropositive subjects were PLA2R seronegative. To explore coexistence of other antibodies, these subjects were tested for autoantibodies against a limited panel of other autoantigens associated with systemic lupus erythematosus and other autoimmune diseases (15). Subject 2 harbored Ro52, Ro60, RNP-A and high level GAD65 autoantibodies and subject 1 with HA20, harbored Ro52, Ro60, RNP-A and interferon-alpha1 autoantibodies. These findings highlight the diversity of other autoantigen targets seen in these subjects (**Table 1**). Lastly, treatment and outcomes of these patients are shown in Table 1.

THSD7A AB LIPS Testing a Validation Cohort

The LIPS assay was next used to assess an independent, validation MN cohort. Based on the cut-off value above 30,000 LU, 25 cases were assigned as THSD7A seropositive and 10 cases as seronegative (**Figure 3**). After unblinding, the THSD7A serological status in these samples demonstrated 100% sensitivity and specificity as compared to Western blot analysis. Additional comparisons revealed LIPS closely tracked (Pearson $R=0.58$, $p=0.017$) the THSD7A IgG4 levels determined by ELISA (data not shown).

Immunoreactive Regions of THSD7A Protein

A previous study identified an important antigenic region located in the N-terminus of the THSD7A molecule (14). To investigate immunoreactivity of different regions of THSD7A, two additional smaller fragments, THSD7A- $\Delta 1$ (amino acids 1-416), and THSD7A- $\Delta 2$, (amino acids 1-671), were tested with the validation cohort and the results was compared with full-length THSD7A protein (**Figure 4A**). A heat map normalized to the 10 seronegative cases was used to visualize immunoreactivity in the 25 MN subjects (**Figure 4B**). The smallest fragment

THSD7A- Δ 1 fragment detected only 33% (8/25) of the MN samples as seropositive. The larger THSD7A- Δ 2 fragment was slightly more informative detecting 45% (11/25) as seropositive. While many of the THSD7A- Δ 1 and THSD7A- Δ 2 seropositive cases overlapped, three seropositive subjects detected by the smaller THSD7A- Δ 1 protein fragment were seronegative with the larger THSD7A- Δ 2 fragment. Overall, these results suggest there are multiple antigenic regions within the protein, yet the near full-length THSD7A is the most informative, showing 100% sensitivity, because it likely captures important immunoreactive targets in the C-terminal part of the molecule.

Monitoring Treatment Response by THSD7A AB Detection

THSD7A AB were next evaluated in longitudinal samples from three MN subjects (2, 4 and 5) in the pilot cohort who receiving treatment. Before treatment all three patients had sustained high levels of THSD7A antibody and proteinuria. Treatment of subject 2 with cyclophosphamide and rituximab led to reduced THSD7A autoantibodies over 12 months paralleling the decrease in proteinuria (**Figure 5A**). By 8 months, THSD7A AB levels decreased below the cut-off value, corresponding to partial remission of proteinuria and proteinuria nadired at 0.355 g/day by 12 months. Subject 4 responded to rituximab monotherapy and showed a similar pattern of antibody depletion in advance of proteinuria improvement (**Figure 5B**). In subject 5, THSD7A AB was fully depleted below the cut-off value by six months after rituximab and cyclosporine which preceded complete remission (based on proteinuria) by three months (**Figure 5C**). Autoantibody reemergence occurred at 48 months and retreatment with rituximab led to clinical and immunologic remission. Two additional relapses occurred in subject 5 with similar patterns in THSD7A AB and proteinuria (**Figure 5C**). Immunological relapse was apparent five months in

advance of clinical relapse. It is notable that seropositivity at the earliest time of presentation was detectable with large THSD7A protein, as well as the two fragments, but with relapse, autoantibodies were only detectable with near full-length protein (**data not shown**). Overall, these results demonstrate that the LIPS THSD7A immunoassay is a sensitive method for monitoring clinically informative changes in autoantibodies.

THSD7A AB Before Disease Diagnosis

The emergence of PLA2R AB was previously studied in retrospective, biobanked serum MN samples from DODSR (12). In the current study, previously identified PLA2R seropositive patients from the DODSR cohort were excluded, and the remainder were evaluated for THSD7A AB. Of 371 PLA2R seronegative samples, fifteen sera were THSD7A seropositive and after decoding, were found to comprise five subjects from screening 75 “primary” MN, and 35 secondary MN. Thus 4.5% (5/110) of the PLA2R-negative MN cohort were THSD7A seropositive. One case was classified as “primary” MN and another was classified as “cancer-associated secondary MN and three (2.7%) had lupus membranous nephropathy (LMN) and had been classified at “secondary MN”. Serum albumin levels in serial samples were determined and plotted over time with the THSD7A AB levels (**Figure 6**). The subjects with primary (**Figure 6A**) and cancer-associated MN (**Figure 6B**), were seropositive for THSD7A AB only in the last longitudinal sample (also confirmed positive by THSD7A IgG4 ELISA) less than one month prior to histologic diagnosis and showed co-occurrence of hypoalbuminemia. In contrast, seropositive THSD7A AB were detected in three subjects with LMN up to 5 years before histologic diagnosis (**Figure 6C-E**). Appearance of THSD7A AB preceded hypoalbuminemia in LMN subjects 8 and 10. In LMN subject 9, THSD7A seropositivity fluctuated over time (**Figure**

6D) appearing to show a subclinical presentation of LMN manifesting as emergence of THSD7A AB with hypoalbuminemia, followed by immunologic and clinical remission and then “relapse” prior to biopsy diagnosis of LMN. Autoantibodies against three lupus-associated autoantigens were assessed in subjects 8-10 (**Figure 7**). In subject 8, Ro52, Ro60 and THSD7A AB were present at the first time point and remained positive. Subject 9 was seronegative for Ro52 and Ro60 autoantibodies at the first measured time point, but seropositive in the second and last time point concurrent with the appearance of THSD7A AB (**Figure 7**). Subject 10 was seronegative for the three circulating lupus autoantibodies.

Discussion

In this study, we developed and validated a LIPS THSD7A AB immunoassay based on luciferase fusion proteins. A near full-length THSD7A recombinant protein was chosen for the immunoassay after detailed investigation. While the exact form, intact versus proteolyzed and immunoreactive regions of THSD7A are not well-established, mapping revealed that multiple regions are targeted by autoantibodies. An N-terminal THSD7A fragment containing the first 416 amino acid showed only limited sensitivity (33%) for detecting seropositivity and a larger N-terminal fragment only improved sensitivity marginally to 45%. The highest sensitivity of 100% required the entire extracellular domain. These findings are partly consistent with Seifert et al. (16), which used Western blotting and found 87% of subjects had autoantibodies that recognized amino acids 48–192 of THSD7A. One potential explanation for this discrepancy is the more denaturing methods such as Western blot or ELISA may expose epitopes not available to antibody binding to the native THSD7A molecule. Further evidence for the importance of the C-terminus region in LIPS testing was detected in a THSD7A treated patient, in which autoantibodies were present early in the course of the disease against near full-length and two protein fragments, but later in the course of disease, immunoreactivity was only seen against the full-length protein. These results suggest that epitope spreading may occur during disease progression and/or response to treatment.

THSD7A AB showed similar dynamics to PLA2R AB, in which levels declined with immunosuppressive treatment, disappeared with clinical remission, and reappearance preceded proteinuria and predicted relapse indicating that THSD7A AB levels are a good clinical marker of disease activity consistent with other studies (6, 17, 18). By analyzing longitudinal DODSR

samples, we provide the first description of the natural history of THSD7A AB emergence before diagnosis of MN. THSD7A AB was detected only within one month of the diagnosis for both “primary” MN and malignancy-associated MN, but the limited patient number and timing of samples precludes firm conclusions. In contrast, THSD7A AB were detected in three patients with LMN years before diagnosis and were elevated prior to prediagnostic hypoalbuminemia. In two LMN cases, the appearance of THSD7A AB was concurrent with other lupus-associated antibodies. In one subject, subclinical disease “flare” manifesting as transient THSD7A seropositivity and hypoalbuminemia was evident years before formal diagnosis. We previously reported similar episodes of undiagnosed subclinical flares in PLA2R-associated MN (12).

THSD7A seropositivity was 5.2% in the pilot cohort and 4.5% of PLA2R seronegative patients in the DODSR cohort. Among these ten THSD7A seropositive MN cases, 70% had evidence of a secondary comorbidity or systemic autoimmunity. There was one case of MN (0.9%) associated with malignancy in the DODSR cohort. THSD7A-MN has been associated with an increased risk of cancer compared to PLA2R-associated MN (19). It is notable that THSD7A AB were not detected earlier in this case of cancer-associated MN, which might have been expected if a link existed between a THSD7A-expressing tumor and autoantibody generation.

THSD7A positive MN has been associated with a variety of other diseases (20, 21). In the pilot cohort, several MN cases were considered secondary to another disease. We report the first case of THSD7A-associated MN potentially related to haploinsufficiency in A20 (HA20), an autoinflammatory condition manifesting as genital, oral and/or gastrointestinal ulcers (22), related to enhanced NFκB (23). Treatment of the underlying disease led to remission of

proteinuria supporting a link between HA20 and development of MN. One subject also had psoriasis, which has been associated with MN. Treatment of the underlying skin disease did not lead to improvement in proteinuria. This may reflect the severe, refractory nature of the skin disease or suggests coincidental occurrence of two diseases. In support of the latter, a study of 24 MN cases with psoriasis did not show evidence of glomerular expression of PLA2R or THSD7A or circulating autoantibodies (24). Interestingly in the pilot cohort, two cases of THSD7A-associated MN were diagnosed shortly after pregnancy. THSD7A-associated MN during pregnancy has been reported (25). Since THSD7A is expressed in placental vasculature and umbilical vein endothelial cells (13), these sites could be a source of extrarenal expression leading to autoantibody production, but this is purely speculation.

Four of the ten THSDA seropositive cases were LMN. THSD7A antibodies have only rarely been associated with class V LN (5), whereas exostosins 1 and 2, NCAM1, and TGF- β receptor 3 are well recognized target autoantigens in MLN (26-28). Reasons for the higher-than-expected prevalence of THSD7A AB in LMN in our study are unclear but may be due to misclassification of cases in the DODSR cohort or differences in detection method (THSD7A renal antigen expression versus LIPS), as well as assay error. Nevertheless, the simultaneous appearance of lupus autoantibodies with THSD7A AB supports the validity of our findings.

There are several limitations of this study mainly due to inherent drawbacks of the DODSR cohort (12). As it relies on historical charting, there is potential for misclassification of MN cases as primary versus secondary and misattribution to the secondary cause. The time intervals between samples and diagnosis were variable for each case and limited, which may have led to

underestimation of seropositive cases. Another limitation is the small percentage of detectable THSD7A seropositive cases, similar to previous reports. Further studies are needed to better understand THSD7A AB contribution to primary MN, LMN, and other secondary forms of MN, as well as describe how genetics and the dynamics of the autoantibody may impact disease presentation and outcome. Overall, the LIPS technology appears well-suited for the development of a panel of MN autoantigens in a high-throughput format that could serve as non-invasive biomarkers to facilitate diagnosis without a kidney biopsy, an approach currently supported by the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines for PLA2R associated MN.

Disclosures

P. Burbelo reports the following: Patents or Royalties: NIH, HHS. L. Beck reports the following: Consultancy: Alexion, Novartis, Visterra, Ionis; Research Funding: Pfizer; Honoraria: UpToDate, Inc.; Patents or Royalties: I am a co-inventor on and receive royalties related to the US patent "Diagnostics for Membranous Nephropathy"; and Advisory or Leadership Role: Kidney Medicine editorial board. J. Keller reports the following: Employer: BioNTech SE. G. Lambeau reports the following: Patents or Royalties: Euroimmun DE. S. Olson reports the following: Employer: Novartis employee (4/2021). D. Schwartz reports the following: Other Interests or Relationships: I have an agreement to consult through Guidepoint Consulting on intracellular signal transduction and cytokines. I have not consulted through this company or other companies in the last 12 months. The remaining authors have nothing to disclose.

Funding

This work was supported by the intramural research programs of the National Institute of Dental and Craniofacial Research and National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (Z99 DE999999), as well as an extramural grant DK097053 to LHB. This work was supported by the Agence Nationale de la Recherche (ANR-20-CE14-0024-01 to G.L.) and the Fondation de la Recherche Médicale (DEQ20180339193 to GL).

Author Contributions

Peter Burbelo: Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review and editing. Stephen Olson: Conceptualization; Data curation; Supervision; Writing - review and editing. Jason Keller: Methodology; Writing - review and editing. Megha Joshi:

Investigation; Resources Daniella Schwartz: Resources; Writing - review and editing. Yung-Jen Chuang: Resources; Writing - review and editing. Gérard Lambeau: Investigation; Writing - review and editing. Laurence Beck: Investigation; Writing - review and editing. Meryl Waldman: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Project administration; Supervision; Writing - original draft; Writing - review and editing.

Data Sharing Statement

All data is included in the manuscript and/or supporting information.

Supplemental Materials

Luciferase Immunoprecipitation Systems (LIPS) Methodology

Fig 1A. Schematic of the construction and expression for generating the THSD7A-luciferase recombinant protein.

Fig. 1B Schematic overview of the LIPS assay.

Supplemental references

References

1. Couser, W.G. Primary Membranous Nephropathy. *Clin J Am Soc Nephrol*, 2017. 12(6): 983-997. doi: 10.2215/CJN.11761116
2. Glassock, R.J. The pathogenesis of idiopathic membranous nephropathy: a 50-year odyssey. *Am J Kidney Dis*, 2010. 56(1): 157-67. doi: 10.1053/j.ajkd.2010.01.008
3. Ronco, P. Debiec, H. Membranous nephropathy: current understanding of various causes in light of new target antigens. *Curr Opin Nephrol Hypertens*, 2021. 30(3): 287-293. doi: 10.1097/MNH.0000000000000697
4. Beck, L.H., Jr., Bonegio, R.G., Lambeau, G., Beck, D.M., Powell, D.W., Cummins, T.D., et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. *N Engl J Med*, 2009. 361(1): 11-21. doi: 10.1056/NEJMoa0810457
5. Tomas, N.M., Beck, L.H., Jr., Meyer-Schwesinger, C., Seitz-Polski, B., Ma, H., Zahner, G., et al. Thrombospondin type-1 domain-containing 7A in idiopathic membranous nephropathy. *N Engl J Med*, 2014. 371(24): 2277-2287. doi: 10.1056/NEJMoa1409354
6. Zaghrini, C., Seitz-Polski, B., Justino, J., Dolla, G., Payre, C., Jourde-Chiche, N., et al. Novel ELISA for thrombospondin type 1 domain-containing 7A autoantibodies in membranous nephropathy. *Kidney Int*, 2019. 95(3): 666-679. doi: 10.1016/j.kint.2018.10.024
7. Ren, S., Wu, C., Zhang, Y., Wang, A.Y., Li, G., Wang, L., et al. An update on clinical significance of use of THSD7A in diagnosing idiopathic membranous nephropathy: a systematic review and meta-analysis of THSD7A in IMN. *Ren Fail*, 2018. 40(1): 306-313. doi: 10.1080/0886022X.2018.1456457
8. De Vriese, A.S., Glassock, R.J., Nath, K.A., Sethi, S., Fervenza, F.C. A Proposal for a Serology-Based Approach to Membranous Nephropathy. *J Am Soc Nephrol*, 2017. 28(2): 421-430. doi: 10.1681/ASN.2016070776
9. Reinhard, L., Thomas, C., Machalitz, M., Lattwein, E., Weiss, L.S., Vitu, J., et al. Characterization of THSD7A-antibodies not binding to glomerular THSD7A in a patient with diabetes mellitus but no membranous nephropathy. *Sci Rep*, 2021. 11(1): 16188. doi: 10.1038/s41598-021-94921-y
10. Hoxha, E., Beck, L.H., Jr., Wiech, T., Tomas, N.M., Probst, C., Mindorf, S., et al. An Indirect Immunofluorescence Method Facilitates Detection of Thrombospondin Type 1 Domain-Containing 7A-Specific Antibodies in Membranous Nephropathy. *J Am Soc Nephrol*, 2017. 28(2): 520-531. doi: 10.1681/ASN.2016010050
11. Burbelo, P.D., Beck, L.H., Jr., Waldman, M. Detection and monitoring PLA2R autoantibodies by LIPS in membranous nephropathy. *J Immunol Methods*, 2017. 444: 17-23. doi: 10.1016/j.jim.2017.02.001
12. Burbelo, P.D., Joshi, M., Chaturvedi, A., Little, D.J., Thurlow, J.S., Waldman, M., et al. Detection of PLA2R Autoantibodies before the Diagnosis of Membranous Nephropathy. *J Am Soc Nephrol*, 2020. 31(1): 208-217. doi: 10.1681/ASN.2019050538
13. Wang, C.H., Su, P.T., Du, X.Y., Kuo, M.W., Lin, C.Y., Yang, C.C., et al. Thrombospondin type I domain containing 7A (THSD7A) mediates endothelial cell migration and tube formation. *J Cell Physiol*, 2010. 222(3): 685-94. doi: 10.1002/jcp.21990
14. Burbelo, P.D., Ching, K.H., Klimavicz, C.M., Iadarola, M.J. Antibody profiling by Luciferase Immunoprecipitation Systems (LIPS). *J Vis Exp*, 2009(32). doi: 10.3791/1549
15. Ching, K.H., Burbelo, P.D., Tipton, C., Wei, C., Petri, M., Sanz, I., et al. Two major autoantibody clusters in systemic lupus erythematosus. *PLoS One*, 2012. 7(2): e32001. doi: 10.1371/journal.pone.0032001
16. Seifert, L., Hoxha, E., Eichhoff, A.M., Zahner, G., Dehde, S., Reinhard, L., et al. The Most N-Terminal Region of THSD7A Is the Predominant Target for Autoimmunity in THSD7A-Associated

- Membranous Nephropathy. *J Am Soc Nephrol*, 2018. 29(5): 1536-1548. doi: 10.1681/ASN.2017070805
17. Wang, J., Cui, Z., Lu, J., Probst, C., Zhang, Y.M., Wang, X., et al. Circulating Antibodies against Thrombospondin Type-I Domain-Containing 7A in Chinese Patients with Idiopathic Membranous Nephropathy. *Clin J Am Soc Nephrol*, 2017. 12(10): 1642-1651. doi: 10.2215/CJN.01460217
 18. Zhang, D., Zou, J., Zhang, C., Zhang, W., Lin, F., Jiang, G. Clinical and Histological Features of Phospholipase A2 Receptor-Associated and Thrombospondin Type-I Domain-containing 7A-Associated Idiopathic Membranous Nephropathy: A Single Center Retrospective Study from China. *Med Sci Monit*, 2018. 24: 5076-5083. doi: 10.12659/MSM.909815
 19. Hanset, N., Aydin, S., Demoulin, N., Cosyns, J.P., Castanares-Zapatero, D., Crott, R., et al. Podocyte Antigen Staining to Identify Distinct Phenotypes and Outcomes in Membranous Nephropathy: A Retrospective Multicenter Cohort Study. *Am J Kidney Dis*, 2020. 76(5): 624-635. doi: 10.1053/j.ajkd.2020.04.013
 20. Matsumoto, A., Matsui, I., Namba, T., Sakaguchi, Y., Mizuno, H., Shirayama, Y., et al. VEGF-A Links Angiolymphoid Hyperplasia With Eosinophilia (ALHE) to THSD7A Membranous Nephropathy: A Report of 2 Cases. *Am J Kidney Dis*, 2019. 73(6): 880-885. doi: 10.1053/j.ajkd.2018.10.009
 21. Chen, M., Zhang, L., Zhong, W., Zheng, K., Ye, W., Wang, M. Case Report: THSD7A-Positive Membranous Nephropathy Caused by Tislelizumab in a Lung Cancer Patient. *Front Immunol*, 2021. 12: 619147. doi: 10.3389/fimmu.2021.619147
 22. Schwartz, D.M., Blackstone, S.A., Sampaio-Moura, N., Rosenzweig, S., Burma, A.M., Stone, D., et al. Type I interferon signature predicts response to JAK inhibition in haploinsufficiency of A20. *Ann Rheum Dis*, 2020. 79(3): 429-431. doi: 10.1136/annrheumdis-2019-215918
 23. Aeschlimann, F.A., Batu, E.D., Canna, S.W., Go, E., Gul, A., Hoffmann, P., et al. A20 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly recognised NF-kB-mediated autoinflammatory disease. *Ann Rheum Dis*, 2018. 77(5): 728-735. doi: 10.1136/annrheumdis-2017-212403
 24. Ge, Y.C., Jin, B., Zeng, C.H., Zhang, M.C., Chen, D.C., Yin, R., et al. PLA2R antibodies and PLA2R glomerular deposits in psoriasis patients with membranous nephropathy. *BMC Nephrol*, 2016. 17(1): 185. doi: 10.1186/s12882-016-0407-3
 25. Iwakura, T., Fujigaki, Y., Katahashi, N., Sato, T., Ishigaki, S., Tsuji, N., et al. Membranous Nephropathy with an Enhanced Granular Expression of Thrombospondin Type-1 Domain-containing 7A in a Pregnant Woman. *Intern Med*, 2016. 55(18): 2663-8. doi: 10.2169/internalmedicine.55.6726
 26. Caza, T.N., Hassen, S.I., Kenan, D.J., Storey, A., Arthur, J.M., Herzog, C., et al. Transforming Growth Factor Beta Receptor 3 (TGFB3)-Associated Membranous Nephropathy. *Kidney360*, 2021. 2(8): 1275-1286. doi: 10.34067/KID.0001492021
 27. Caza, T.N., Hassen, S.I., Kuperman, M., Sharma, S.G., Dvanajscak, Z., Arthur, J., et al. Neural cell adhesion molecule 1 is a novel autoantigen in membranous lupus nephritis. *Kidney Int*, 2021. 100(1): 171-181. doi: 10.1016/j.kint.2020.09.016
 28. Ravindran, A., Casal Moura, M., Fervenza, F.C., Nasr, S.H., Alexander, M.P., Fidler, M.E., et al. In Patients with Membranous Lupus Nephritis, Exostosin-Positivity and Exostosin-Negativity Represent Two Different Phenotypes. *J Am Soc Nephrol*, 2021. 32(3): 695-706. doi: 10.1681/ASN.2020081181

Table

Table I Characteristics of THSD7A AB seropositive subjects

Subject	Age (yrs)	Gender	Race	SCr mg/dl	eGFR ml/min/1.73m ²	Proteinuria g/day	THSD7A Ab titer LU (LIPS) [‡]	Coexisting Disorders	Selected serologies (LIPS) ^Δ	Treatments/Outcome
1	29	F	White	0.7	120	4.2	35,036	*HA20	Ro52 Ro60 RNP-A IFN α	anakinra, tofacitinib PR; relapse after self discontinuation of medications
2	48	M	Black	1.1	83	16.9	215,884	Lupus	Ro52 Ro60 RNP-A GAD65	cytoxan, rituximab, corticosteroids; PR
3	46	M	White	0.8	111	13.4	221,519	refractory generalized pustular psoriasis	Negative	cyclosporine, anakinra NR, died
4	36	F	Black	1.3	55	13.7	135,806	eclampsia	Negative	Rituximab CR, sustained
5	51	F	Asian	1.5	42	9.8	313,858	-	Negative	rituximab + cyclosporine; CR, followed by relapse

F: female; M: male; SCr: serum creatinine; eGFR: CKD-EPI Creatinine (2021); ‡: cut-off value for LIPS assay seropositivity: 30,000 LU; *HA20: haploinsufficiency of A20 due to heterozygous loss of function mutation in TNFAIP; Δ: Selected serologies performed by LIPS: Ro52, Ro60, RNP-A, GAD65, IFN-alpha(α); PR: partial remission defined as >50 % reduction in peak proteinuria and <3.5 grams/day; CR: complete remission defined as proteinuria <300mg/day; NR: no response.

Figure Legends

Figure 1. Characteristics of the three cohorts screened for THSD7A AB by LIPS.

This study screened 502 patient serum samples for THSD7A AB from three different cohorts. The characteristics of each cohort is shown above. THSD7A AB status was assigned for each sample before unblinding. Three THSD7A seropositive cases from the pilot cohort who were treated were also monitored longitudinally for changes in autoantibody levels and clinical response.

Figure 2. LIPS detection of THSD7A AB in MN subjects and kidney disease controls.

Kidney disease controls (Kidney CTRLS), PLA2R seropositive and seronegative MN subjects were tested by LIPS with the near full-length THSD7A-*Gaussia* fusion protein. Each circle represents the antibody level in light units (LU) in individual serum sample and were plotted on the Y-axis using a \log_{10} scale. The cut-off value for the assay for determining THSD7A seropositive and seronegative status is shown by the dotted line.

Figure 3 Validation of the LIPS assay for detecting THSD7A AB in an independent cohort.

LIPS detection of THSD7A AB were evaluated in a validation cohort of blinded samples from known seronegative and seropositive MN patients. Classification status of seronegative (-) or seropositive (+) was based on THSD7A ELISA antibody testing and/or Western blot analysis. Each circle represents antibody levels in light units (LU) derived from individual subjects plotted on the Y-axis using a \log_{10} scale. The cut-off value is shown by the dotted line and LIPS testing showed 100% sensitivity and specificity.

Figure 4 Detection of patient seropositivity against multiple epitopes of THSD7A.

A. Schematic of THSD7A- Δ 1, THSD7A- Δ 2 and near full-length THSD7A (FL) chimeric Gaussia luciferase (Luc) fusion proteins used for testing the different MN serum samples. The location of the transmembrane region (amino acids 1608-1628) of THSD7A is shown in THSD7A-FL by the blue box. **B.** A heat map is shown of the autoantibody responses to the three different THSD7A proteins from the validation cohort, in which each row represents a different MN sample. To construct the heatmap, the autoantibody values for the 10 seronegative controls (not shown) and the THSD7A seropositive MN subjects were color-coded based on a Z-score scale shown on the right representing the number of standard deviations above the mean of the controls for that antigen. Coloring in the heat map indicates that the relative antibody levels are at least greater than the mean of the controls plus three standard deviations. The signal intensities range from white to dark red indicating low and high autoantibody levels, respectively.

Figure 5. Association between THSD7A AB and clinical disease activity in three MN subjects receiving immunosuppressive treatment. THSD7A AB levels (blue circles and lines) and proteinuria (black squares and lines) are shown over time. The red arrow denotes the start of treatment, in which all three patients had sustained high levels of THSD7A AB and proteinuria for months before treatment. The X-axis is the time in months after initiation of immunosuppressive treatment. The left Y-axis represents the scale of the autoantibody levels in LU determined by LIPS with the near full-length protein. The right Y-axis denotes the level of urinary protein excretion expressed as grams per day. The blue dotted line represents the

seropositive cut-off value for LIPS; the black dotted line represents complete remission status (proteinuria <300 mg/day).

Figure 6. Prediagnostic appearance of THSD7A AB before diagnosis. Autoantibody analysis of DODSR cohort identified five THSD7A seropositive subjects. The five cases included one with “primary” MN (A), one cancer-associated MN (B) and three with lupus membranous nephropathy (C-E). THSD7A autoantibody levels in LU derived from serial samples of the seropositive cases are plotted in blue. The blue, horizontal dotted line is the cut-off for determining THSD7A seropositivity. The right Y-axis denotes the serum albumin levels, and the cut-off value for determining hypoalbuminemia is shown by the black dotted line. Time zero represents biopsy-proven diagnosis of MN.

Figure 7. Prediagnostic appearance of THSD7A AB relative to appearance of Ro52 and Ro60 AB. From testing the three subjects with lupus membranous nephropathy, two (Pt 8 and Pt 9) were also seropositive for Ro52 and Ro60 AB. THSD7A autoantibody levels are plotted in blue. The Ro52 and Ro60 AB are shown in black, and the approximate cut-off value for seropositivity is shown by the red dotted line. The cut-off value for THSD7A AB seropositivity is shown with the dotted blue line.

Figure 1

27 Glomerular disease controls
34 PLA2R positive MN
35 PLA2R negative MN

Pilot Cohort (blinded) n=96

25 THSD7A positive
10 THSD7A negative

Validation Cohort (blinded) n=35

DODSR longitudinal Cohort (blinded)
from 75 PLA2R negative "Primary" MN
and 35 "Secondary" MN

Third Cohort (blinded) n=371

Figure 3

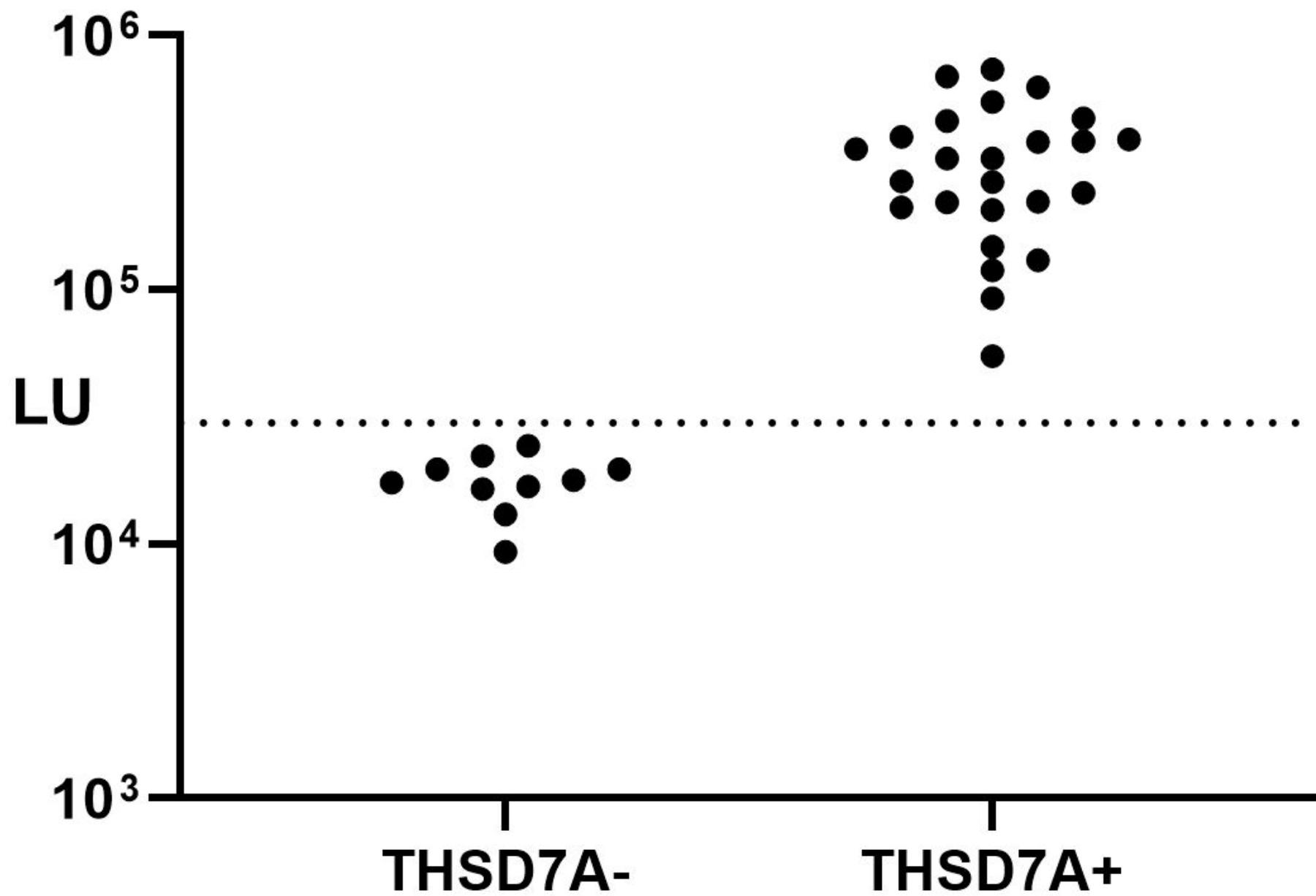


Figure 4

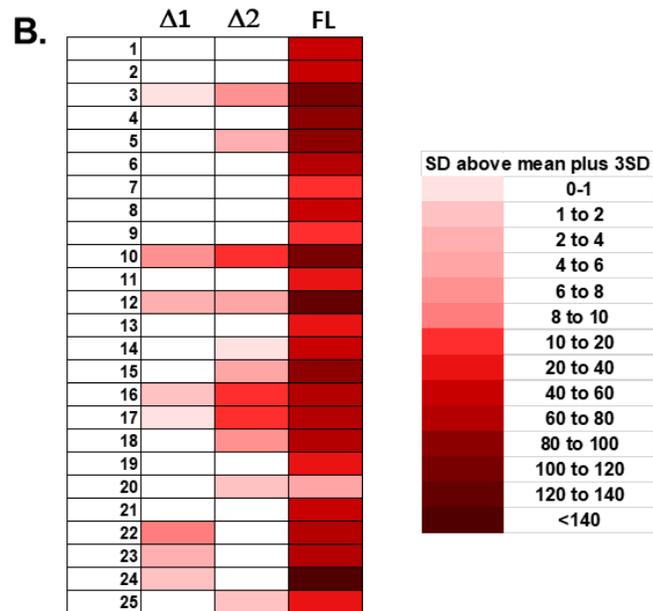
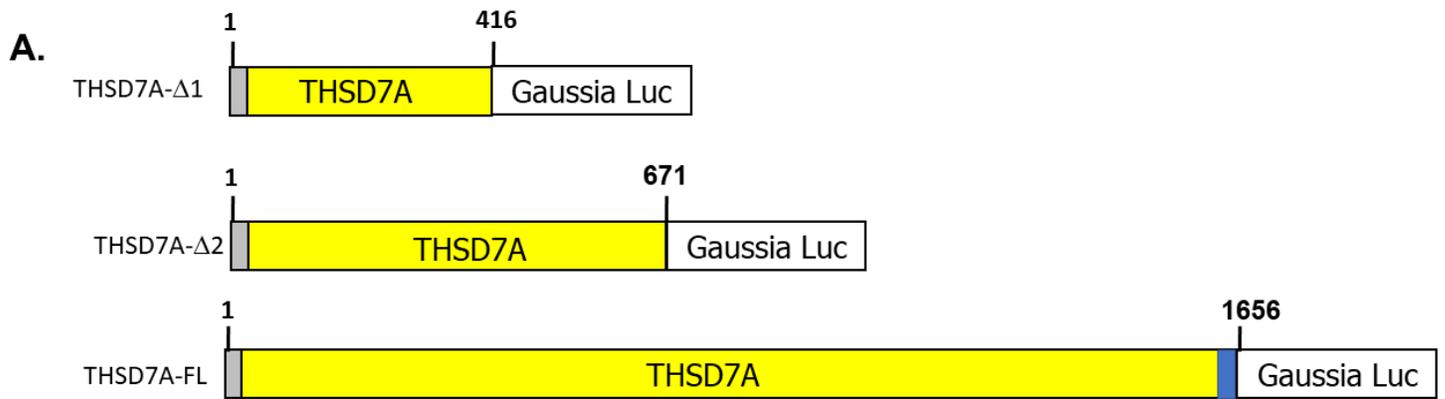
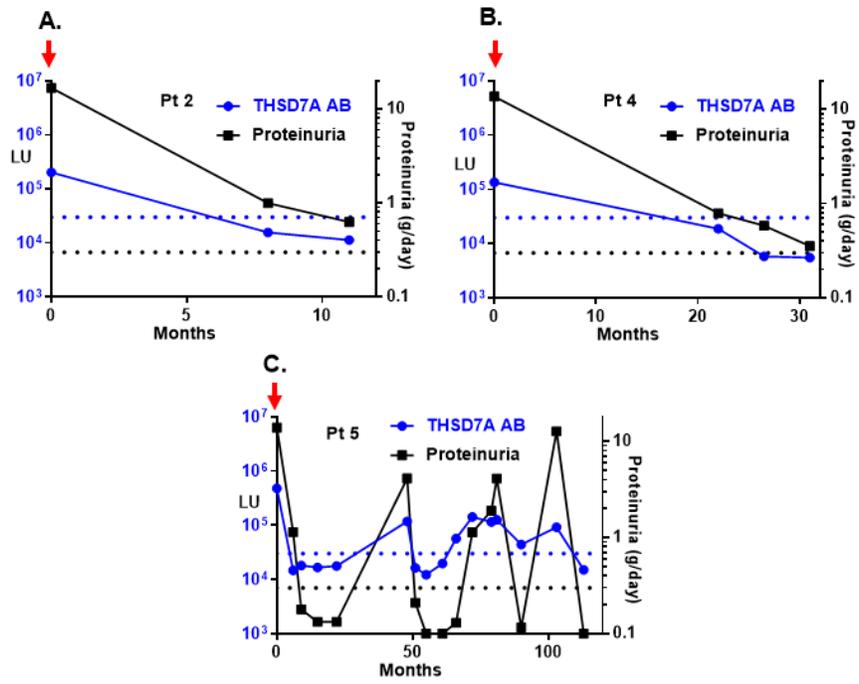


Figure 5



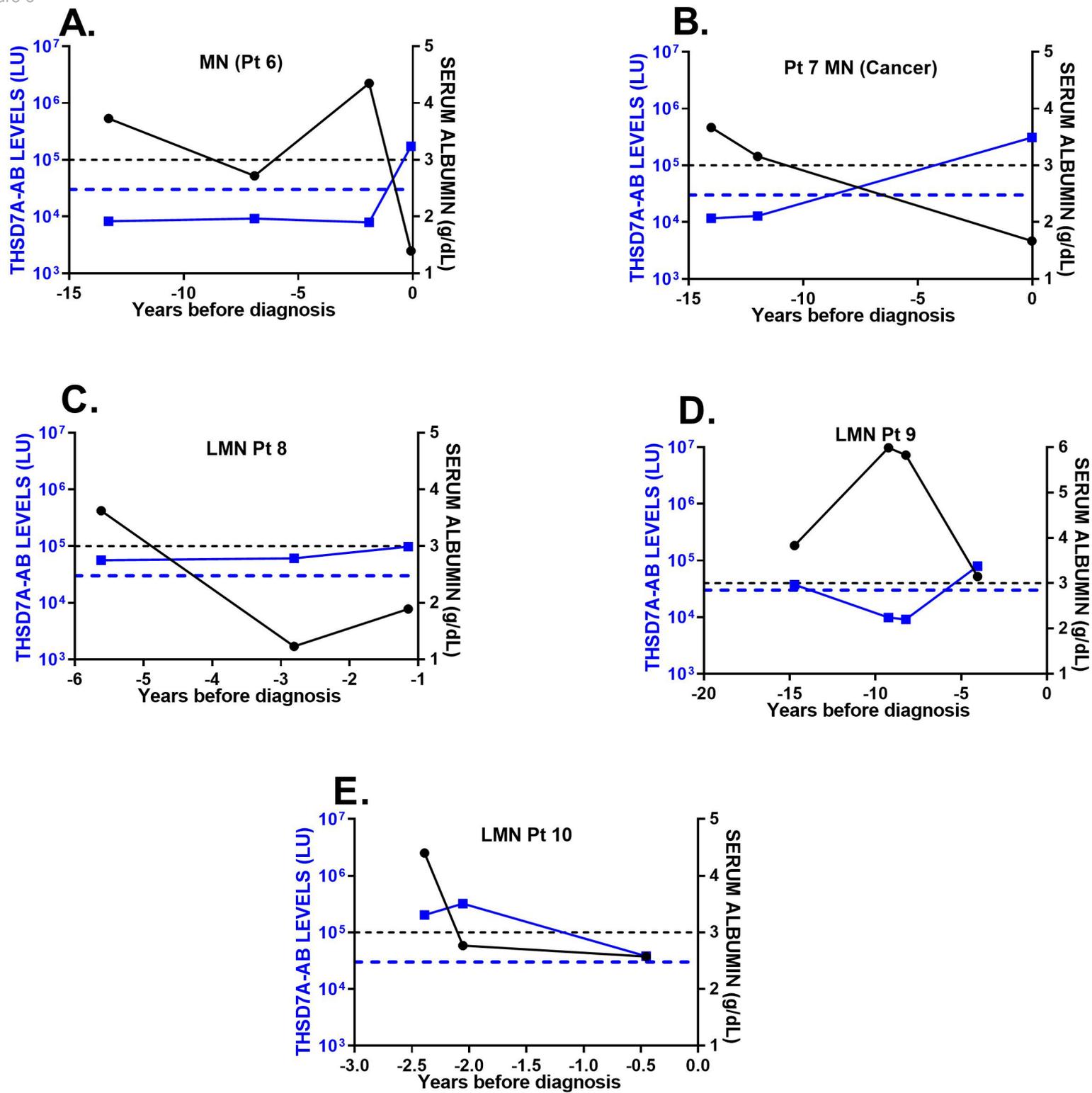
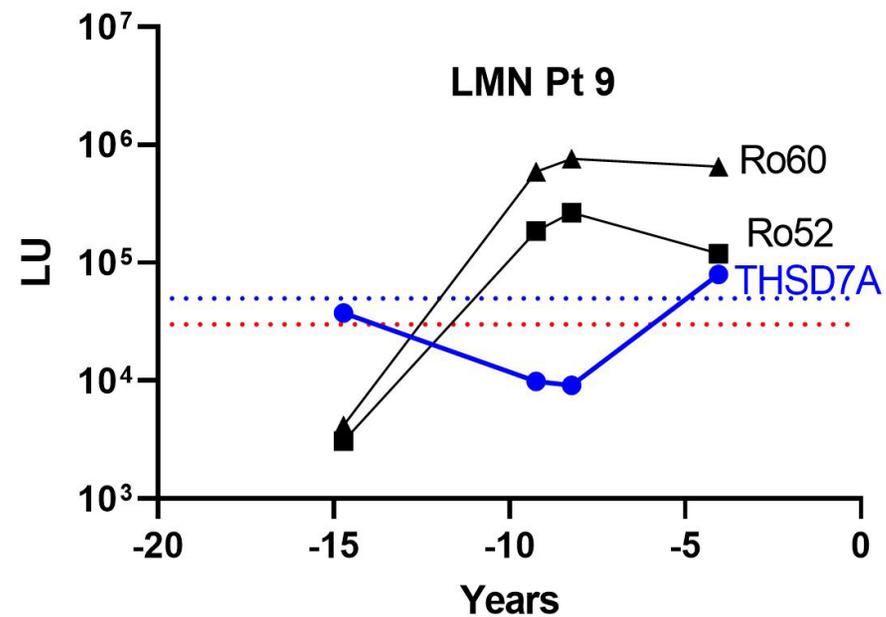
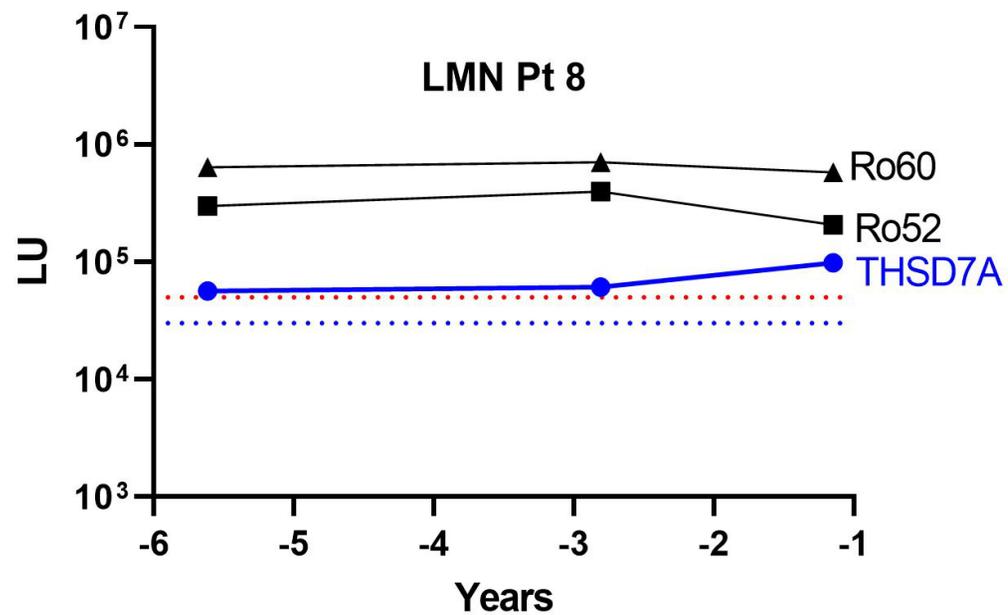


Fig.7



One Supplementary File-

Supplementary Material 1

Luciferase Immunoprecipitation Systems (LIPS) Methodology

LIPS assays were designed to detect IgG autoantibodies that can precipitate THSD7A protein. The general methodology in a 96-well plate format performed at room temperature has been described previously and in the corresponding video (1). In our current study of autoantibodies against THSD7A, we first generated mammalian vectors to express the full-length or fragments of THSD7A as luciferase fusion proteins (**Fig. 1A**). For constructing plasmids, a clone containing the entire human THSD7A cDNA (1) was used as the template. PCR with restriction enzyme adapter primers was used to generate two THSD7A fragments, encompassing amino acid residues 1–416 (THSD7A- Δ 1) and 1–671 (THSD7A- Δ 2). Those N-terminal THSD7A fragments included the start methionine and signal peptide and were subcloned into the pGAUS3 vector (2) positioned N-terminal to *Gaussia* luciferase (GLuc). Due to the large size of THSD7A, the near full-length coding sequence for the THSD7A protein (1656 of 1657 amino acids designated THSD7A-FL) was cloned into the pGAUS3 vector using a combination of PCR and fragment subcloning. High quality DNA was then prepared from each plasmid construct using a Qiagen Midiprep kit.

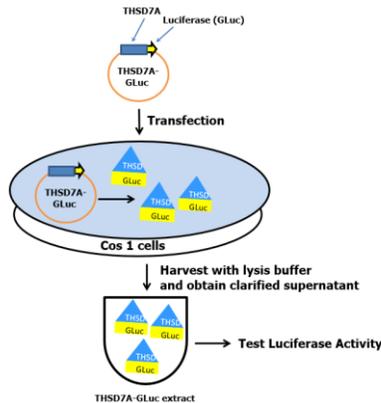


Fig 1A. Schematic of the construction and expression for generating the THSD7A-luciferase recombinant protein. DNA sequences encoding the THSD7A protein are genetically fused to the N-terminus of *Gaussia* luciferase (GLuc). These recombinant plasmids are then used to transfect Cos1 cells and cell lysate is harvested 48 hours later without purification.

Using Lipofectamine 2000 (Invitrogen), Cos-1 cells were transfected with the plasmids to produce recombinant light-emitting fusions of full-length THSD7A protein and fragments. Forty-eight hours post-transfection, crude lysates containing the recombinant proteins were harvested (**Fig. 1A**). This was performed by rinsing the adherent cells once with room temperature PBS before scraping and harvesting the monolayer in cold lysis buffer A (20 mM Tris, pH 7.5, 150 mM NaCl, 5mM MgCl₂, 1% Triton X-100 containing protease inhibitors). The lysates were centrifuged two times at 13,000×g to remove particulate matter. The clarified supernatants were collected and either processed immediately or stored frozen at -80° C in buffer A containing 50% glycerol. A tube luminometer (20/20 from Turner Scientific) was used to

measure the luciferase activity in the lysates with a coelenterazine substrate mix (Promega, Madison, WI).

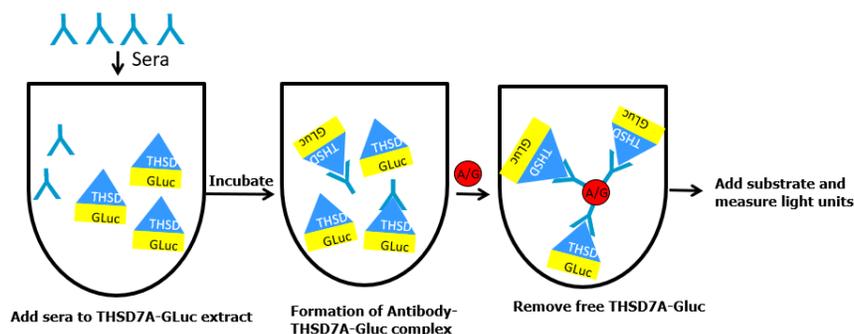


Fig. 1B Schematic overview of the LIPS assay. The LIPS assay is initiated by adding THSD7A-GLuc fusion protein extract directly to serum samples. The THSD7A-GLuc immunoglobulin complex is then captured by protein A/G beads. After washing the beads, bound THSD7A-GLuc is measured in a luminometer using coelenterazine as the substrate.

A 96-well “master plate” is first set up with patient sera by diluting sera 1:10 in assay buffer A containing 0.001% bromophenol red dye as tracker. For measuring antibody levels, a separate “working plate” is set up that contains 40 μ l buffer A, 10 μ l diluted serum (1 μ l equivalent) and 50 μ l 2×10^7 mix of the THSD7A-*Gaussia* luciferase extract in each well. Next, 7 μ l of a 30% suspension of Ultralink protein A/G beads (Thermo Scientific) in PBS was added to the bottom of each well of a 96-well filter HTS plate (Millipore, Bedford, MA). The 100- μ l antigen-antibody reaction mixture was then transferred to this filter protein A/G bead plate and incubated for 1 hour at room temperature on a rotary shaker. The protein captured by the beads was then washed using a vacuum manifold via an automated Tecan Hydroflex plate washer. After the final wash, luminescence was measured in light units (LU) on a Berthold LB 960 Centro microplate luminometer (Berthold Technologies, Bad Wilbad, Germany) using a coelenterazine substrate. All data represent the average of at least two independent experiments. Selected THSD7A seropositive serum samples from the pilot cohort (cohort1) were used to standardize the assay and for determining cut-off values for the validation cohort and cohort 3.

1. Burbelo, P.D., Ching, K.H., Klimavicz, C.M. Iadarola, M.J. Antibody profiling by Luciferase Immunoprecipitation Systems (LIPS). *J Vis Exp*, 2009(32). doi: 10.3791/1549.
2. Burbelo, P.D., Ferré, E.M.N., Chaturvedi, A., Lionakis, M.S., Chiorini, J.A. and Warner, B.M. Profiling Autoantibody Responses Against Salivary Proteins in Sicca Conditions. *Journal of Dental Research*, 98 (7):772-778, 2019.