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TRANSLATIONAL SCIENCE

Dissecting the histological features of lupus nephritis highlights new common patterns of injury in class III/IV

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ABSTRACT

Objective The International Society of Nephrology/Renal Pathology Society classification is the gold standard for the characterisation of lupus nephritis (LN) on renal biopsy, with therapeutic repercussions. Its recent revision simplified the current class subdivisions, eliminating the S/G forms of class IV, although data on a possible pathogenetic/clinical value of this subdivision are still contradictory.

Methods 353 renal biopsies from Belimumab International Study in LN were assessed through central pathology review. Univariate logistic models and a decision tree were performed on 314 adequate biopsies to evaluate the impact of histological features on focal/diffuse classes. Removing class I/II (n=6) and 'pure' class V (n=34), principal component analysis (PCA) and heatmap were used to explore similarities among III, IVS and IVG biopsies either incorporating or not the mixed classes (+V, n=274). Finally, a method aimed at partitioning the cases into k clusters based on their similarity (KMeans), was used to study features from the cohort of 'pure' class III/IVS/IVG cases (n=214) to determine alternative subdivisions based on phenotypic data.

Results Segmental endocapillary hypercellularity (EH) was prevalent in class III, global EH, wire loops, hyaline thrombi and double contours were hallmarks of class IVG, with IVS cases showing intermediate characteristics. Heatmap and PCA confirmed the segregation of these features among classes, showing better segregation for focal/diffuse LN as compared with the mixed classes (+V). KMeans revealed the presence of two main clusters, membranoproliferative-like (n=83) or vasculitis-like (n=131).

Conclusions This study reveals new phenotypic forms of LN surpassing the traditional classes as determined by the current classification. Future validation and confirmation are required to confirm these findings.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The International Society of Nephrology/Renal Pathology Society classification is the gold standard for the evaluation of renal biopsies with lupus nephritis (LN).
⇒ The LN classification is an important tool for classifying various forms of renal involvement in SLE, and consequently, an important tool for therapeutic decisions.

WHAT THIS STUDY ADDS

⇒ By cluster analysis, two main groups were distinguished labelled as membranoproliferative-like and vasculitis-like.
⇒ The clusters found here share similarities with previously diagnosed class III and IVG.
⇒ Cases from previously diagnosed class IVS may be reassigned to one of the two clusters.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The proposed clusters may have significant pathogenetic meaning, considering that the patterns of injury (MPGN vs vasculitic) are known to be linked to different underlying disease processes.
⇒ The simplification of the current classification (MPGN-like vs vasculitis-like) could potentially increase interobserver reproducibility.
⇒ This may be the starting point of further investigations focused on how to incorporate new subdivisions into a classification system that would better guide patient- tailored therapies in the near future.

INTRODUCTION

Renal biopsy is a cornerstone for the management of patients with lupus nephritis (LN), as their treatment is strongly guided by the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification system.[1] The classification system was recently modified and updated. It was advised to more actively report on the Activity (AI) and Chronicity Index (CI), the definitions of lesions were updated,[2–4] and the subclassification of class IV into segmental and global forms was abandoned.[5] By doing so, it was hoped to make the classification scheme more useful for international usage in studies and to decrease the degree of interobserver variability that had been described previously.[6]

Although lupus classification is a useful tool in the guideline of therapeutic decisions of patients with LN, it does not exemplify possible pathogenetic mechanisms behind the lesions as evaluated in the renal biopsy. By re-evaluating the histological findings in detail, we have tried in this study to work towards new categories hitherto unknown. This may give new insights into a possible subdivision of

Table 1 Clinical characteristics of the cohort

Clinical characteristics	Overall (n=306)
Age	
Median (IQR)	37 (31–47)
Range	24–70
Sex (n, %)	
Male	39 (13)
Female	267 (87)
Ethnicity (n, %)	
African-American	52 (17)
Asian	150 (49)
Caucasian	104 (34)
Creatinine	
Mean (SD)	0.92 (0.43)
Range	0.31–3.20
UPCR	
Mean (SD)	4.06 (3.30)
Range	0.58–20.81
Activity	
Mean (SD)	6.57 (4.18)
Range	0–17
Chronicity	
Mean (SD)	2.45 (2.27)
Range	0–10
LN classes (n, %)*	
Class I/II†	6 (2)
Class III	83 (27)
Class IVS	104 (34)
Class IVG	82 (27)
Class V	31 (10)

*Integrating each class with the mixed +V, LN classes only in patients with complete clinical characteristics.
†Renal biopsy samples were initially analysed locally to confirm eligibility (biopsy-proven class III, IV, V or combination of these, as per BLISS-LN enrollment criteria[7]). Additional evaluation of the available renal biopsy samples was performed by a central pathology review board at the University of Milano-Bicocca, during which six cases have been assigned to class I/II.
BLISS-LN, Belimumab International Study in LN; LN, lupus nephritis; UPCR, urine protein/creatinine ratio.

LN that goes by unnoticed by using the classical scheme in terms of lupus classes I–VI.

The Belimumab International Study in LN (BLISS-LN)[7] collected 353 renal biopsies, an invaluable opportunity to test whether data obtained through systematic assessment of histological lesions would reveal new levels of LN phenotypes, other than those already known through the traditional classification criteria.

METHODS

Patients

This multicentre study included 353 cases, enrolled in the setting of the clinical trial BLISS-LN,[7] with ISN/RPS classes certified by a central pathology review board (IB, JAB, HTC, FF, L-HN). From the initial cohort, cases with less than five evaluable glomeruli[8] as per experts judgement were excluded (n=39), leading to a final cohort of 314 cases. Baseline clinical data (age, sex, ethnicity, serum creatinine and urinary proteine/creatinine ratio (UPCR)) were provided by the BLISS-LN study GSK team for 306/314 cases.[7]

Digital histopathology

All the original renal biopsy glass slides were received from July 2012 to July 2017 at the Pathology Unit, Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy. H&E, periodic acid–Schiff, Masson trichrome and periodic acid methenamine silver stains were available. A subset of cases (16%, n=59/353) were received as Whole-Slide Images (WSI). For the remaining cases, glass slides were scanned to obtain WSI through the Aperio CS2 scanner (Leica Biosystems, Nussloch, Germany) at ×40 magnification (0.247μm per pixel) and cases were uploaded on the online platform Spectrum after a careful quality control of each virtual slide.[9 10] Each biopsy was independently evaluated by two renal pathologists of the board, after a randomisation process that assigned two pathologists to each case. A detailed histology scoring was performed. Biopsies were classified according to the ISN/RPS classification[1] with class III defined as the presence of active/inactive glomerular focal (<50% of biopsy glomeruli) segmental or global lesions, class IV as the presence of active/inactive diffuse (>50% of biopsy glomeruli) lesions, either segmental (IVS) or global (IVG) if less or more of 50% of the tuft was involved, and class V when a membranous pattern was noted (holes and spikes of the glomerular basement membrane at the Jones stain along with reported diffuse/global granular positivity of the capillary walls in immunofluorescence). Mixed classes were defined as the coexistence of a class V with either class III or IV LN. Moreover, subsequent evaluation of AI and CI was performed, as per recent recommendations.[2] To minimise interobserver variability and facilitate critical discussion, glomeruli were numbered on the WebScope plugin for WSI visualisation.[11] After a first review, discordances in terms of final ISN/RPS class was recorded in 88 out of 314 cases between the two panel nephropatologists assigned to each case. In these cases, regular consensus meetings of the board were organised to reach consensus.[12] Detailed glomerular, tubulointerstitial and vascular parameters were systematically recorded case by case in a dedicated scoring sheet (multipage Excel file, Microsoft, Redmond, Washington, USA) with pull-down menus for data entry, in order to maintain data uniformity throughout the database (online supplemental table 1). The AI and CI were automatically calculated from the histological variables scored by the pathologists. In online supplemental material 1 are reported exemplificative WSI that recapitulates the class III, IVS and IVG cases with systematic annotation of the principal lesions that have been scored by the renal pathologists during the review process.

Statistical analysis

Relative frequencies of histological features were calculated in order to standardise by the number of glomeruli evaluated by the pathologist. For continuous variables, mean and SD or quartiles (Q1, median and Q3), were calculated, as appropriate, while qualitative variables were reported as count and frequency. For the descriptive analysis, histological data from all biopsies were included, while subsequent analyses focused on the subgroup of 274 cases from III (focal) or IV (diffuse) LN class (ie, excluding 6 cases from class I/II and 34 'pure' class V patients).

Univariate logistic models were performed to evaluate the impact of each histological feature on classes (ie, III vs IVS, III vs IVG, IVS vs IVG, incorporating the mixed classes+V to each group). Multiple tests were adjusted by Holms at 5% level of significance.

A decision tree was applied on histological components to explore their relevance in differentiating classes. Exploratory

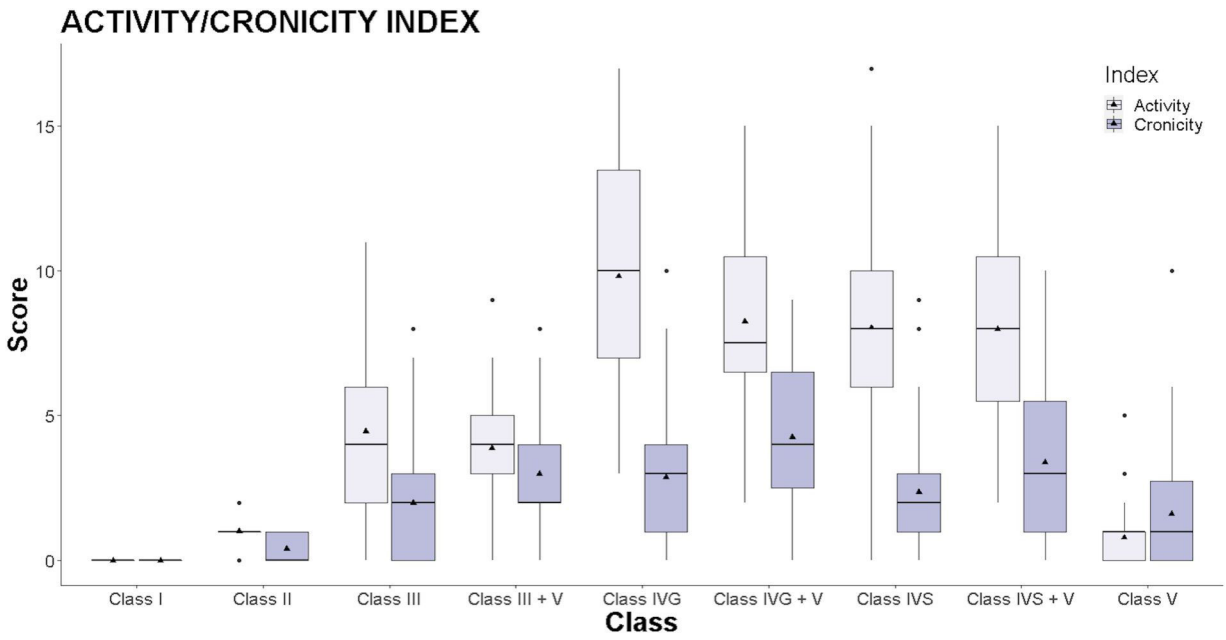

Figure 1 Box plot showing the distribution of median values of AI and CI among the different classes and subclasses. A difference can be noted in mixed as compared with 'pure' focal/diffuse classes in terms of median values of CI. Class I n=1, class II n=5, class III n=61, class III+V n=25, class IVG n=70, class IVG+V n=12, class IVs n=83, class IVS+V n=23, class V n=34. AI, activity index; CI, chronicity index.

analyses were performed to assess similarities among III, IVS and IVG biopsies either incorporating or not the mixed classes (+V) through principal component analysis (PCA) and heatmap. To investigate the putative presence of different clustering with respect to LN classes among biopsies, a method aimed at partitioning the observations (renal biopsy cases) into two groups in which each observation belongs to the group with the nearest mean (KMeans), has been performed on histological features. The internal validation of the cluster analysis was based on the silhouette index and the t-test was used to compare the two groups identified by the KMeans procedure in terms of AI and CI mean values. Statistical analyses were performed using the open-source R software V.3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Clinical characteristics

The clinical and demographic characteristics of the broader cohort of patients enrolled in the BLISS-LN trial have already been reported elsewhere.[7] In this study, 87% of patients were female, with a median age of 37 years (IQR 31–47). Ethnicity was Caucasian (34%), Asian (49%) and African-American (17%). The median number of glomeruli was 15 (IQR 11–23). Details on creatinine and UPCR are reported in table 1. No significant association was found among serum creatinine and UPCR with either AI or CI (ranging between 0.13 and 0.36). The distribution of AI and CI among classes and subclasses showed slightly higher values of CI in mixed as compared with 'pure' focal/diffuse classes (figure 1).

Histological lesions in LN

The distributions of a subset of histological features according to LN classes are shown in figure 2. Global glomerulosclerosis was prevalent in mixed classes, while no significant differences were noted for segmental glomerulosclerosis. Endocapillary hypercellularity (EH) was mostly segmental (<50% of the tuft) in class III, and typically involved more than 50% of the tuft in class IVG, with class IVS demonstrating a variable representation of segmental/global EH. A similar trend was noted for other histological features, such as wire loops (WL), double contours (DC), hyaline thrombi (HT) and tuft necrosis, the presence of which was more typical of class IVG as compared to class III, while IVS cases showed intermediate characteristics. The results of the univariate analysis identified a subgroup of histological features, mainly related to glomerular active lesions, which showed a statistically significant difference among class III, IVG and IVS (table 2). Results were confirmed by decision tree analysis (online supplemental figure 1). Heatmap and PCA on histological features, excluding those mainly related to glomerular chronic lesions based on the results of univariate analysis, revealed that focal and diffuse LN demonstrated a good segregation (figure 3B–D) as compared with the mixed classes (figure 3A), suggesting that the presence of an underlying class V could partially 'contaminate' the histological data. Results confirmed that the most impactful features were reconducible to those with a statistically significant value in univariate analysis (EH, WL, DC and HT).

Clustering cases from histological features

A different unsupervised clustering analysis (KMeans) proposed a better grouping for all 'pure' cases starting from their histological similarities (in online supplemental figure 2, the silhouette indices and the two identified clusters are shown). Translating this subdivision to the heatmap, a new grouping was proposed by the algorithm, as can be appreciated by the heatmap in figure 4A: the majority of class IVG is reassigned to the 'violet' group (global EH, WL, DC, the so-called membranoproliferative (MPGN)-like pattern) and the majority of class III cases to the 'orange' group (segmental HE, so-called vasculitis-like pattern). The IVS cases demonstrated a slight prevalence in clustering with the 'orange' cases (vasculitis-like, similar to class III), although there was a subset assigned to the 'violet' group (MPGN-like, similar to class IVG). A comparison of cases assigned to new groups through the PCA (figure 4B) with the ISN/RPS classes

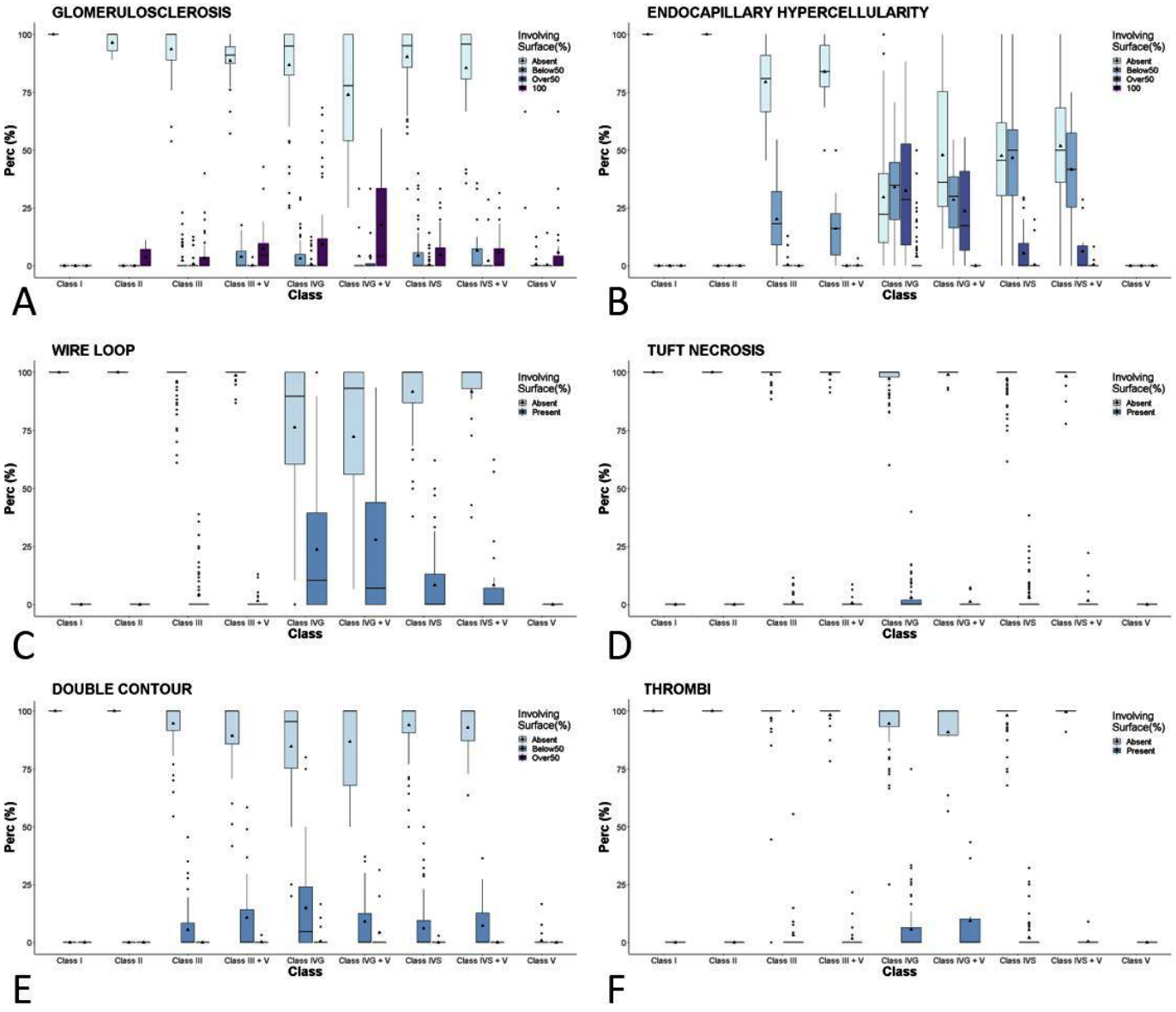

Figure 2 Box plots depicting the distribution of some of the histological features demonstrating substantial graphical differences among the different classes, especially class III, IVs and IVG cases: glomerulosclerosis (A), endocapillary hypercellularity (B), wire loops (C), tuft necrosis (D), double contours (E) and hyaline thrombi (F). Features are expressed either in a four tiered way (absent, involving less, more of the 50% or the 100% of the tuft) or as present/absent (see wire loops, double contours, tuft necrosis and hyaline thrombi). Class I n=1, class II n=5, class III n=61, class III+V n=25, class IVG n=70, class IVG+V n=12, class IVS n=83, class IVS+V n=23, class V n=34.

(figure 4C) demonstrates better segregation of cases with the newly proposed clustering. The revision of WSI from cases assigned to the 'vasculitis-like' group and classified as class III by two independent renal pathologists (VL'I and FP) highlighted the recurrent presence of segmental EH, eventually associated with segmental sclerosis or EH/crescents without DC, WL and HT. On the other hand, the analysis of WSI from cases clustered as 'MPGN-like' and belonging to the IVG group demonstrated a completely different histology, with global EH, often devoid of crescents and with invariable presence of DC, WL and HT. To further validate these observations, WSI belonging to pure class IVS were revised blindly from the new assignment to the 'MPGN-like' or 'vasculitis-like' group (figure 4D). Finally, to corroborate the proposed partition, we compared the AI and CI mean values among these two groups and found a statistically significant difference in terms of AI ($p<0.001$; 10.52 ± 3.18 vs 5.72 ± 2.85, for the 'MPGN-like' and 'vasculitis-like' group, respectively), but not for CI ($p=0.088$; 2.11 ± 1.74 vs 2.60 ± 2.39) (figure 4E).

DISCUSSION

LN is characterised by a wide spectrum of lesions, and historically, the LN classification has been used to distinguish various patterns of renal involvement. The LN classification provides an important tool in patient management and therapeutic decisions. Its usage has become so intricately linked to therapeutic decisions, that the basis on which it was created originally, is almost lost. The subdivision of class III and IV, for instance, was originally based on a difference in histology that later on was converted into a complex system of multiple glomerular lesions that were either active/chronic; segmental/global; present in more or less than 50% of the glomeruli in the biopsy. Although useful for clinical purposes, it may be questioned whether other ways of

Table 2 Univariate analysis depicting the impact of each histopathological feature in discriminating class III, IVS and IVG in the cohort

	Classes*			Univariate analysis†		
Histological characteristics (n=274)	**III (n=86)**	**IVS (n=106)**	**IVG (n=82)**	**III versus IVS**	**III versus IVG**	**IVS versus IVG**
Endocapillary hypercellularity						
Absent	80.77 (13.87)	48.40 (24.05)	32.24 (27.42)	<0.001	<0.001	0.003
<50%	18.90 (13.69)	45.54 (20.54)	33.14 (18.13)	<0.001	<0.001	0.003
≥50%	0.30 (1.73)	5.62 (8.12)	31.13 (25.17)	0.002	<0.001	<0.001
100%	0.04 (0.36)	0.44 (2.57)	3.48 (9.05)	1	0.6264	0.1862
Glomerulosclerosis						
Absent	92.10 (10.16)	89.20 (15.39)	84.89 (20.93)	1	0.1404	1
<50%	2.73 (5.07)	4.90 (9.37)	3.29 (6.98)	1	1	1
≥50%	0.61 (2.46)	0.89 (3.82)	1.25 (4.57)	1	1	1
100%	4.56 (8.45)	5.02 (8.48)	10.58 (17.88)	1	0.1734	0.178
Extracapillary hypercellularity (cellular)						
Absent	93.31 (9.39)	78.82 (19.72)	79.49 (20.59)	<0.001	<0.001	1
≤25%	5.26 (7.67)	12.10 (10.66)	10.04 (9.93)	<0.001	0.0273	1
>25%; <50%	0.94 (2.71)	6.22 (10.21)	6.05 (7.66)	<0.001	<0.001	1
≥50%	0.36 (1.71)	2.32 (5.24)	2.90 (6.91)	0.1276	0.056	1
100%	0.12 (0.83)	0.54 (1.89)	1.52 (5.16)	1	0.5642	1
Extracapillary hypercellularity (fibrous)						
Absent	94.49 (8.57)	90.97 (13.96)	93.38 (9.53)	0.8517	1	1
≤25%	3.47 (5.11)	5.04 (8.88)	3.39 (5.89)	1	1	1
≥25%; <50%	1.26 (3.57)	2.39 (5.09)	1.87 (4.57)	1	1	1
≥50%	0.78 (2.46)	1.30 (4.22)	1.22 (2.94)	1	1	1
100%	0.00 (0.00)	0.29 (1.62)	0.14 (0.64)	1	1	1
Wire loop						
Absent	96.49 (8.12)	91.67 (14.63)	75.67 (31.01)	0.2394	<0.001	0.003
Present	3.51 (8.12)	8.33 (14.63)	24.33 (31.01)	0.2394	<0.001	0.003
Thrombi						
Absent	97.26 (12.61)	98.35 (5.40)	93.91 (12.37)	1	1	0.0858
Present	2.74 (12.61)	1.65 (5.40)	6.09 (12.37)	1	1	0.0858
Double contour						
Absent	93.01 (12.96)	93.67 (11.32)	84.92 (21.29)	1	0.1026	0.024
≤50%	6.95 (12.87)	6.30 (11.33)	14.04 (20.59)	1	0.1856	0.0621
≥50%	0.04 (0.36)	0.03 (0.28)	1.04 (4.62)	1	1	1
Tuft necrosis						
Absent	99.11 (2.49)	97.37 (6.46)	97.37 (6.08)	0.5966	0.3375	1
Present	0.89 (2.49)	2.63 (6.46)	2.63 (6.08)	0.5966	0.3375	1
Karyorrhexis						
Absent	90.52 (15.14)	80.43 (19.92)	72.98 (25.39)	0.01	<0.001	0.4932
Present	9.48 (15.14)	19.57 (19.92)	27.02 (25.39)	<0.001	<0.001	0.4932

Mean (SD) and p value resulting from univariate logistic models were reported.
*Integrating each class with the mixed +V.
†P value with Holms adjustment for multiple comparisons are reported.

categorisation that better reflect pathogenic mechanisms, could be useful or even surpass the classification as we know it. In this study, we investigated whether an analysis of detailed histopathology data obtained from a large cohort of cases enrolled in the BLISS-LN trial would reveal new differences among classes and subclasses of LN.

Our results showed higher values of CI in mixed as compared with isolated III/IV classes, suggesting that the presence of a concurrent class V can worsen the chronic changes of the kidney. This is in line with the most recent evidence in the literature pointing out a worse outcome of mixed as compared with 'pure' cases.[13 14] Most interestingly, an alternative clustering for pure focal/diffuse classes was found after supervised and unsupervised analysis, with a dichotomous subdivision in MPGN-like and vasculitis-like forms based on morphological features. This goes back to an original division which could never be substantiated in terms of clear-cut histological parameters, but is characterised by a prevalence of global lesions, lower frequency of segmental lesions and higher presence of hyaline deposits on the one hand (MPGN-like pattern) and the prevalence of more segmental lesions, crescents and fibrinoid necrosis (vasculitis-like) on the other hand. In the LN classification, a subtle distinction by light microscopy described as IVG as compared with IVS, alludes to this difference.[15] Also other studies have alluded to this distinction, for instance by reporting more commonly WL in the IVG group.[16] Conversely, combined lesions with segmental endocapillary proliferation and fibrinoid necrosis were reported more frequently in class IV-S LN, and the percentage of glomeruli with cellular crescents was also reported to be more common in the IVS group.[16] However, in these studies the prevalence of cellular

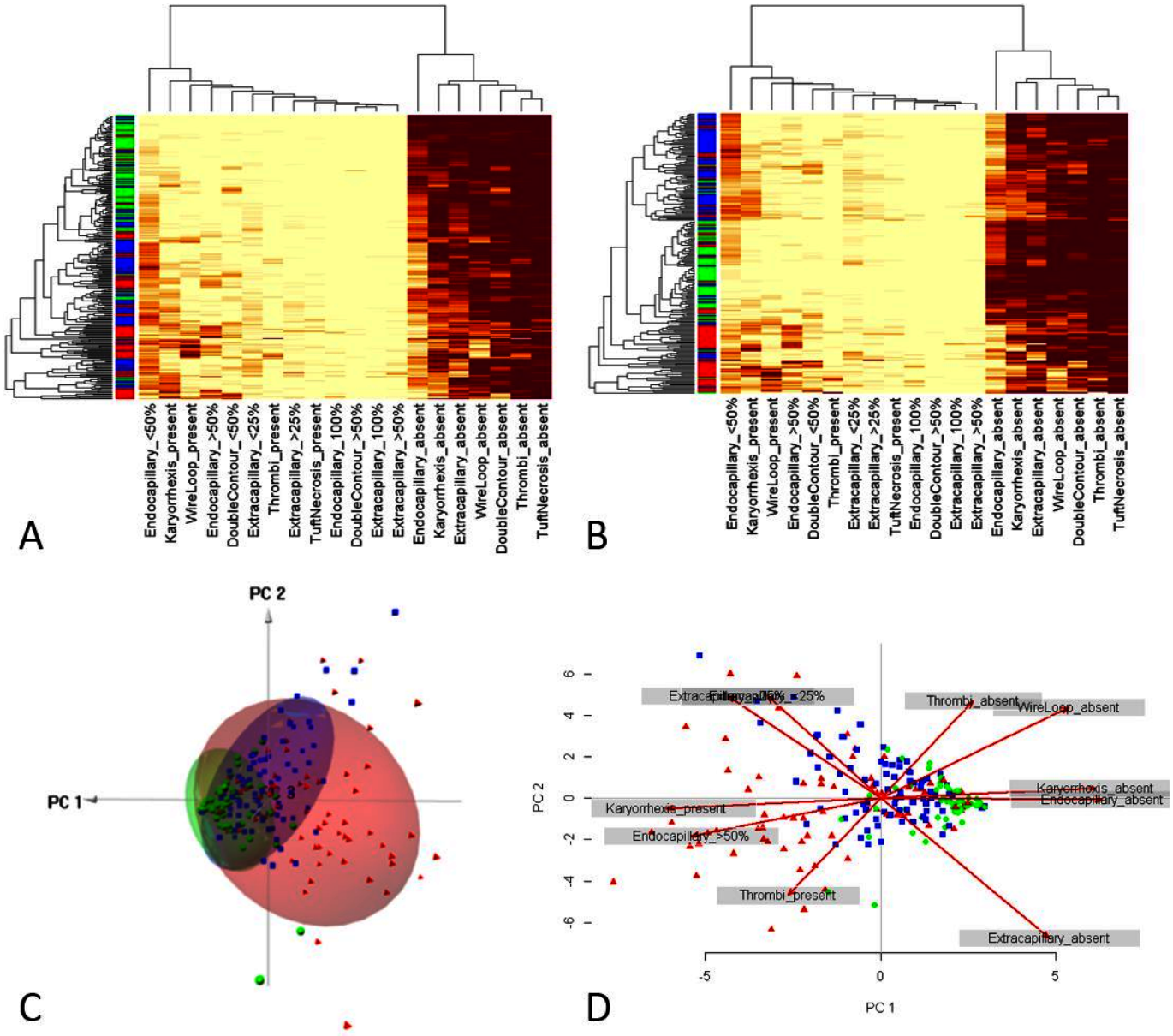

Figure 3 Heatmap of the different histological variables evaluated in the subset of cases with a final diagnosis of class III (green, n=86), IVS (blue, n=106) and IVG (red, n=82) incorporating (A) or not (B–D) the mixed classes (+V). In this last group, the distribution of the cases in PCA analysis demonstrated a compartmentalisation of green dots (pure class III, n=61) and blue (pure class IVS, n=83) with a spread distribution of red dots (pure class IVG, n=70), with some cases intersecting among classes, especially for class IVS (C). The same PCA analysed for the variables that mainly dictates the distribution of the cases shows that the most impactful histological features overlap with those that better differentiated the class III, IVS and IVG in the univariate regression analysis (D). PCA, principal component analysis.

crescents and EH did not reach a statistical relevance. The notion of different morphological aspects in LN with focal and segmental lesions led some authors to postulate a more 'vasculitic' nature of LN[17] in comparison to an MPGN-like pattern. In this setting, a re-evaluation of the classification in 2015 by the ISN/RPS working group stressed the need of new data to shed light on potential differences between either vasculitic-like or MPGN-like patterns, in order to propose eventual modifications in the classes and subclasses.[18] In this study, we found evidence through a cluster analysis for such a division, which was confirmed by re-evaluation of the biopsies. Moreover, as compared with the currently used ISN/RPS classification that did not show significantly different distribution of the AI among classes/subclasses, the application of the newly proposed clusters demonstrated a better segregation of cases with highly active lesions (MPGN-like), suggesting a better capability of this proposal to identify patients with potentially better responses to immunosuppressive therapies. However, cluster analysis per se cannot investigate whether such a division has clinical relevance, which is an important limitation of this study. Moreover, there is increasing evidence pointing to an active role for tubulointerstitial lesions in LN as crucial for the final prognosis, as already demonstrated by other large and independent cohorts,[19] which is also reflected by the revised 2018 ISN/RPS classification emphasising the employment of AI/CI for every biopsy in the report. It should be taken into account, however, that in this study, none of the tubulointerstitial histological features evaluated by the board demonstrated its relevance under both supervised and unsupervised analysis, maybe due to a relative lack of detail in the scoring process. For this reason, in the

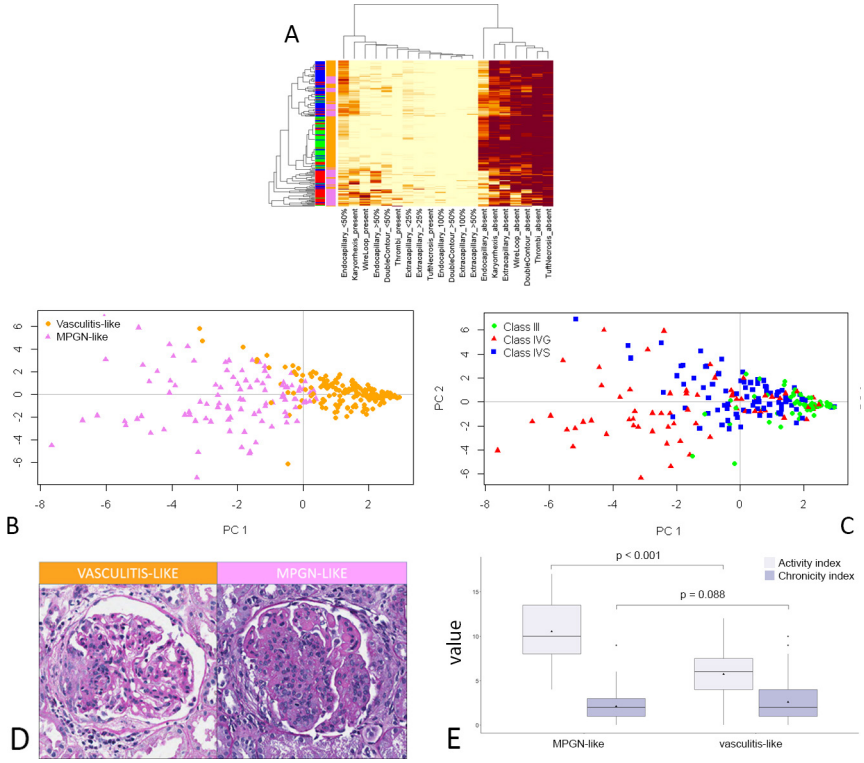

Figure 4 The heatmap (A) shown recapitulate the findings reported in figure 3B with a further subdivision of cases following the clustering suggested by KMeans analysis, for which the majority of 'pure' class III reclassified in orange, class IVG in violet and class IVS being containing a mixture of orange/violet cases. The comparison of the PCAs with the new clustering (B) and ISN/RPS classification (C) demonstrates a better segregation of cases with the former grouping. The histological aspects that characterise the newly defined group are depicted in (D), with the orange group characterised by mainly segmental EH in the absence of WL, DC and HT (vasculitis-like aspects, n=131) as opposite to the violet group, characterised by the presence of global EH, WL, DC and HT (MPGN-like aspects, n=83). Finally, a comparison of the AI and CI between the newly discovered clusters is reported in (E). DC, double contour; EH, endocapillary hypercellularity; HT, hyaline thrombi; ISN/RPS, International Society of Nephrology/Renal Pathology Society; PCA, principal component analysis; WL, wire loops.

future, a further and more detailed additional analysis of this compartment could allow a more in depth understanding of the pathobiological relationship between interstitial infiltrates, their composition and the clusters found here.

For its potential introduction in the routine assessment of LN renal biopsies, a validation involving clinical data including clinical follow-up data is required, to further look into the superiority/non-inferiority of the traditional LN classes. Moreover, the application of the proposed clustering on an alternative and independent cohort of cases by other expert renal pathologists would further strengthen the value of the proposed subdivision. This is of paramount importance, since such a change in the current paradigm would certainly imply a modification of the mindset in clinical practice, moving towards a specific study of different phenotypes. Finally, this would significantly impact on the design of future LN clinical trials, stressing the need for a more 'pathogenesis oriented' look at the therapeutic approach.

CONCLUSIONS

This study gives new insights into different phenotypic forms of LN thereby challenging the traditional classification scheme. This may be the starting point of further investigations focused on how to incorporate new subdivisions into a classification system that would better guide patient-tailored therapies in the near future.

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