Original paper

Morphological assessment of thin basement membrane disease

Jakub Żurawski[1], Paweł Burchardt[1], Jerzy Moczko[2], Monika Seget[3], Katarzyna Iwanik[3], Jacek Sikora[1], Aldona Woźniak[3], Wiesława Salwa-Żurawska[3]

[1]Department of Immunobiochemistry, Chair of Biology and Environmental Protection, Poznan University of Medical Sciences, Poznan, Poland
[2]Department of Computer Science and Statistics, Poznan University of Medical Sciences, Poznan, Poland
[3]Chair and Department of Clinical Pathology, Poznan University of Medical Sciences, Poznan, Poland

Thin basement membrane disease is more common than IgA nephropathy or Alport syndrome, which are also associated with the presence of erythrocyturia. Very few reports on the disorder are available in the Polish literature.
The objective of this work was to analyze the results from 83 patients with thin basement membrane syndrome as well as to formulate a proposal of strict morphological assessment criteria for the disorder. Attention was drawn to the requirement of thickness of the lamina densa rather than the entire basement membrane thickness and a sufficiently high number of loops featuring thinned lamina densa, namely at least 80% of loops, being taken into account. Occurrence of other morphological changes associated with the disorder and clinical symptoms other than erythrocyturia was also highlighted.

Key words: thin glomerular basement disease.

Introduction

Thin basement membrane disease was finally characterized by Rogers *et al*. in 1973 [1]. The term "thin basement membrane disease" replaced the old name "mild and curable hemorrhagic nephritis" introduced by G. Baehr in 1926 [2]. Fourty years after Baehr's report, i.e. in 1966, McConville and McAdams observed familial and non-familial occurrence of mild erythrocyturia [3]. In the case of the familial form, an autosomal dominant inheritance pattern was identified.

Initially, some authors deemed that the disease did not lead to renal insufficiency, and family histories lacked information on cases of uremia in patients' families [4]. However, Dische *et al*. [5] described cases in which proteinuria was observed along with erythrocyturia, with the development of renal insufficiency in some patients. This was later confirmed in other studies by other authors, some of whom [6, 7, 8, 9] ascertained that renal insufficiency could develop, albeit infrequently, in the natural history of thin basement membrane disease [10].

Finally, the disease was defined as being characterized by varied clinical courses, usually mild, but sometimes progressive, of both familial and spontaneous occurrence, and affecting ca. 1% of the population [11].

No family history is identified in more than 30% of patients [12]. Besides the most common symptom of erythrocyturia, rare episodes of hematuria may occur. It is slightly more common after physical exercise or as a consequence of infection. Over time, some patients may develop proteinuria. It is usually of mild or moderate intensity [5, 6, 7, 8, 9].

Histological images are non-characteristic. Glomerular lesions are usually undetectable [6]. A relatively common change is the presence of erythrocytes within the lumina of renal tubules [13]. In exceptional cases (involving extreme thinning), reduced silver absorption may be observed [12, 14, 15, 16, 17]. Sometimes, an increase in the number of mesangial cells with matrix growth is observed.

In adults, various degrees of glomerulosclerosis, foci of interstitial fibrosis and lesions within interstitial vessels may be observed [7]. These lesions are most commonly attributed to hypertension or the elderly age of patients; however, in some adults, focal segmental sclerotization develops before hypertension and proteinuria. It has been suggested that such premature glomerulosclerosis may constitute a risk factor for disease progression [18].

In most patients, immunofluorescence assays reveal no presence of deposits [18, 19, 20, 21], although small, isolated deposits of IgG, IgM, IgA, and C3 have been observed in rare cases [18, 21].

The diagnosis of thin basement membrane disease is made on the basis of the thinning of the lamina densa, extensively involving the renal glomerulus as seen in electron microscopic examination. The diagnosis appears easy, although some difficulties are also encountered [18].

Different methodologies are used by different laboratories for the assessment of the basement membrane thickness. Most authors use data from multiple measurements made within the peripheral parts of numerous capillary loops. Most often, the basement membrane thickness is measured from the cellular membrane of the basal podocyte layer to the cellular membrane of endothelial cells; thus, the measurement pertains to the entire membrane. However, some authors recommend that only the lamina densa should be measured as the thinning of this layer is of decisive importance for the diagnosis of thin basement membrane disease [4, 22].

Besides the significant thinning in the natural history of the disorders, segmental changes in membrane structure are also occasionally observed, including membrane loosening and discontinuity in extremely rare cases. The changes facilitate the transfer of erythrocytes into the ultrafiltrate.

Genetic studies are performed on extremely rare occasions. As shown by the presented data, the diagnosis of thin basement membrane disease is, against all appearances, quite difficult. Most commonly, the disorder is not taken into account in clinical considerations or morphological diagnostics.

Objective

1. To compare the results of the measurements of the thickness of the lamina densa of the glomerular basement membrane using MATLAB software with those obtained by the manual method.
2. To analyze the morphological diagnostic criteria of thin basement membrane disease. Identification of potential presence of other lesions (mainly glomerulosclerosis) accompanying the disorder.
3. To assess potential differences in clinical and morphological symptoms of the disorder in pediatric and adult patients.

Material and methods

The study material consisted of the results from 83 patients: 57 children and 26 adults with thin basement membrane disease.

For the purpose of comparison of the measurements of the thickness of the lamina densa of the basement membrane, specimens from puncture biopsies collected from 10 patients with minimal change disease were also included in the study.

Some of the puncture biopsies preserved in neutral buffered 10% formalin solution were subjected to routine preparation. Sections were subjected to hematoxylin and eosin staining, Jones' silver staining, and PAS staining reaction.

Immunofluorescence assays

Immunofluorescence slices of 5-6 μm were fixed in a cold 1 : 1 mixture of alcohol and acetone for 10 minutes, dried and rinsed three times in PBS. Next, the specimens were incubated with sera containing antibodies against IgA, IgG, IgM, the complement proteins C3, C4, C1q, and fibrinogen, labeled with fluorescein 5-isothiocyanate (FITC). Following incubation, the slices were rinsed three times in PBS and covered in glycerol under cover slips. The presence, composition and locations of the deposits of immunoglobulins, complement proteins and fibrinogen were assessed by fluorescence microscopy.

Electron microscopy examinations

The material collected for ultrastructural studies was routinely fixed in a 3.6% buffered glutaraldehyde solution with pH of 7.4. Next, the material was embedded in Epon 812 epoxide resin. Photographic documentation was carried out using an Opton 900 transmission electron microscope.

Measurement of thickness of the lamina densa

The thickness of the lamina densa of the basement membrane was measured by two methods.

The dense plaque thickness was measured and the results in millimeters were divided by the actual magnification of the electron microscopic image (manual method). Three measurements were taken at the same time, using the MATLAB computer software.

The measurements were taken at various peripheral segments of glomerular capillary loops and in segments directly adjacent to the mesangial regions.

Morphometric analysis was carried out on all glomeruli within the puncture biopsy (1 to 4 glomeruli), with 5 segments being examined on average in all vascular loops. Microphotographs were taken at magnifications of 3500×, 4400×, 5600×, 7000×, 8750×, 10 500×, and 11 500×. The obtained results were submitted to statistical analysis.

Statistical analysis

Due to the non-normal distribution of certain variables (Shapiro-Wilk test, $p < 0.05$) and the low sample sizes, statistical analyses were based solely on nonparametric methods including median tests. The differences in the results of the measurements of lamina densa thickness between the groups of data acquired by individual methods were analyzed using the Kruskal-Wallis test, which is a nonparametric alternative to unifactorial analysis of variance. Dunn's conservative multiple comparisons test was used as the post-hoc analysis method. Wilcoxon's paired rank test was used for comparison of the lamina densa measurement results obtained by the manual and the semiautomatic methods. The analysis of internal relationships between the three measurements made by means of the semiautomatic method was based on Spearman's coefficient of rank correlation. The significance level was defined as $p < 0.05$. Levels of $p < 0.01$ and $p < 0.001$, if observed, were identified in the description.

Table I. Clinical signs

Clinical signs	Children (57) ♀29, ♂28	Adults (26) ♀14, ♂12
Isolated erythrocyturia	33	6
Hematuria => isolated erythrocyturia	1	–
Acute renal insufficiency => isolated erythrocyturia	1	–
Erythrocyturia and proteinuria	4	8
Nephrotic syndrome and erythrocyturia	2	–
Nephrotic syndrome => erythrocyturia and proteinuria	–	2
Hematuria and proteinuria	–	1
Proteinuria	2	2
Nephrotic syndrome	14	7

Results

General data, clinical signs

The pediatric patient group consisted of 29 girls and 28 boys. At the time of diagnosis, the 3 youngest girls were 2 years old, the oldest girl was 16 years old, the youngest boy was 2 years old and the oldest boy was 17 years old. Family history of renal disease was reported in 13 children.

In the group of adults consisting of 14 females and 12 males, the patients' age ranged from 19 to 70 years.

No family history of renal diseases was observed in either of the adult patients.

Clinical signs are included in Table I.

Erythrocyturia was found only in 41 patients including 1 child following an initial episode of hematuria and in another after short-term acute renal failure. Erythrocyturia was accompanied by other symptoms in an additional 17 patients. Overall, it was reported in 58 patients.

Results of morphological examinations

In both the pediatric and the adult population, the optical microscopic image of renal puncture biopsies was non-characteristic. Most commonly, presentations were referred to mesangial hypercellularity of non-specific lesions due to their low intensity. In the pediatric population, possible mesangial glomerulonephritis was considered in 4 patients (3 girls and 1 boy): IgA nephropathy in 3 patients and IgM nephropathy in 1 patient due to the presence of IgA deposits in 1 case, IgA and IgM deposits in 1 case, IgA, IgM, and C3 deposits in 1 case, and IgM and C1q deposits in 1 case. In none of these cases did the electron microscopic study identify the presence of deposits or mesangial hypercellularity. Otherwise, deposits identified in immunofluorescence studies appeared in varying amounts in the individual mesangial areas.

Signs of glomerulosclerosis were observed in 15 children, including 11 in whom the signs were revealed only in electron microscopic imaging. The clinical symptoms observed in these patients were quite varied, with nephrotic syndrome being diagnosed in 3 cases, isolated erythrocyturia in 9 cases, isolated proteinuria followed by nephrotic syndrome in 1 case and erythrocyturia and proteinuria also in 1 case. As mentioned above, 1 patient presented the initial symptoms of Schoenlein-Henoch syndrome.

In 5 cases, Jones' silver staining revealed basement membrane thinning already in the optical microscopy examination.

The results of repeated biopsies were varied. In 1 girl with erythrocyturia in whom the electron microscopy examination of the first biopsy specimen revealed the features of sclerotization, the intensity of sclerotization in the repeated biopsy taken 5 years later was as low as that in the first biopsy. In another girl, the first biopsy did not provide a sufficient amount of material to be used for electron microscopy. Repeated biopsy was performed after 4 years due to treatment-refractory nephrotic syndrome. The biopsy revealed only the signs of thin basement membrane disease. In another girl, a repeated biopsy performed 13 years after the first biopsy revealed features of Alport syndrome. It should be noted that the first biopsy performed in this patient revealed small foci of interstitial fibrosis and foam cells, albeit with glomerular lesions consisting only of extensive thinning of the lamina densa of the basement membrane. The patient had a family history of erythrocyturia, and a biopsy performed in a sister revealed only the features of thin basement membrane disease.

Signs of glomerulosclerosis were observed in 6 adults, including 2 in whom the signs were revealed only in electron microscopic imaging. Trace immunocomplex deposits were observed in 4 patients. They included deposits of IgM and C3 in 2 cases, IgA, IgG, IgM, C3, C1q, and C4 in another case, and IgA, IgM, C3, and C4 in the last case. Electron microscopy could not confirm the presence of deposits in either of these cases. No increase in the number of mesangial cells was observed either.

As in the case of 1 child, 3 patients in the group were suspected of possibly having thin basement membrane disease on the basis of optical microscopy examination following Jones' silver staining.

In all cases, thinning of the lamina densa of the basement membranes was observed in at least 80% of capillary loops. Entire loops were involved (Fig. 1). Only in selected loops were the membranes slightly thicker in the direct vicinity of the mesangial regions. The increase in the quantity of mesangial matrix in the reported 15 pediatric and 6 adult patients was slight, also when identified by optical microscopy. In 16 children and 3 adults, electron microscopy examination revealed isolated, short segments of loosening of the lamina densa structure (Fig. 2). In 3 children and 1 adult, the lesions were accompanied by signs of glomerulosclerosis (Fig. 3).

Results of thickness of lamina densa measurement

Statistically significant differences were observed in membrane thickness measurements depending on the selected method of measurement. Manual measurements led to the thickness of the basement membrane in children and adults with thin basement membrane disease as well as patients with minimal

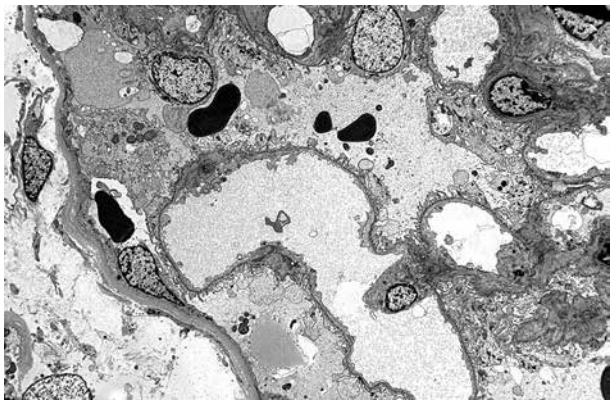


Fig. 1. Thin basement membrane disease. Capillary loops with uniform thinning of lamina densa. El-mi, magnification 4000×

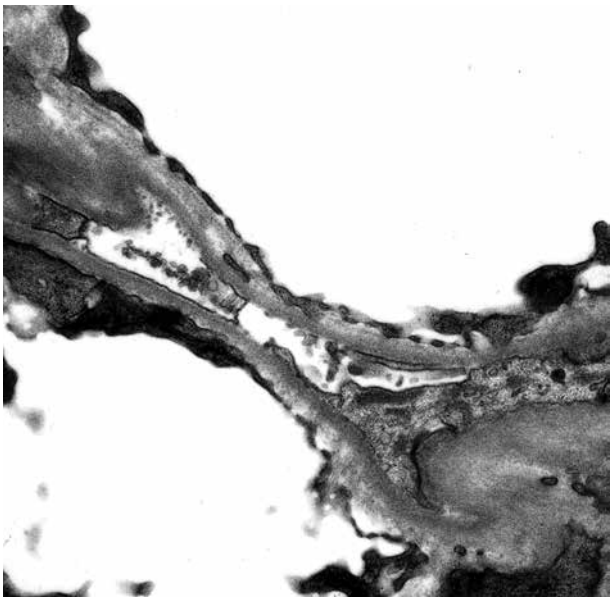


Fig. 2. Thin basement membrane disease. Loosing of lamina densa structure over short segments. El-mi, magnification 10 500×

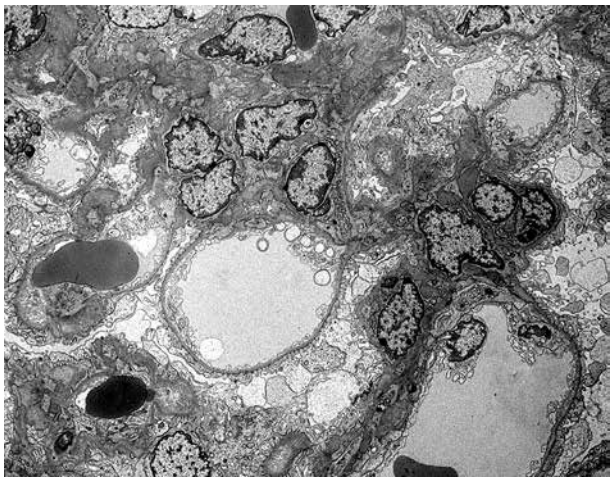
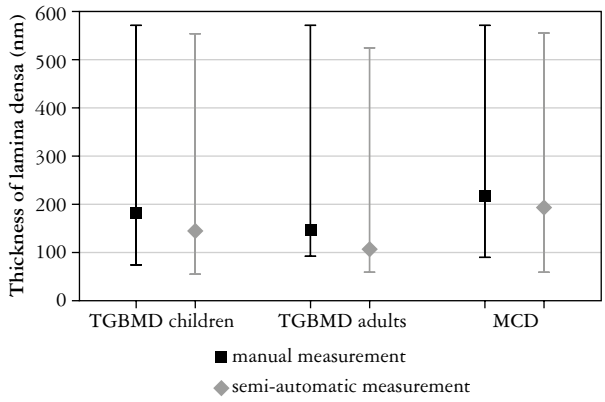


Fig. 3. Thin basement membrane disease. Lamina densa thinning within all capillary loops. Mesangial enlargement with increased cellularity and slight increase in the mesangial matrix volume. El-mi, magnification 4000×



TBMD – thin basement membrane disease, MCD – minimal change disease

Fig. 4. Comparison of the lamina densa thickness values depending on the selected measurement method

change disease being greater than that determined by the semiautomatic method (Wilcoxon, $p < 0.01$). In addition, lower nominal values of dense plaque thickness were observed for the semiautomatic methods in all groups (Fig. 4).

In addition, the analysis of differences in the lamina densa thickness between the study groups depending on the measurement method showed that the semiautomatic method led to a lower diversity of results as compared to the manual method. The manual method revealed statistically significant differences in the dense plaque thickness between the materials collected from adult patients with thin basement membrane disease (Table II). Differences in the thickness of lamina densa values in children with thin basement membrane disease were also revealed by the semiautomatic method (Table III).

Considering the mean differences between individual measurements taken as part of the semiautomatic method, it seems justifiable to use triplicate measurements of the lamina densa thickness. The mean difference between triplicate measurements was small and did not exceed 1.64 nm, with the lowest differences being observed in pediatric patients with thin basement membrane disease.

These results may prove the accuracy and repeatability of the semiautomatic method of measurement of lamina densa. In addition, the analysis of the results between individual measurements revealed a high degree of internal consistency of $Rs \geq 0.99$ for each group (Spearman, $p < 0.05$ for each group).

Discussion

The study material was collected over a period of 27 years. In this period, a total of 4840 renal biopsy specimens were assessed in our material. The diagnoses of thin basement membrane disease accounted for 1.7% of this number. The value is relatively low. Netzer *et al.* [23] estimated this percentage to be 5-10%; Suh *et al*. [24] diagnosed a total of 25 patients in study material of 680 biopsy specimens, accounting for 3.7%. Higher percentages were cited by Lee *et al*. [25]. Of a group of 461 children aged 9.3 ±2.7 years in whom changes were detected in the urine, as many as 127 (27.5%) were diagnosed with thin basement membrane disease.

It should be noted that in our study population of 4840 cases, no material was collected for electron microscopy, which is the decisive examination in the diagnosis of thin basement membrane disease.

Differences in the percentages cited may be due to different study methodologies. According to most authors, extensive thinning of basement membranes is a prerequisite for the diagnosis of thin basement membrane disease [26, 27, 28]. In our material, the thin basement membrane disease diagnosis was made when membrane thinning was observed in more than 80% of loops with complete loop involvement. In other cases (not included in this study) when the thinning of the lamina densa affected the loops to a lesser extent it always coexisted with other glomerulopathies.

Different numbers of measurements are reported depending on the number of glomeruli within the biopsy specimen [8, 17, 24, 29, 30, 31]. The number of measurements in our material was determined by the number of glomeruli within the puncture biopsy.

Most authors report that the membranes were measured from the base of the podocyte layer to the endothelial cells, i.e. the measurements included the internal and external lamina rara. Although the external lamina rara is usually very thin or even

Table II. Descriptive statistics – manual measurements of thickness of the lamina densa [nm] depending on the group

	N	MEAN	MEDIAN	SD	MIN.-MAX.
TBMD children	57	215.6	178.0	114.5	71-571
TBMD adults	26	176.2	142.0	119.2	89-571
MCD	10	273.0	213.5	177.6	86-571

TBMD – thin basement membrane disease; MCD – minimal change disease

Table III. Descriptive statistics – semiautomatic measurements of thickness of the lamina densa [nm] depending on the group

	N	MEAN	MEDIAN	SD	MIN.-MAX.
TBMD children	57	179.0	141.0	103.6	51.7-553.0
TBMD adults	26	147.1	102.3	113.9	55.7-523.7
MCD	10	248.2	189.2	180.4	56.3-555

TBMD – thin basement membrane dwisease; MCD – minimal change disease

invisible, the internal lamina rara may be quite thick in segments. Therefore, only the lamina densa was measured in our material. In a similar fashion, the measurements were limited to the lamina densa in the study by Nasr *et al*. [31], who also pointed to the commonly observed artifacts due to the separation of podocytes from the basement membrane and poor conservation of the lamina rara. The fact of basement membrane thinning being limited to the lamina densa was also highlighted by Dische *et al*. [5], Vogler *et al*. [32], and Basta-Jovanovic *et al*. [33].

Most authors use computer-assisted methods to assess the membrane thickness. Some authors, for example Das *et al*. [34], use both methods for better reliability of results. Following their example, we decided to use the computer-assisted method as well as the so-called "manual" method as used e.g. by Osawa *et al*. [35], Foster *et al*. [16] and Nasr *et al*. [31]. The term "manual method" is adapted from Kanenetsky *et al*. [36]. The measurements were always made in the same segments of the membrane. The manual method proved as useful as the computer-assisted method. Of note is the fact that some studies in which the "manual" method was used as the only method date back to 2005 (Foster) and 2007 (Nasr), i.e. to a time when computer-assisted computational methods were already widely known.

Agreement is also observed in the evaluation of histopathological results by various authors. Most commonly, no significant changes are observed in histological examinations other than the quite common different grades of increased mesangial cell counts. Sometimes, features of glomerulosclerosis are also observed. In the present material, presence of glomeruli with thin basement membranes was noted in five cases of Jones' silver staining, as reported in histological examination summaries. In these cases, the result of staining allowed for the diagnosis of thin basement membrane disease being proposed already on the basis of optical microscopy examination. This is important as silver staining is routinely used in nephropathology and thus may be helpful in initial differential considerations. Usually, not much attention was paid to the possible diagnostic usefulness of this method. Similar results were reported by Foster *et al*. [16], van Breda Vriesman [17], Nieuwhof *et al*. [7], and Tryggvason and Patrakka [15].

When discussing the results of morphological studies, one must mention the control groups. Not all authors provided information on this topic, and the material in papers reporting controlled studies is quite diverse. Control materials included cases of IgA nephropathy [5, 37], autopsy materials [38], cases of tubulointerstitial nephritis, renal tubule necrosis, material from kidneys resected due to cancer [39], and material of kidneys resected due to trauma [40, 41]. Lang *et al*. [37], Ivanyi *et al*. [42], and Basta-Jovanovic *et al*. [33] used control material diagnosed with minimal change disease. Thus, we considered it justifiable to use the cases of minimal change disease as a control group in the study.

Clinical symptoms are of little usefulness in the diagnosis of thin basement membrane disease, as are the results of optical microscopic examinations. Similar to the literature reports, the most common symptom in our study material was erythrocyturia. Most commonly (in 41 cases) it was isolated; in another 17 cases it was accompanied by proteinuria and in isolated cases by nephrotic syndrome. The second most common symptom was nephrotic syndrome. The least common initial symptom (occurring in 4 cases) was isolated proteinuria. Patients with thin basement membrane disease were relatively frequently diagnosed with proteinuria. The high percentage of patients with thin basement membrane disease diagnosed with proteinuria may be explained by the fact that, in the case of proteinuria, material for electron microscopic tests is usually secured, whereas in the case of isolated microhematuria often no material is secured for such tests. If such tests were carried out in all the relevant cases, the number of diagnosed cases of thin basement membrane disease would be significantly higher and the percentage of patients with proteinuria consequently correspondingly lower.

Of note is the presence of nephrotic syndrome or proteinuria in the natural history of thin basement membrane disease. Farquhar believes that proteinuria may be related to the basement membrane thinning as such, being accompanied by damage to the membranes in podocytes [43]. However, relevant cases were not numerous in the current study material. Besides, the sclerotic lesions were of low intensity. One may therefore assume that they develop quite slowly and thus the course of the disease is relatively mild in a vast majority of patients. The slow course of thin basement membrane disease may also be confirmed by the repeated biopsy results, non-numerous though they may be in the present study group.

The issue of glomerulosclerosis has been discussed by various authors. In most cases, however, only the presence of glomerulosclerosis was reported [5, 24, 28, 44, 45, 46, 47]. Only a few authors associated glomerulosclerosis with the presence of proteinuria [9, 48, 49]. An interesting report was presented by Choi *et al*. [50], pertaining to the presence of sclerotic lesions in kidney donors with thin basement membrane disease and erythrocyturia symptoms. Also worth mentioning is the study by common incidence of glomerulosclerosis in cases when the membrane thinning was Nogueira *et al*. [8], who characterized the interstitial lesions that accompany glomerulosclerosis in the natural history of thin basement membrane disease. Ivanyi *et al*. [42] pointed out the more extensive as compared to cases of segmental-on-

ly thinning. It is difficult to take a stance on this statement, as in all cases examined in this study, the thinning extensively involved the glomerular loops. Voskarides *et al*. [51] suggested that the presence of sclerotic lesions in thin basement membrane disease might be due to genetic modifications within the COL4A3/COL4A4 gene. Sue *et al*. [26] claim that thin basement membrane disease is not dangerous unless it is accompanied by other glomerulopathies, FSGS being suggested as an example.

The first papers on thin basement membrane disease put particular focus on its familial occurrence, hence the name "mild familial erythrocyturia". Today, we are aware that the disorder is not always determined by genetic factors and its occurrence is not always familial [52]. In the present study material, familial occurrence was observed in 13 children from 9 families. No family history of renal diseases was reported for any of the adult patients with thin basement membrane disease.

Of note is the occurrence of the first symptoms of the disorder in adult patients, including elderly patients. One may not exclude that these individuals had not been examined previously or that the initial symptoms of low-intensity erythrocyturia had been ignored.

Some authors doubt the plausibility of biopsies being performed in children with asymptomatic erythrocyturia [9, 53]. However, the fact that this initially mild symptom may sometimes be associated with potential risks is suggestive not only of the necessity of biopsies in such cases, but also of the absolute necessity of electron microscopy scans being performed on each specimen.

Conclusions

1. Our results suggest that the diagnosis of thin basement membrane disease requires precise diagnostic criteria consisting of the assessment of the thickness of the lamina densa rather than the entire basement membrane and identification of membrane thinning in at least 80% of capillary loops with complete loop involvement.
2. The measurements of the thickness of the dense plaque of the basement membrane obtained with the computer-assisted method differ from those obtained by the manual method. However, they confirm the usefulness of this method in preliminary diagnostic assessment. Sometimes, Jones' silver staining proves useful in the initial assessment by means of optical microscopy.
3. Persistent isolated erythrocyturia should constitute an indication for renal biopsy, with an electron microscopy scan of the specimen being mandatory in these cases.
4. Glomerulosclerosis, sometimes observed in the course of the disease, should be considered a consequence of thin basement membrane disease rather than concomitant primary focal segmental glomerulosclerosis (FSGS), as its intensity is usually mild even over a long-term course of the disorder.

The authors declare no conflict of interest.

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Address for correspondence

Jakub Żurawski
Department of Immunobiochemistry
Chair of Biology and Environmental Protection
Poznan University of Medical Sciences
Rokietnicka 8
60-806 Poznan, Poland
e-mail: zurawski@ump.edu.pl