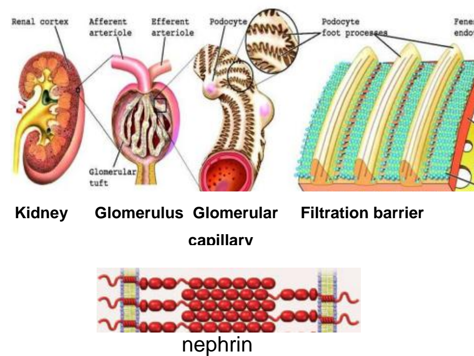
Thesis for doctoral degree (Ph.D)

2011

Structure and function of glomerular epithelial cells in experimental lupus nephritis



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Ιατρός

Ηράκλειο 2011

Πρόλογος

Η εκπόνηση της παρούσας διδακτορικής διατριβής αποτελεί έργο ομαδικής εργασίας που επιτελέστηκε με μεράκι και αγάπη για τον άνθρωπο και την επιστήμη, στο εργαστήριο Νεφρολογίας του Πανεπιστημίου Κρήτης. Μου έμαθε να μελετώ σε βάθος και πλάτος, να θέτω ερωτήματα, να μεθοδεύω την εξεύρεση απάντησης, να αποτυγχάνω και να μην απογοητεύομαι αλλά να ξαναπροσπαθώ. Ακόμη πιο σημαντικό μου έδωσε τη δυνατότητα να συναναστραφώ σημαντικούς επιστήμονες και αγαπητούς συνεργάτες, φίλους που στάθηκαν κοντά μου πάντοτε στην καθημερινή και προσωπική ζωή και μοιράστηκαν μαζί μου απλόχερα τις επιστημονικές τους γνώσεις.

Η νεφρολογία είναι για μένα συναρπαστική γιατί συνδυάζει γνώσεις, δεξιότητες και ερωτήματα της κλινικής ιατρικής, της βιοχημείας, της μοριακής βιολογίας, της γενετικής, της ανοσολογίας. Η νεφρίτιδα του λύκου αποτελεί πρότυπο μοντέλο σπειραματονεφρίτιδας ανοσολογικής αρχής και συγκεντρώνει πάντα μεγάλο επιστημονικό και ερευνητικό ενδιαφέρον.

Θα ήθελα καταρχήν να ευχαριστήσω τον καθηγητή Νεφρολογίας κ Δαφνή που μου εμπιστεύτηκε την εκπόνηση αυτής της διατριβής σε μία περίοδο που το εργαστήριο Νεφρολογίας ήταν ακόμα πολύ νέο σε χρόνια και εμπειρία. Στον κ Δαφνή οφείλεται η σύλληψη του θέματος. Πολλά από όσα έγιναν δεν θα είχαν επιτευχθεί χωρίς την προσωπική επίμονη, ακούραστη συνδρομή και καθοδήγησή του.

Ιδιαίτερη ευχαριστία και ευγνωμοσύνη για την εκπόνηση και ολοκλήρωση αυτής της μελέτης, για την κατάρτισή μου αλλά και για τη στάση μου απέναντι στον ασθενή και την επιστήμη οφείλω στον καθηγητή Παθολογίας κ. Μπούμπα. Ο κ. Μπούμπας αποτελεί τον πνευματικό πατέρα όλων εμάς που ξεκινήσαμε την παθολογία στο Πανεπιστημιακό Νοσοκομείο Ηρακλείου μετά το θάνατο του καθηγητή Εμμανουήλ. Μας έμαθε να αναζητούμε πάντοτε την αριστεία στην καθημερνή κλινική μας πράξη, να αγαπούμε την έρευνα και να νοιαζόμαστε πάνω απ' όλα για τον άνθρωπο.

Θερμές ευχαριστίες στον καθηγητή Δημήτριο Καρδάση που έσκυψε με ενδιαφέρον στα προβλήματα της μελέτης και η υποστήριξη του ήταν καθοριστική για την αποπεράτωση της διατριβής.

Ιδιαιτέρως θα ήθελα να ευχαριστήσω την καθηγήτρια Παθολογικής Ανατομικής του Πανεπιστημίου Αθηνών κ. Νακοπούλου, τη συνεργάτιδά της, κ Γ. Γιαννοπούλου και τους τεχνολόγους του εργαστηρίου κ Κ. Σκεμπάρη και κ Σ. Μαβίδη. Οι συζητήσεις μαζί τους ήταν πάντοτε πολύ βοηθητικές και οι εικόνες πολύ σημαντικές για την κατανόηση των αποτελεσμάτων των πειραμάτων και την αρτιότερη εικόνα του άρθρου.

Δεν θα μπορέσω ποτέ να ξεχάσω τη συμβολή του Δρ Κυριάκου, διευθυντή του Τμήματος Ηλεκτρονικού Μικροσκοπίου /Μοριακής Παθολογοανατομίας του Ινστιτούτου Νευρολογίας και Γενετική της Κύπρου από την

αρχή της μελέτης ως το τέλος. Η βοήθειά του ήταν πολύ σημαντική και έμπρακτη, αφού πολλές φορές μας επισκέφτηκε στο εργαστήριό μας και δουλέψαμε μαζί παρασκευάζοντας τις βιοψίες.

Ευχαριστώ ακόμη τους αγαπητούς συναδέλφους, και φίλους Δημήτρη Μωυσιάδη και Γιώργο Μπερτσιά. Με τον κ Μωυσιάδη συνεργαστήκαμε αρμονικά τις ατελείωτες ώρες των πειραμάτων και αργότερα στη συγγραφή του άρθρου. Στον κ Μπερτσιά οφείλω την εύστοχή επιμελή και ακούραστη κριτική και καθοδήγηση στη συγγραφή του άρθρου. Χωρίς τη βοήθεια και των δύο η διατριβή δε θα είχε ολοκληρωθεί.

Ευχαριστώ επίσης τον Δρ Rakesh Verma και τον καθηγητή Larence B. Holzman από το τμήμα Ιατρικής του Πανεπιστημίου του Michigan που μας δώρισαν το αντίσωμα κατά τη νεφρίνης και μας δίδαξαν τις μεθόδους επεξεργασίας των νεφρικών σπειραμάτων.

Ευχαριστώ το εργαστήριο Ογκολογίας, την υπεύθυνη κ Μαρία Περάκη, τις ερευνήτριες κ. Αλεξάνδρα Βουτσινά και κ Μαρία Σπηλιωτάκη αλλά και όλο το προσωπικό. Αποτέλεσε για μένα μία δροσερή όαση γνώσεων στις πειραματικές τεχνικές. Περάσαμε πολλές όμορφες ώρες μαζί κατά τη διάρκεια των πειραμάτων όσο και στα διαλλείματα μεταξύ αυτών.

Ευχαριστώ το εργαστήριο Ρευματολογίας, Κλινικής Ανοσολογίας και Αλλεργιολογίας του Πανεπιστημίου Κρήτης και ιδιαιτέρως την ερευνήτρια κ. Χριστιάνα Χουλάκη η οποία μας δίδαξε την real time RT-PCR και σχεδίασε τους primers των νεφρίνης και GAPDH. Ακόμη ευχαριστώ την κ Χριστίνα Καστρινάκη για το σχεδιασμό των primers της ποδοσίνης

Θα ήταν παράλειψη να μην αναφερθώ στην Ελληνική Νεφρολογική Εταιρεία που μας έδωσε μεγάλο κουράγιο να συνεχίσουμε, αναγνωρίζοντας και επιβραβεύοντας την ερευνά μας με το πρώτο βραβείο στο 15° Πανελλήνιο Συνέδριο Νεφρολογίας όπου παρουσιάσαμε τα πρώτα ερευνητικά μας αποτελέσματα.

Ένα μεγάλο ευχαριστώ στους συναδέλφους από τη Νεφρολογική Κλινική του Γενικού Νοσοκομείου Ρεθύμνης, κ. Νικόλαο Νικολακάκη, πρώην διευθυντή, κ Ιωάννη Παρασύρη, διευθυντή, κ Ειρήνη Νταουντάκη, επιμελήτρια Α, κ Παναγιώτα Παπαδάκη, κ Ανδρέα Πάσσαμ νεφρολόγους, κ Αντώνιο Παπουτσάκη, κ. Κων/νο Τσουλνάρα αγροτικούς ιατρούς. Με περιέβαλαν με αγάπη και συναδελφικότητα. Προσπάθησαν να μου εξασφαλίζουν συνεχώς ευκαιρίες για να εστιαστώ στην ολοκλήρωση της διατριβής, αναλαμβάνοντας πολλές φορές δυσανάλογο μέρος του κλινικού έργου.

Τέλος ιδιαιτέρως θα ήθελα να ευχαριστήσω τα παιδιά μου Σοφία και Χρήστο και το σύζυγό μου ιατρό παθολόγο κ. Συμεών Παναγιωτάκη για την ανοχή, υποστήριξη και αγάπη που έδειξαν όλα αυτά τα χρόνια μέχρι την αποπεράτωση του πονήματος αυτού. Χωρίς τη δική τους άδεια και ενίσχυση δε θα είχα καν αρχίσει. Ευχαριστώ επίσης τους γονείς μου Σάββα και Μαρία για την ακούραστη βοήθεια και φροντίδα τους τόσο απέναντί μου όσο και προς τα παιδιά μου κατά την πολύωρη απουσία μου στο εργαστήριο, καθώς και τον πνευματικό μου πατέρα για την ουσιαστική ενίσχυση και συμπαράστασή του.

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1. ABSTRACT

The slit diaphragms (SD) that cover the slit pores among adjacent foot processes (FP) of the podocytes are crucial constituent of the primary barrier for ultrafiltration of plasma in renal glomeruli.

A putative distraction of the SDs, of its main components, nephrin and podocin is involved in the immune mediated glomerulonephritis with proteinuria. We undertook an animal and human study to determine the expression of nephrin and podocin in the various forms of lupus nephritis (LN), a prototype of autoimmune disease with a various histological and clinical picture with proteinuria been a major clinical manifestation.

The kidneys were examined by light microscopy (LM), and IgG immunofluorescence in order to determine the LN characteristics. Electron microscopy was also performed. In view of electron microscopy findings, we next assessed the glomerular expression of total nephrin and podocin at the protein level by western blot analysis in normal (n=11), MMLN: mild mesangial hyperplasia (n=5), FPLN: focal (n=6) and DPLN: diffuse (n=4) proliferative nephritis in NZB/W females and of age and sex matched C57BL/6 controls (n=24) as well as in NZB (n=5) and NZW (n=4) mice. Nephrin expression was also examined by immunofluorescence in kidney biopsy specimens. To determine whether alterations to protein expression of nephrin and podocin are due to altered transcription of the corresponding genes, we performed quantitative real-time PCR in the same kidney samples. In order to verify our findings in murine LN we performed immunohistochemistry for nephrin expression in renal tissue from patients with various classes of LN: class II (n=5), IV (n=4), V (n=7) and healthy controls (n=2).

Data are expressed as the mean ± standard error of the mean (SEM) value. Comparisons were performed with the Mann–Whitney U-test (numerical data) or the Fisher's exact test (categorical data).

The SD alterations determined by electron microscopy correlate with histology in NZB/W F1 mice. The severity of mesangial, subendothelial and subepithelial deposits, as well as FP effacement findings were significantly associated with 24-hr proteinuria (ρ = 0.85, p<0.003)

and IgG intensity (ρ = 0.90, p<0.001). Western blot and immunofluorescence showed significantly reduced nephrin protein expression in mice with FPLN or DPLN (p<0.05 for all pair-wise comparisons). Podocin protein expression was also significantly decreased in the same groups (p<0.05). Both proteins were significantly reduced in NZB/W mice with extensive FP effacement (p<0.05 for both). Glomerular nephrin and podocin mRNA levels were significantly reduced in DPLN - but not in FPLN - (p=0.034). We found a good correlation between glomerular nephrin and podocin mRNA levels in all NZB/W mice (ρ = 0.77, p<0.001). One NZB mouse showed focal proliferative nephritis while the rest NZB mice and all the NZW mice showed normal LM findings however, they exhibit significant 24-hr proteinuria. Podocin protein was reduced in the NZB and in the NZW as compared to NZB/W with normal LM findings (p= 0.024, p=0.030 respectively) and with MMLN (p= 0.034 for both NZB and NZW). Significantly more patients with DPLN had reduced nephrin expression as compared to patients with class II or V LN (p < 0.05).

Our study represents the first comprehensive analysis of podocin and nephrin expression at protein and mRNA level coupled with immunofluorescence findings in murine and human LN. Glomerular nephrin protein levels were reduced by almost 50% in mild LN (MMLN) and became diminished at more advanced stages (FPLN/DPLN). Podocin is affected only in the most serious forms of the disease (FPLN/DPLN). Nephrin and podocin protein expression correlate with the severity of the disease histology. This coupled with the significant reduction of nephrin and podocin glomerular mRNA only in the most advanced stages (DPLN) may imply that the SD alterations at the protein level precede the alterations in the mRNA level. The mRNA level is finally affected only in the most sever forms of the disease. The previously regarded healthy ancestors of the NZB/W, especially the males NZW were found with ultrastructural SD alterations regarding reduced podocin which might is a co-factor contributing to NZB/W predisposition to renal disease. We found significantly reduced nephrin expression in patients with class IV proliferative disease as compared to class V patients, despite both groups having comparable levels of proteinuria. These results confirm our findings in murine LN, suggesting that nephrin expression correlates better with the histological class than with the level of proteinuria. Our findings suggest a novel role of podocytes and their structures in immune-mediated nephritis.

ΠΕΡΙΛΗΨΗ

Οι λεπτές μεμβράνες (ΛΜ) που καλύπτουν τους πόρους μεταξύ των ποδικών εκβλαστήσεων (ΠΕ) γειτονικών ποδοκυττάρων είναι βασικές δομές του σπειραματικού ηθμού όπου γίνεται η υπερδιήθηση του πλάσματος στο νεφρικό σπείραμα.

Πιθανή διαταραχή στις ΛΜ και στα βασικά συστατικά τους, τη νεφρίνη και την ποδοσίνη, ενέχεται στην παθογένεια της σπειραματονεφρίτιδας με πρωτεϊνουρία που οφείλεται σε ανοσολογικούς μηχανισμούς. Διεξήγαμε μελέτη σε πειραματόζωα και σε ανθρώπους με σκοπό να καθορίσουμε της έκφραση της νεφρίνης και της ποδοσίνης στις διάφορες ιστολογικές μορφές της νεφρίτιδας του λύκου (ΝΛ), μια νόσο πρότυπο για τις νεφρίτιδες με αυτοάνοση αρχή, της οποίας χαρακτηριστική κλινική εκδήλωση είναι η πρωτεϊνουρία.

Οι νεφροί εξετάστηκαν με φωτονικό μικροσκόπιο (ΦΜ) και με ανοσοφθορισμό IgG για να προσδιοριστούν τα χαρακτηριστικά της ΝΛ. Η εξέταση συμπληρώθηκε και με ηλεκτρονική μικροσκοπία. Βασιζόμενοι στα ευρήματα από την εξέταση με το ηλεκτρονικό μικροσκόπιο, προσδιορίσαμε στη συνέχεια τη σπειραματική έκφραση της ολικής νεφρίνης και ποδοσίνης σε πρωτεϊνικό επίπεδο με ανάλυση western blot σε θηλυκά NZB/W ποντίκια, φυσιολογικά στο ΦΜ (n=11), με ήπια μεσαγγειακή υπερπλασία (ΗΜΝΛ) (n=5), με εστιακή (ΕΥΝΛ) (n=6) και διάχυτη υπερπλαστική νεφρίτιδα (ΔΥΝΛ) (n=4) καθώς και σε ποντίκια C57BL/6 (n=24) αντίστοιχης ηλικίας και φύλου που αποτέλεσαν την ομάδα ελέγχου, όπως επίσης και σε NZB (n=5) και NZW (n=4) ποντίκια. Η έκφραση της νεφρίνης εξετάστηκε επίσης με ανοσοφθορισμό στα παρασκευάσματα του νεφρικού ιστού. Για να προσδιορίσουμε εάν οι μεταβολές της νεφρίνης και της ποδοσίνης οφείλονται σε μεταβολή της μεταγραφής των αντίστοιχων γονιδίων, προσδιορίσαμε το mRNA στα ίδια δείγματα, με ποσοτική αντίστροφη real time PCR. Προκειμένου να επιβεβαιώσουμε τα ευρήματα μας στα πειραματόζωα εξετάσαμε με ανοσοϊστοχημεία την έκφραση της νεφρίνης σε ασθενείς με διάφορους τύπους ΝΛ: τάξης ΙΙ (n=5), IV (n=4), V (n=7) και σε υγιείς μάρτυρες (n=2).

Τα αποτελέσματα εκφράζονται ως μέση τιμή ± τυπικό σφάλμα της μέσης τιμής (SEM). Για τις συγκρίσεις χρησιμοποιήθηκαν οι έλεγχοι Mann-Whitney U-test (αριθμητικές μεταβλητές) και το Fisher’s exact test (κατηγορικές μεταβλητές).

Οι μεταβολές στα ΛΔ, όπως προσδιορίστηκαν με την εξέταση στο ηλεκτρονικό μικροσκόπιο συσχετίζονται με την ιστολογική εικόνα στα θηλυκά NZB/W ποντίκια. Η σοβαρότητα των μεσαγγειακών, υπό-ενδοθηλιακών και υπό-επιθηλιακών εναποθέσεων, καθώς και η εξάλειψη των ΠΕ συσχετίζονταν σημαντικά με την 24ωρη πρωτεϊνουρία. (ρ = 0.85, p<0.003) και την ένταση του ανοσοφθορισμού IgG (ρ = 0.90, p<0.001). Η ανάλυσεις με Western blot και ανοσοφθορισμό ανέδειξαν σημαντικά μειωμένα επίπεδα νεφρίνης στα ποντίκια με ΕΥΝΛ ή ΔΥΝΛ (p<0.05 για όλες τις ανά ζεύγη συγκρίσεις). Τα επίπεδα ποδοσίνης ήταν επίσης σημαντικά μειωμένα στις ίδιες ομάδες ποντικιών (p<0.05). Τα επίπεδα και των δύο πρωτεϊνών ήταν σημαντικά μειωμένα στα ποντίκια NZB/W με εκτεταμένη εξάλειψη ΠΕ (p<0.05 και για τις δύο). Τα επίπεδα του σπειραματικού mRNA της νεφρίνης και της ποδοσίνης ήταν σημαντικά μειωμένα στην ΔΥΝΛ – αλλά όχι στη ΕΥΝΛ (p=0.034). Βρήκαμε καλή συσχέτιση μεταξύ των επίπεδων mRNA νεφρίνης και ποδοσίνης σε όλα τα ποντίκια NZB/W (ρ = 0.77, p<0.001). Από τα ποντίκια γονείς, ένα NZB ποντίκι παρουσίασε ΕΥΝΛ ενώ όλα τα υπόλοιπα NZB και όλα τα NZW ποντίκια παρουσίασαν φυσιολογικά ευρήματα στο φωτονικό μικροσκόπιο. Εντούτοις όλα εκδήλωσαν σημαντική πρωτεϊνουρία στο 24ώρο. Τα επίπεδα ποδοσίνης ανευρέθηκαν μειωμένα στα NZB και NZW σε σύγκριση με τα NZB/W που έχουν φυσιολογική ιστολογία στο φωτονικό μικροσκόπιο (p= 0.024 , p=0.030 respectively) και εικόνα με ΗΜΝΛ (p= 0.034 για NZB και NZW). Στις ανθρώπινες βιοψίες, σημαντικά περισσότεροι ασθενείς με ΔΥΝΛ παρουσίαζαν μειωμένη έκφραση νεφρίνης σε σύγκριση με τους ασθενείς με ΝΛ τάξης II ή V LN (p < 0.05).

Η μελέτη μας αποτελεί την πρώτη συνοπτική ανάλυση που αφορά την έκφραση της νεφρίνης και της ποδοσίνης σε πειραματόζωα και ανθρώπους με ΝΛ. Οι παραπάνω πρωτεΐνες προσδιορίζονται σε πρωτεϊνικό επίπεδο και σε επίπεδο mRNA και τα ευρήματα διασταυρώνονται με ανοσοφθορισμό στις βιοψίες. Βρήκαμε ότι τα επίπεδα της νεφρίνης στο σπείραμα ήταν μειωμένα κατά σχεδόν 50% στην ήπια ΝΛ (ΗΜΝΛ) και πολύ μειωμένα σε σοβαρότερες μορφές της νόσου (ΕΥΝΛ/ΔΥΝΛ). Τα επίπεδα της ποδοσίνης είναι επηρεασμένα μόνο στις πιο σοβαρές μορφές της νόσου (ΕΥΝΛ/ΔΥΝΛ). Οι μεταβολές στη νεφρίνη και στην ποδοσίνη σχετίζονται με τη σοβαρότητα της ιστολογικής εικόνας. Τα παραπάνω ευρήματα σε συνδυασμό με τα εξίσου σημαντικά μειωμένα επίπεδα σπειραματικού mRNA των αντίστοιχων πρωτεϊνών στα μόνο πιο προχωρημένα στάδια της

νόσου (ΔΥΝΛ), ίσως υποδηλώνουν ότι οι μεταβολές στις ΛΜ συμβαίνουν καταρχήν σε επίπεδο πρωτεΐνης και ακολούθως σε επίπεδο μεταγραφής της πρωτεΐνης. Οι μέχρι τώρα θεωρούμενοι φυσιολογικοί γονείς των NZBW και ιδιαιτέρως τα άρρενα ποντίκια NZW παρουσίαζαν στη μελέτη μας δομικές αλλοιώσεις των ΛΜ που σχετίζονταν με μειωμένα επίπεδα ποδοσίνης. Το εύρημα αυτό πιθανά σχετίζεται με την προδιάθεση των απογόνων τους NZB/W για νεφρική βλάβη. Βρήκαμε επίσης σημαντικά μειωμένη έκφραση νεφρίνης σε ασθενείς με τάξης IV υπερπλαστική νόσο σε σχέση με ασθενείς με τάξης V νεφρίτιδα παρά το γεγονός ότι και οι δύο έχουν συγκρίσιμα ποσά πρωτεϊνουρίας. Τα αποτελέσματα αυτά επιβεβαιώνουν τα ευρήματά μας στα πειραματόζωα προτείνοντας ότι τα επίπεδα της νεφρίνης συσχετίζονται καλύτερα με την ιστολογική τάξη της νεφρίτιδας παρά με το βαθμό της πρωτεϊνουρίας. Τα ευρήματά μας υπαινίσσονται ένα νέο ρόλο των ποδοκυττάρων και των δομών τους στη σπειραματονεφρίτιδα ανοσολογικής αρχής.

Abbreviations

ANCA	anti-neutrophil cytoplasmic antibody
AT	angiotensin II receptors type
ATRA	all-trans-retinoic acid
CD2AP	CD 2 associated protein
CDK	cyclin dependent kinase
CFH	complement factor H
CNF	congenital nephrotic syndrome
CR	complement receptor
DAF	decay accelarating factor
DNA	deoxyribonucleic acid
DPLN	diffuse proliferative lupus nephritis
EMT	epithelial-mesenchymal transition
FP	foot process
FPLN	focal proliferative lupus nephritis
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
HCN	hepatitis C virus
HIVAN	HIV associated nephropathy
IC	immune complex
ICAM	intercellular adhesion molecule
IgAN	IgA nephropathy
IL	interleukin
LN	lupus nephritis
MAC	membrane attack complex
Mapk	mitogen-activated protein kinase
MCD	minimal change disease
MCP	membrane cofactor protein
MCP-1	macrophage chemoattractant protein 1
MIF	macrophage inhibitory factor
MMLN	mild mesangial lupus nephritis
MN	membranous nephropathy
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
Nck	adaptor protein, known to associate with proteins of the actin polymerization complex
PAN	puromycin aminonucleoside nephrosis
PHN	passive Heymann nephritis
RANTES	Regulated upon Activation Normal T cell Expressed and Secreted
ROS	reactive oxygen species
SD	slit diaphragm
SLE	systemic lupus erythematosus
SV2B	synaptic vesicle protein 2B
TGF	transforming growth factor (-β)
TLR	toll-like receptor
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor

1. INTRODUCTION

3.1 Basics of kidney anatomy

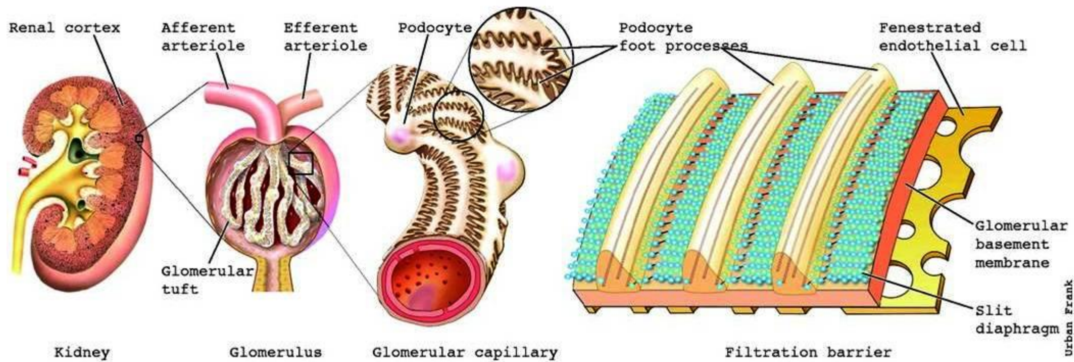

Figure 3.1 Basics of kidney anatomy

The kidneys are two bean-shaped organs lying in the retroperitoneal space, each weighing about 150g. They consist of highly specialized cells. The outer layer of the kidney is called cortex. The inner section is called medulla. The functional unit of the kidney is called nephron. There are 1 million nephrons in one human kidney. Each nephron consists of a glomerulus and a long tubule. The tubule is segmented into distinct parts – proximal tubule, loop of Henle, distal tubule, collecting duct. Glomeruli are spherical 'bags' of capillaries. The capillaries are partially attached to the mesangium, consisting of mesangial cells and the matrix. The free wall of glomerular capillaries across which filtration takes place, consists of a basement membrane covered by visceral epithelial cells (podocytes) with individual foot processes (FP) and lined by endothelial cells. The blood is filtrated through the glomerular capillary wall which is consisted of three layers: the fenestrated endothelial cell, the glomerular basement membrane (GBM) and the slit diaphragm (SD) that bridge the slit pores between the FPs of adjacent podocytes. Each glomerulus is enclosed within an epithelial cell capsule (Bowman's capsule) that is continuous with the proximal convoluted tubule. The glomeruli, much of the proximal and distal tubules are located in the cortex. The loops of Henle and the collected ducts are located in the medulla.

The renal artery carries the one-fifth of the cardiac output. The renal artery bifurcates several times after in enters the kidney and then brakes into the arcuate arteries which give rise, at right angles, to interlobular arteries from which come off the afferent arterioles. Blood reaches glomerulus through the afferent arteriole that forms the glomerular capillaries (hemi-arterioles of the glomerular capillary tuft) consecutively emanating to efferent arteriole. The efferent arterioles reform and collect to form the post-glomerular circulation (peritubular capillaries, venules and renal vein) [1-3]. (**Figure 3.1**)

There is a broad range of kidney diseases which are categorized according to the primary affected structures (e.g. glomerulonephritis, tubulointerstitial nephritis when glomerulus or the tubules respectively are is the primary affected structures). They are also categorize primary and secondary according to their etiology [e.g. minimal change disease (MCD) is a primary glomerulonephritis but lupus nephritis (LN) is a secondary one caused by systemic lupus erythematosus].

We will focus on kidney disease affecting the glomerulus, LN with special interest for the podocyte SD.

3.2 Basics of kidney function

The main function of the kidneys can be categorized as follows: (1) Maintenance of body composition (e.g. volume of body fluid its osmolarity, electrolyte content, restriction the passage of large molecules such as albumin). (2) Excretion of metabolic end products and foreign substances (e.g urea, toxins, drugs) (3) Production and secretion of enzymes and hormones (e.g. rennin, Angiotensin, erythropoietin, 1,25-dihydroxyvitamin D_3) [1].

3.3 Glomerulonephritis

3.3.1 Pathogenesis of glomerulonephritis

The term glomerulonephritis refers to a broad range of disease with different etiology, clinical and histological features characteristic for each type (**Figure 3.2, Box 3.1**) [4]. The histological and laboratory findings suggest that immune mediated mechanisms are implicated in the pathogenesis of glomerulonephritis. The presence of

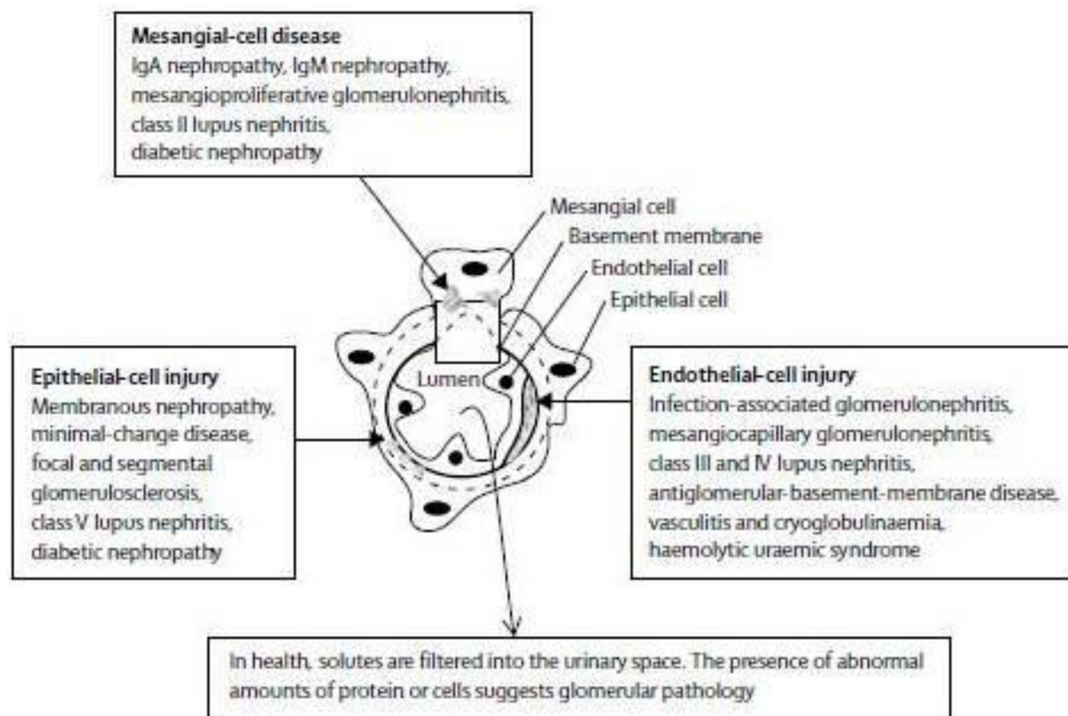

Figure 3.2 Cellular location of injury during glomerulonephritis

immunoglobulins and complement is prominent glomerulonephritis referred as immune mediated [5]. Additionally, in pauci-immune glomerulonephritis lymphocyte and macrophage infiltrations are observed. Thus, both innate and adaptive immunity mechanisms are involved. Typical examples of innate immunity activation are the postinfectious glomerulonephritis, the IgA glomerulonephritis (IgAN), the membranous nephropathy (MN), the LN. Examples of adaptive immunity activation are the MCD, the focal segmental glomerulosclerosis (FSGS), the crescentic glomerulonephritis. Although the type of insult remains mostly unknown, it is

becoming increasingly apparent that most glomerulonephritis are autoimmune in nature [6]. Antibodies against normal glomerular structures or against exogenous antigens are involved. Loss of self tolerance [7-8] and mechanisms of epitope spreading are induced and cause glomerulonephritis. It has been suggested that genetic predisposition plays also a central role. Finally the progressive course of kidney disease is due to non-specific factors, such as glomerular hypertension and proteinuria, that might continue kidney damage despite resolution of the intrinsic insult [4].

Glomerular involvement
All glomeruli (diffuse) or only some (focal)
Extent of disease within involved glomeruli: patchy (segmental) or general

Cell involvement
Increases in cell number (proliferative)
Neutrophil accumulation (exudative)

Cell damage
Cell necrosis visible by light microscopy (necrotising)
Ultrastructural damage visible only by electronmicroscopy (foot-process effacement, membrane thinning)

Changes in non-cellular glomerular components
Matrix accumulation (hyalinosis) or immune deposits
Site of deposition (mesangial, subendothelial, subepithelial)

Box 3.1 Pathological classification of GN

In the following paragraphs we shall describe the main immunological and biochemical pathways have been described for the pathogenesis of glomerulonephritis. We shall not refer to the progression of kidney damage to end stage renal disease (ESRD).

3.3.1.a Immune complexes (antibody - antigen)

It is well documented the etiologic role of immune complex (IC) deposition as first event in various forms of glomerulonephritis. Passive trapping of circulating ICs regards the mesangial and subendothelial deposits while in situ formation of ICs regards the subepithelial deposits as well. Typical examples of the first case is human LN and of the second case is the Heymann nephritis (experimental MN) [9-10]. The ICs can attract and activate infiltrating leukocytes or intrinsic glomerular cells to release many local mediators of inflammation, including complement, growth

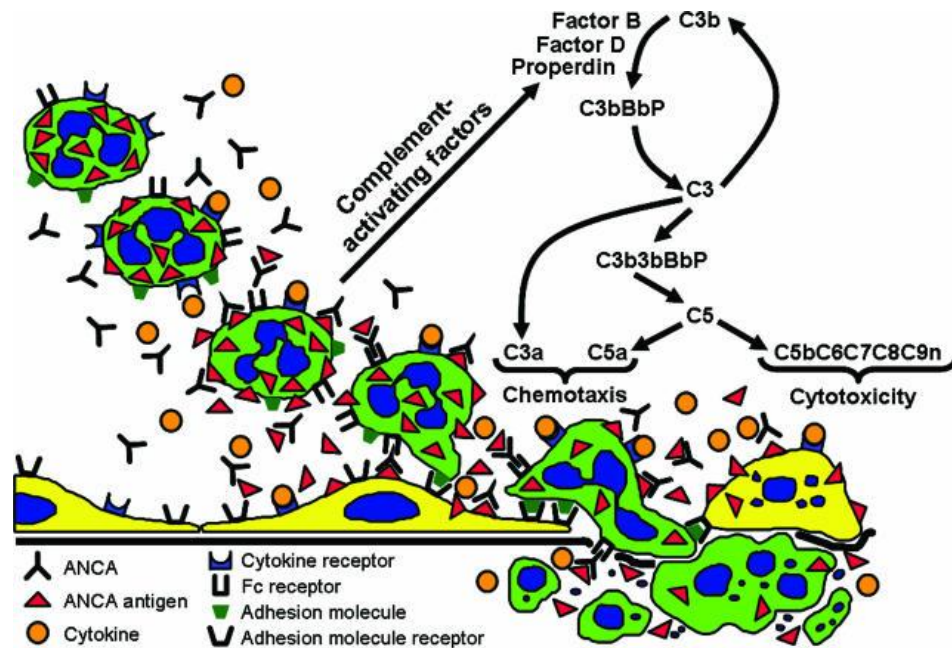

Figure 3.3 Diagram depicting a putative pathogenic mechanism for ANCA GN. Antigens are primed by cytokines to express more ANCA antigens at the surface were they can interact with ANCA antibodies. This results in neutrophil activation both by Fc receptors engagement and Fab´2 binding. ANCA activated neutrophils release factors (e.g. properdin, ROS, proteases) that activate the alternative complement pathway with the generation of the neutrophil chemoattractant C5α and the membrane attack complex C5b-9. This complement activation amplifies neutrophil influx, neutrophil activation, and vessel damage, resulting in aggressive necrotizing inflammation (For the complement activation in the pathogenesis of GN see next paragraph)

factors, vasoactive substances, cytokines and activators of coagulation. Especially the Fc receptors initiate a number of responses. Medium sized ICs that are less cleared than small ICs, and that can activate complement and bind to Fc receptors tend to deposit in tissues and initiate glomerular injury. Goodpasture's disease, cryoglobulinaemic glomerulonephritis due to hepatitis C virus (HCV) infection, anti-neutrophil cytoplasmic antibody (ANCA) vasulitis, IgAN and post-infectious glomerulonephritis are typical examples. Some antibodies bind directly to intrinsic glomerular Ags. Polymeric aberrantly glycosylated IgA1 deposition [11-14] in the mesangium is the hallmark of IgAN. Formation of complexes with soluble IgA Fc receptor I (FcαRI) and priming of monocytes, the mesangial activation by interaction

of IgA1 with mesangial receptors (e.g. TfR) are the essential events in the pathogenesis of IgAN [15-21]. Similarly ANCA activate cytokine primed neutrophils and monocytes through both Fab´2 binding and Fc receptor engagement [22]. Activated neutrophils adhere to and kill endothelial cells. It is speculated that the ANCA antigen may be introduced into the body by an infectious pathogen [23] (**Figure 3.3**). In LN the antigen is endogenous [24] while the MN antigens are still unidentified. Hepatitis B and C, helicobacter pylori Ags, tumor Ags, and thyroglobulin have been detected in subepithelial deposits but there is no proof that they are pathogenic [25-26]. Megalin and neutral endopeptidase both podocyte sole membrane proteins have been recognized as autoantigenic target in Heymann nephritis and neonatal MN respectively [27-29] of a circulating antibody [30-31]. Only recently a conformation-dependent epitope in M-type phospholipase 2 receptor (PLA_2R) has been recognized as the target of IgG4 in idiopathic human MN [32-33]. The NCI domain of α3 chain of type IV collagen is the primary target for circulating and tissue bindings autoantibodies and T-cells in Goodpasture’s disease [34-36]

3.3.1.b Complement activation

Complement is an important component of the innate immune system. Kidney is a passive target for circulating complement and also an active participant in its own injury, with most types of resident cells been able to synthesize complement. Complement causes tissue injury through the generation of chemotactic factors (e.g. C3a, C5a) and mainly through the activation of resident glomerular cells following C5b-9 insertion. Complement components of the classical and of the alternative pathway as well as complement regulatory proteins (CR1, DAF, MCP, CD59) have been detected by immunofluorescence and immunohistochemistry in kidney biopsies as well as by biochemical methods in the serum and urine of patients and animals exhibiting glomerulonephritis (**Figure 3.4**) [37]. Activation with sublytic quantities of C5b-9 results in mesangial cell proliferation [38] and podocyte injury [39], generation

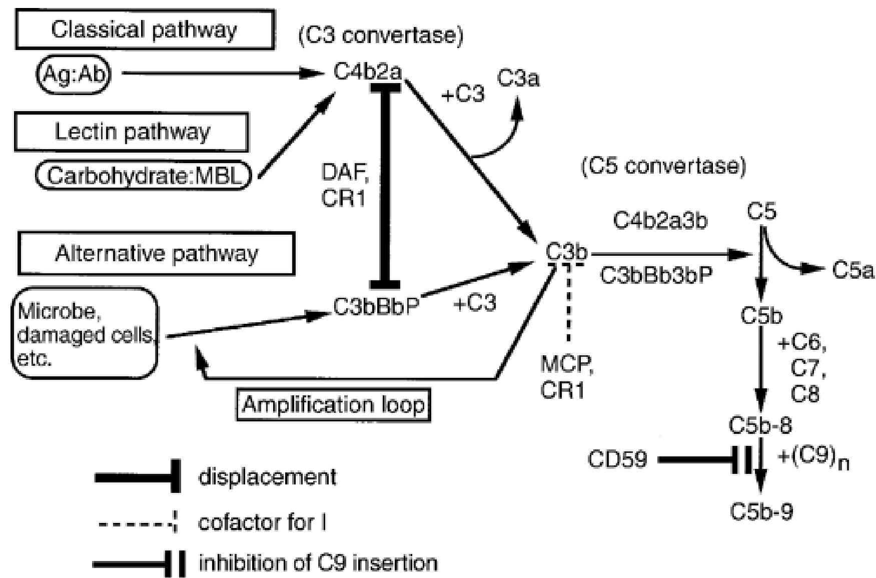

Figure 3.4 Schematic view of complement activation during GN

of inflammatory mediators such as oxidants, proteases, growth factors, prostanoids, and increased production of extracellular matrix. Decay accelerating factor (DAF), membrane cofactor protein (MCP), are upregulated in kidney disease states [40-41], perhaps due to their protective role from complement activation [42]. Complement receptor related protein y (Crry) neutralizes antibodies that exacerbate glomerulonephritis [43-44]. Complement receptors (CRs) are located in hematopoetic cells but CR1 is found in human podocytes. Experimental data suggest decreased synthesis of CR1 in IC renal disease.

3.3.1.c Leucocytes (lymphocytes, macrophages, neutrophils)

Inflammation within the glomerulus cause structural and functional damage. Macrophage, T lymphocyte and neutrophil infiltration are observed in most forms of glomerulonephritis.

Activated macrophages are the predominant inflammatory cells in human and experimental glomerulonephritis, especially those exhibit crescents such as in rapidly progressive glomerulonephritis, LN and cryglobulinemic glomerulonephritis. Their role in glomerulonephritis pathogenesis has mainly been studied in models of anti GBM disease. By an adoptive transfer approach were found to be able to cause proteinuria and mesangial proliferation in an experimental model of accelerated anti-GBM disease [45]. They are recruited via interaction with immunoglobulins with chemokines (e.g. MCP-1: chemoattractant protein 1, MIP-1alpha: inflammatory protein-1-alpha, RANTES: regulated upon activation normal T cell expressed and secreted), with lymphocyte derived molecules (MIF: macrophage inhibitory factor) and with leukocyte adhesion molecules (e.g. ICAM-1, VCAM-1, osteopontin) [46-50]. Macrophage proliferation is crucial for glomerular inflammation and is mostly growth factor and rarely cyclin kinase dependent [48, 51-53]. They release tumor necrosis factor α (TNFα), chemotactic molecules (e.g. macrophage chemotactic molecule 1), reactive oxygen species (ROS) and nitrogen oxide (NO) resulting in glomerular inflammation promotion, other leukocyte recruitment and cell death respectively. By metalloproteases secretion they cause GBM disruption, fibroblast accumulation and by transforming growth factor-β (TGF-β) release they contribute to matrix production [54-57].

T cells (CD4-positive and CD8-positive) are crucial for glomerulonephritis development. As it comes up from T-cell depletion studies in Heymann nephritis models [58-59] and from a model of anti GBM disease they are sufficient to produce glomerulonephritis [60]. Activated T-cells release chemokines, mediate macrophage recruitment by lymphocyte-derived molecules (e.g. MIF) initiating injury [48, 61] and induce mesangial cell apoptosis[62]. In addition they secrete the glomerular permeability factor, a non immunoglobulin, yet not identified molecule responsible for

the development of the non inflammatory glomerulonephritis, namely the FSGS and the MCD [63-65].

Neutrophil contribution to glomerulonephritis pathogenesis has been studied in ANCA associated pauci-immune necrotizing glomerulonephritis (human and animal model) and in animal anti-GBM disease. They are present in post infectious, lupus, ANCA associated, membranoproliferative (MPGN), and IgA glomerulonephritides, localized at sites with necrosis. A primary role in the development of MPO positive glomerulonephritis has been illustrated [66-67]. They are recruited by chemoattractants such as IL8 and C5[68] and interact with adhesion molecules of glomerular endothelial cells (e.g. selectins, integrins, ICAM-1)[69-70]. Thus neutrophils become activated and release ROS, mainly the nephritogenic hydrogen peroxide [71]. Myeloperoxidase and proteinase 3, both cationic protease in granules of neutrophils are contributed in the ANCA associated glomerulonephritis pathogenesis [72].

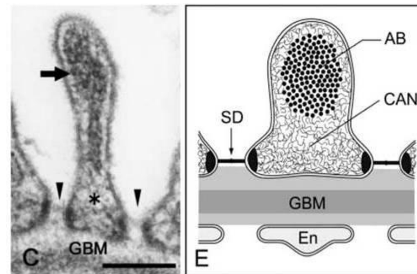

Figure 3.5 In cross-sections of the foot processes, the cortical actin network (CAN) (asterisk) is recognized and the actin bundle (arrow). The electron-dense materials at the insertion site of the slit diaphragms (arrowheads) are in contact with the cortical actin network.

3.3.1.d The Podocyte

The podocytes are highly specialized, terminally differentiated polarized visceral epithelial cells with unique structure and function, attached to the outer aspect of the GBM. They play a key role in maintenance of the glomerular barrier, to protein filtration, synthesis of normal GBM, provision of structural support of the glomerular tuft and immune response to injury [73]. They have a complex cellular organization consisting of a cell body, major processes and FPs that form a characteristic interdigitating pattern with

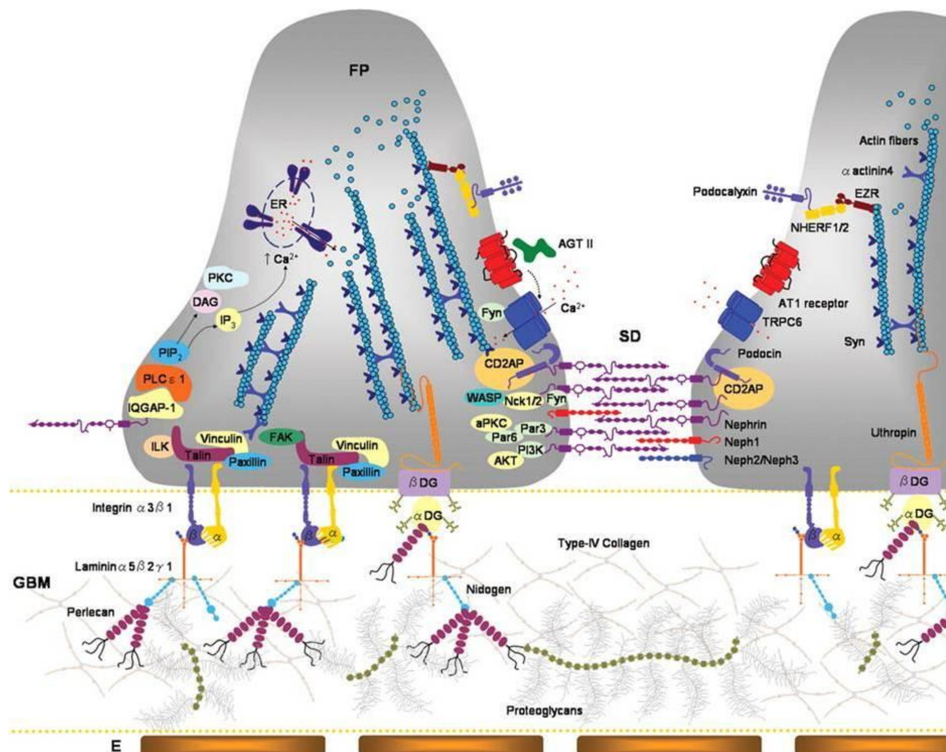

Figure 3.6 Molecular overview of the slit-diaphragm and podocyte cell-matrix interactions. E: fenestrated endothelium. Nephrin mediated signals that control actin cytoskeleton remodeling (NCK1/2, WASp), cell polarity (Par3/6, αPKC) and survival (PI3K, AKT). TRPC6-podocin interactions modulate mechanosensation, whereas angiotensin II type I receptor (ATI) may increase TRPC6 calcium influx upon stimuli by angiotensin II (ANG II). Activation of PLCεI degrade phosphatidyl-inositol-biphosphate (PIP_2) into dyacyl-glycerol (DAG) and inositol-triphosphate (IP3) which leads to protein kinase (PKC) activation and Ca^{2+} efflux from the endoplasmic reticulum. The integrin a3b1-laminin a5b2g1 and dystroglycans-uthropin complexes which connect the GBM components (proteoglycans, nidogen, perlecan, agrin and type-IV collagen) to the cell actin cytoskeleton. The podocalyxin, NHERF 1/2, ezrin (EZR) complex are including in pathways that control actin cytoskeleton remodeling.

FPs of neighboring podocytes, leaving in between 30 - 50nm wide filtration slits that are bridged by the glomerular SD (**Figure 3.1**). FPs are functionally defined by three membrane domains: the apical membrane domain (AMD), the SD and the basal membrane domain (BMD), also known as the sole

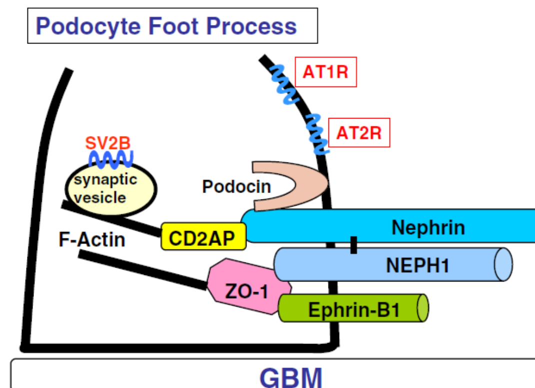

Figure 3.7 Nephrin has an interaction with CD2AP, podocin and NEPH1. NEPH1 has an interaction with ZO-1. Ephrin-B1, a transmembrane protein, is detected at the slit diaphragm. Synaptic vesicle protein 2B (SV2B) plays a role in maintaining the proper localization of CD2AP. Type 1 and type 2 receptors of angiotensin II (AT1R, AT2R) are located at the podocyte surface

plate, which is associated with GBM via α3β1 integrin and α-and β-dystroglycans [74] (**Figure 3.5**). The FPs include an actin-based cytoskeleton that also contains myosin-II, talin and vinculin [75], crucial for the maintenance of podocyte morphology. Multiple membrane proteins are connected to actin through a variety of adaptor and effector proteins [76] responsible along with podoplanin [77] and podoendin for the negative charge of the AMD (**Figure 3.6**). The negative charge has a key role in the normal development of FPs [78]. It is diminished in proteinuric diseases such as in diabetic nephropathy [79] In puromycin aminonucleoside nephrosis (PAN), neutralized podocalyxin leading to disrupted FP architecture due to SD displacement is responsible for the presentation of proteinuria [80]. Uncoupling of podocalyxin from the actin cytoskeleton is also associated with FP effacement [81-82]. Palladin, a cytoskeletal protein with essential functions for stress fiber formation is also expressed in podocytes and plays an important role in actin dynamics [83]. Synaptic vesicle protein 2B (SV2B), known to participate in the calcium-mediated synaptic transmission and to play a role in vesicle trafficking by binding to other cell surface proteins is expressed in rat podocytes. Ephrin B1, a membrane bound protein that function as ligand-receptor pair with ephrs and regulate the paracellular permeability in epithelial cells has also been found in rat podocyte. Both SV2B and Ephrin B1 play an important role in maintenance of the podocyte function. In experimental proteinuric disease they contribute to

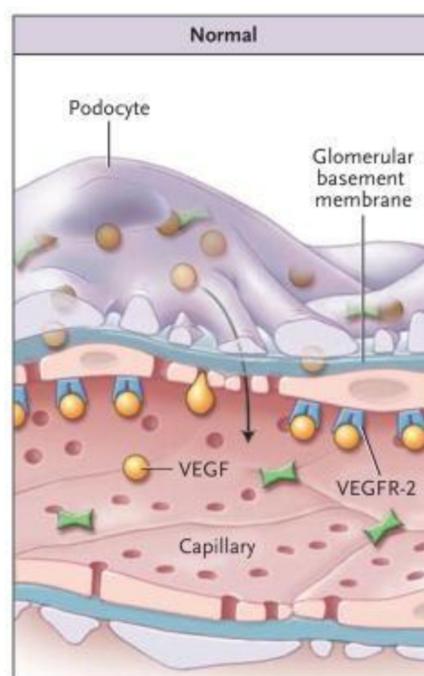

Figure 3.8 Local podocyte production of VEGF

the redistribution of the CD2AP (**Figure 3.7**). Rho guanine dissociation inhibitor (GDI)-α, Nck1 and Nck2 adaptor proteins, FAT1, and synaptopodin regulate the plasticity of the actin cytoskeleton which is important for function of the filtration barrier (Figure 3.12). Additionally local podocyte production of vascular endothelial growth factor (VEGF) is required for glomerular endothelial cells integrity [84] and plays a critical protective role in the pathogenesis of microangiopathic processes accompanied by FP effacement and proteinuria [85] (**Figure 3.8**). Podocytes express receptors for all-trans-retinoic acid (ATRA). ATRA improved proteinuria in experimental anti-GBM disease, by preventing the decrease of nephrin and podocin [86].

The podocyte response to injury.

Podocyte injury occurs in a variety of kidney diseases (Figure 3) (a) Hypertrophy / proliferation, (b) epithelial-mesenchymal transition (EMT), (c) depletion (by apoptosis, detachment-migration, and lack of proliferation). Podocyte response to injury depends on the duration and severity of injury [87] (**Figure 3.9, Table 3.1**). The initial response may be cell hypertrophy in an attempt to compensate for any loss of function. If the injury remains, podocytes will undergo EMT to escape from apoptosis resulting in impairment of filtration barrier and proteinuria. More severe/long injury

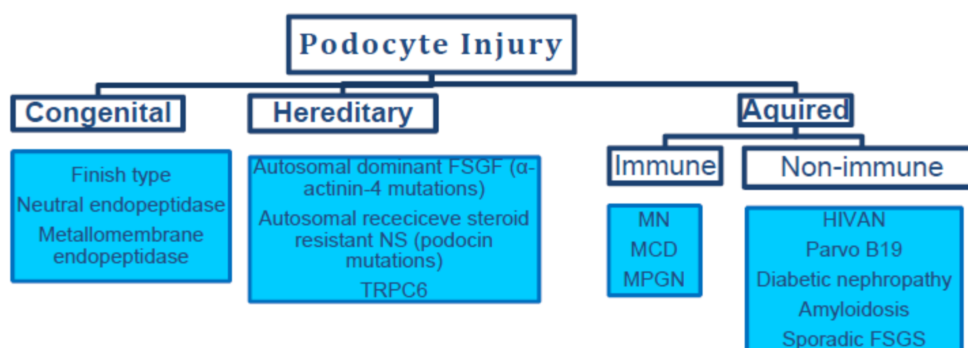

Figure 3.9 Spectrum of diseases characterized by podocyte injury

induces podocyte detachment from GBM and/or apoptosis resulting in podocyte depletion by loss in the urine or migration to glomerular crescents.

(a) Hypertrophy has been described in experimental model of ageing kidney [88]. It is initially an adaptive response to cover the denuded GBM by a cell incapable for proliferation. Increase in cyclin dependent kinase (CDK) inhibitors (p21, p27) is found in experimental hypertrophy. Angiotensin II and mechanical stress induces podocyte hypertrophy mediated by CDK inhibitors [89]. In the end hypertrophy becomes maladaptive. Podocyte proliferation has been described in experimental crescentic glomerulonephritis [90] and in human immunodeficiency virus-associated nephropathy (HIVAN). Scr-dependent activation of Stat3, mitogen-activated protein kinase 1,2 and hypoxia inducible factor 2α is an important driver of proliferation in HIVAN glomerulosclerosis [91]. Nef antigen of HIV induces proliferation via decrease of p27 and increase of cyclin D1 [92].

(b) EMT has been shown in human diabetic nephropathy [93].

(c) Podocyte depletion precedes glomerulosclerosis. It leads to denuded GBM that due to the not opposed intra glomerular pressure, attaches to the parietal epithelial cells of Bowman's capsule, leading to scar formation. Apoptosis is TGF-β mediated. Activation of the p38 mitogen-activated protein kinase and TGF-β1 causes podocyte apoptosis mediated by specific Smad [94] pathways and in the absence of the CDK-inhibitors p21 and p27 [73]. Upregulation of local angiotensin II (AngII) [95] and angiotensin type 1 receptor [96] expression induced by mechanical strain also induce TGF-β mediated apoptosis. Puromycin induces ROS mediated apoptosis in cultured podocytes [97]. Diminished expression of VEGF observed in active experimental MN contributes to podocyte apoptosis too [98]. CD2AP and nephrin activate the phosphatidyl-inositol 3´-kinase kinase/AKT antiapoptotic signaling

Table 3.1 Markers of podocyte response to injury

Epithelial markers	Mesenchymal markers	EMT-related mediators
Nephrin Podocin ZO-1 P-cadherin	Desmin FSP-1 MMP-9 Collagen 1 Fibronectin	Wnt/β-catenin ILK Snail Jagged/Notch

pathway thus they reduce podocyte apoptosis [99]. Recently activation of the Notch pathway has been reported to contribute to apoptosis through activation of p53 [100]. Podocyte loss in the urine has been observed in IgAN [101], PAN model [102], passive Heymann nephritis (PHN) model of MN [103], and human and experimental diabetic nephropathy [104]. In diabetic nephropathy, loss of α3β1 is followed by podocyte detachment and shedding into the urine [105-106] (**Figure 3.10**). Migration in the crescents has been described in experimental anti-GBM disease [90] (**Figure 3.11**). Animal and human studies verify a marked increase in CDK-inhibitors p21,

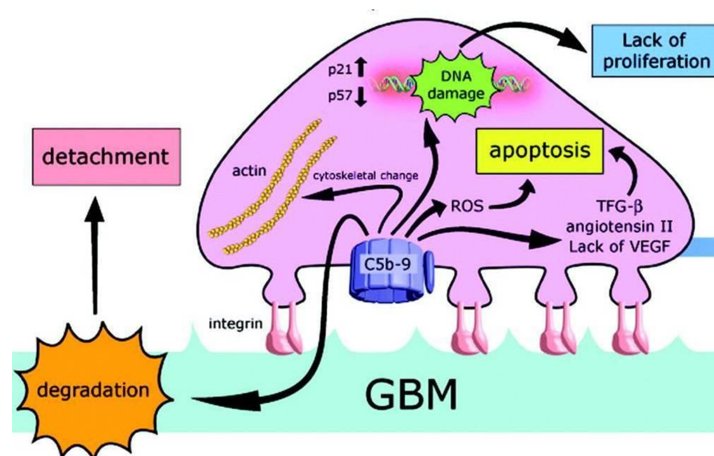

Figure 3.10 C5b-9 attack on podocytes leads to podocyte loss through several processes, including apoptosis, lack of proliferation, and detachment of the cells from the underlying GBM. These events contribute to both proteinuria and development of glomerular sclerosis.

p27, p57, to be critical in *limiting the proliferative response* of podocytes [107-110]. Also the reduction in podocyte mitosis after sublytic C5b-9 induced injury is due to DNA damage [73]. DNA damage prevents proliferation by arresting cells at G_2/M phase, process coordinated by Increase in p53, p21, growth arrest DNA damage (GADD45) and checkpoint kinase-1 and -2 [39]. Additionally secreted protein acidic and rich in cysteine (SPARC) a protein with antiproliferative and counter adhesive properties is found upregulated in experimental MN [111].

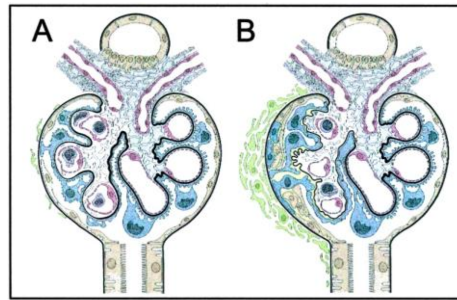

Figure 3.11 The morphologic features of a healthy glomerulus are depicted on the right half of the glomerulus, and alterations are indicated on the left half.
(A) During the early phase of the disease, podocytes (blue) project multiple filopodial protrusions (microvillous transformation). A cell process of a podocyte intrudes between two parietal epithelial cells (brown) and establishes a bridge between the GBM and the parietal basement membrane (PBM) - basement membranes are shown as black lines. (B) Subsequently, galactosidase-positive cells derived from podocytes (blue) loose contact with the GBM and are located close to the PBM. Parietal epithelial cells are preferentially distributed toward the urinary space.

Foot process effacement

FP effacement is a common finding in many proteinuric diseases and the major

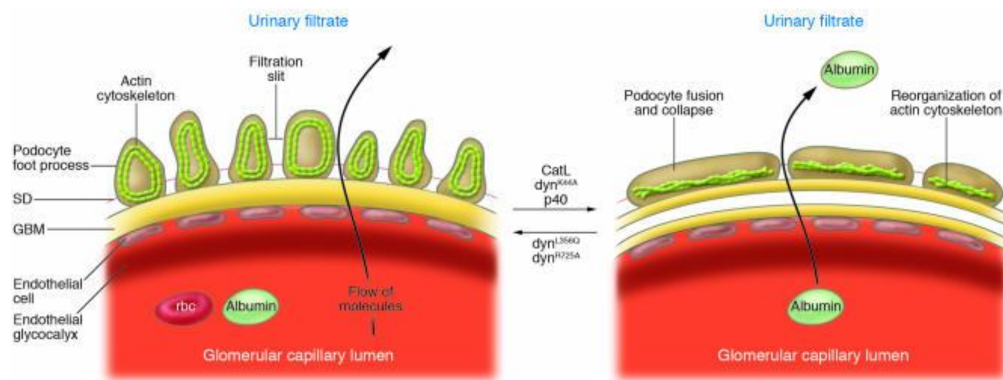

Figure 3.12 The glomerular filtration barrier - FP effacement

alterations underlie podocyte injury. The FP length decreases up to 70% and the width increases up to 60% compared to normal (**Figure 3.12**). Four major causes of FP effacement have been identified:

(a) interference with the SD complex and its lipid rafts [112]

(b) the podocyte - GBM interaction [113-116]. C5b-9 attack on podocytes induces ROS and protease production. ROS production is mediated by NADPH upregulation induced by arachidonic acid release. ROS contribute to collagen IV GBM degradation via lipid peroxidation. In podocytes, ROS also induce apoptosis, DNA damage and FP effacement [117-118]. Metalloproteinase-9 is markedly increased in podocytes following C5b-9 injury in experimental MN [119]. Cathepsin L, metalloproteinase-2 and metalloproteinase-9 are endogenous to podocytes, and their activities are increased by cytokines such as TGF-β [120]. Additionally, in MN and in diabetic nephropathy, upregulation of podocyte produced laminin and collagen IV production and release, as well as increased TGF-β2 and TGF-β receptors result in the GBM accumulation of ‘new’, usually ‘abnormal’ matrix [74, 121-122].

(c) Alterations in the actin cytoskeleton and to alpha-actinin-4 [123]

(d) Alteration in the negatively charged apical membrane domain [81, 124]. Podocytes express angiotensin II receptors type 1 (AT1) and type 2 (AT2) [125-126]. Increased AT1 signaling in podocytes leads to protein leakage and structural podocyte damage progressing in FSGS [127]. In mouse podocytes, insulin activation of the insulin receptor results in the phosphorylation of Akt and Mapk42 or Mapk44, resulting in physiologic remodeling of the actin cytoskeleton and preservation of cell function and survival. Thus in insulin resistance state, the absence of protection mechanism results in FP effacement, cell malfunction, death and proteinuria [128-

129].

Although in many forms of glomerulonephritis, proteinuria is regarded as a result of FP effacement, a finding not invariant in albuminuric diseases, the fact that most of the filtered albumin is returned to the peritubular blood by a retrieval pathway raise the hypothesis that the nephrotic proteinuria is due to retrieval pathway dysfunction [130].

Podocytes play an active role in **immune response** within the kidney. They express receptors for interleukins 4, 10, 13, functional CCR and CXCR chemokine receptors and respond to exogenous chemokines [131]. They are also capable of producing inflammatory mediators such as interleukin 1[132], complement proteins including C3 [133], TGF-β and fibroblast growth factor 2. They also express CR1 [134] and Toll like receptor-4 (TLR-4) [135]. CR1 is localized exclusively in human podocytes on which is capable of binding C3b [42]. Complement factor H (CFH) is uniquely expressed in rodent podocytes and is the functional surrogate for human CR1[136]. In a model of chronic serum sickness, C57BL/6 mice did not develop glomerulonephritis unless deficient in CFH. Locally produced podocyte CFH is important to process ICs in the subepithelial space, where it also limits complement activation. DAF is also primarily a podocyte protein [137]. In conditions with proteinuria and FP effacement the podocytes upregulate B7-1, also called CD80, a transmembrane protein expressed on the surface of B cells and other antigen-presenting cells (APC). B7-1 in podocytes was found in genetic, drug-induced, immune-mediated, and bacterial toxin-induced experimental kidney disease with nephrotic syndrome [138]. B7-1 induces proteinuria through the reorganization of the podocyte FP actin cytoskeleton and disruption of the SD [138-139]. The induction of B7-1 in podocytes by LPS through TLR-4, signaling suggests that the podocyte is a component of the innate immune system, equipped with a danger signaling

machinery [139]. Podocytes express adiponectin receptors in their plasma membrane. Adiponectin is a circulating plasma protein secreted by adipocytes and is decreased in visceral obesity. Adiponectin has a protective role against FP effacement, likely acting through the 5´-AMP activated kinase (AMPK) pathway to modulate oxidant stress in podocytes [140].

The slit diaphragm (SD)

The SD function as a size and charge barrier, as some of its proteins are phosphorylated. Karnovsky et al first proposed a zipper-like organization of the SD structure [141]. The SD represents the only cell-cell contact between podocytes, a modified adherence junction, a complex signal transdunction unit which spans the 30-50nm filtration slits and is connected to the actin cytoskeleton. Unique membrane structural (e.g., nephrin, podocin, Neph1), adherens junctions proteins (e.g. P-cadherin, FAT, catenins), tight junction proteins (e.g., JAM-A, occluding, cingulin) and Par3-Par6-sPKC polarity complex, receptors, signaling adaptors, ion channels, scaffolding proteins are the components of SD and are localized to lipid rafts [142] (**Figures 3.1, 3.6, 3.10 - 3.14**). The tight junction proteins are upregulated in PAN nephrosis and loosened their attachment to the actin cytoskeleton [143]. The polarity complex interacts with Neph-nephrin proteins and govern the appropriate distribution of nephrin and podocin [144-146]. Defects in α-actinin-4, nephrin, phospholipase C epsilon gene, podocin, transient receptor potential cation channel 6 result in

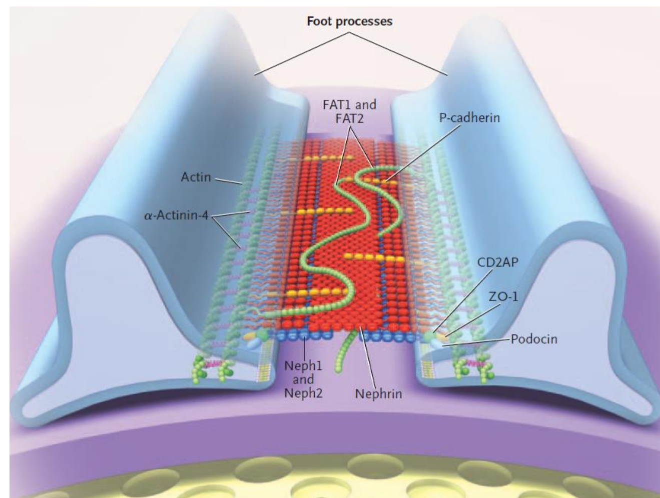

Figure 3.13 Components of the SD protein complex that form a porous Slit-Diaphragm Filter.

rearrangement of the actin cytoskeleton. Nephrin associates with adaptors such as Nck to regulate the actin cytoskeleton [147-148]. Densin, known due its involvement in the synaptic organization, maintenance of cell shape and polarity in nerve cells, has been identified in the SD area and interacts with nephrin [149]. NF-κB activated by cell surface receptor signaling to meet stress and inflammatory responses, regulating innate and adaptive immunity and cell growth and survival is involved in GN. Animal data suggest that nephrin may normally limit NF-κB activity thus discouraging the evolution of glomerular disease [150]. CD2 associated protein (CD2AP) connects the nephrin complex with the actin modifying proteins WASP, CAPZ, cortactin, and the Arp2/3 complex. The protein ZO-1 directly associates with the cortical actin cytoskeleton, and densin binds to a-actinin-4. FAT-1 is also an organizer of actin polymerization [74]. Dendrin, a cytosolic protein is found in FPs of mouse podocytes and associates with the SD complex in normal human kidney. In MCD patients, dendrin and ZO-1 are re-distributed within the podocytes [151].

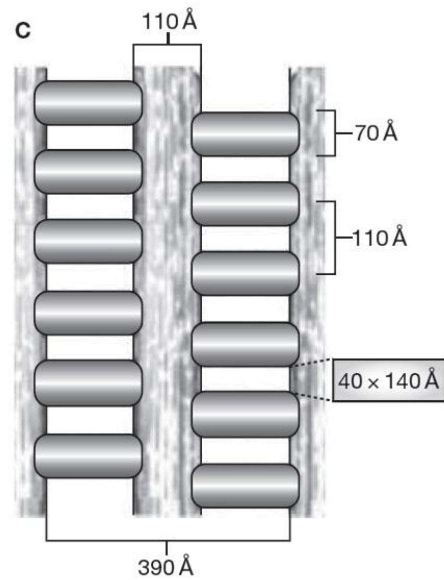

Figure 3.14 The slit diaphragm model of Karnovsky and colleagues. Podocyte foot processes (left and right) with a central filament running in parallel to FP. Perpendicular cross strands form a regular lattice, with rectangular pores between the strands. This lattice forms a molecular sieve.

Nephrin

Studies in families with congenital nephrotic syndrome (CNF) of the Finish type, an autosomal-recessive disorder characterized with heavy proteinuria in utero, led to the identification of **nephrin** as a critical component of the glomerular SD. The respective *NPHS1* gene, conserved through evolution, mutated in CNF, is located in a 150kb

region on chromosome 19q13.1 and was cloned in 1998 [152]. Nephrin is a 185 kDa, 124-residue transmembrane protein of the immunoglobulin superfamily of cell adhesion molecules [153]. It includes a 22-residue N-terminal signal peptide, an extracellular domain containing eight immunoglobulin-like modules, and one fibronectin type III-like module, followed by a single transmembrane domain-like sequence, and a cytocolic C-terminal end [152]. It is not only the main structural component of the podocyte SD, forming a scaffold for intertwining SD molecules, but also a cell surface receptor participating in cell-cell signaling functions. Nephrin molecules interact with one another in a hemophilic fashion (**Figure 3.15**) in the middle of the slit to form a filtering structure [154-155]. Nephrin has shown to interact with CD2AP and podocin linking the SD with the podocyte cytoskeleton. ZO-1 as well as α-actinin, IQ motif-containing GTPase activating protein 1(IQGAP1), membrane-associated guanylate cyclase inverted 2 (MAGI2) and calcium/calmodulin-dependent serine protein kinase (CASK) also couple the nephrin to the actin cytoskeleton [155-157]. Thus alterations in nephrin result in alterations of the podocyte cytoskeleton/shape characterized by FP effacement and vice versa. The signaling functions are enabled by the nine tyrosine residues of the intracellular domain, phosphorylated by Src kinase at ligand binding [158-159]. The Src kinase tyrosine phosphorylation of the cytoplasmic tail of nephrin seems to promote antiapoptotic signaling [160]. Lack of Fyn kinase, a member of the Src tyrosine kinase family, leads in proteinuria and FP effacement in mice. Podocin and Neph1, form a protein complex with nephrin that functions as a

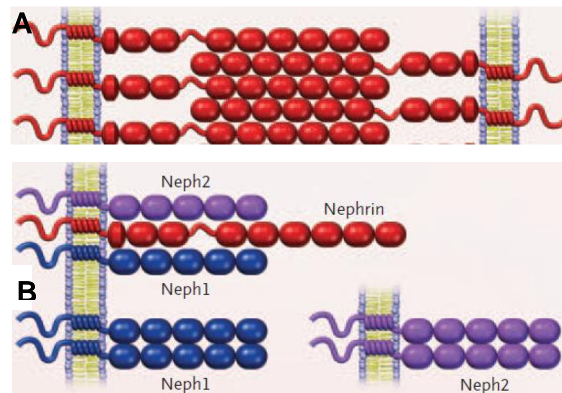

Figure 3.15 A. Homophilic interaction between nephrin molecules from adjacent podocytes. B hemophilic interactions between identical neph molecules and heterophilic interactions with adjacent nephrin molecules. Neph1 and Neph2 do not interact with each other

transmembrane receptor (nephrin receptor complex) crucial for the maintenance of the maintenance of FP architecture and holds FP together in the face of arterial pressure transduced from glomerular capillaries [142]. A basic function of the nephrin receptor complex is to form a polarized junction between two cells [161]. Mutated *NPHS1* gene or anti nephrin antibodies cause loss of SDs, FP effacement and proteinuria.

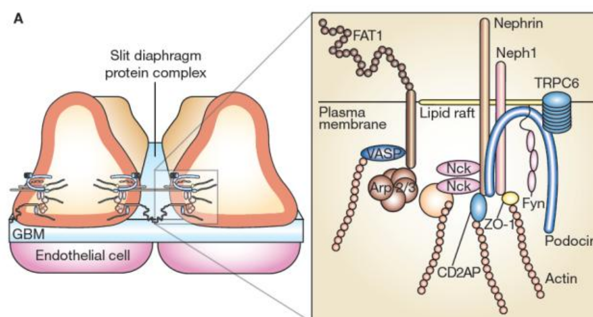

Figure 3.16 The glomerular filter viewed perpendicular to podocyte FP. Filtrate passes from within the glomerular capillary lumen (at bottom) through fenestrate between endothelial cells, across the glomerular basement membrane, and through the slit diaphragm between podocyte FP into the Bowman's space. Inset: slit diaphragm components are localized to lipid rafts. Podocin serves as a scaffolding molecule to localize nephrin and Neph1 to lipid rafts, which bring multiple proteins together in a small area of membrane to create a signaling platform.

Podocin

Podocin was first isolated from families with an autosomal recessive, steroid-resistant nephrotic syndrome (SRNS) that affects children within the first few years of life [162] and has histological characteristics of MCD or FSGS. It is encoded by the *NPSH2* gene, located in 1q25-31, which is mutated in the SRNS [162]. Podocin is a member of the stomatin family of proteins. It is a hairpin-like membrane protein with intracellular N- ans C- termini (**Figure 3.16**). Podocin associates with lipid rafts and recruits [163-164] transmembrane receptors to these rafts such as the nephrin complex receptor. It acts as a scaffolding protein, serving the structural organization of the SD. Several studies have reported a modulation or correlation of nephrin and podocin expression with levels of proteinuria in PAN, diabetes and MCD [165-167]. Recent data support a critical role of the podocyte SD and its protein component nephrin and podocin in the pathogenesis and of immune mediated

glomerulonephritides such as the PHN and the experimental anti-GBM glomerulonephritis [168-169].

3.4 Lupus nephritis (LN)

3.4.1 Overview

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease which can affect any organ of the body and thus have a broad array of manifestations such as rash, arthritis, anemia, serositis, nephritis, seizures and psychosis. The prevalence of lupus ranges from 40 to more than 200 cases per 100000 persons. The life expectancy of patients with SLE has improved from 4-year survival rate of 50% in the 1950s' to a 15-year survival rate of 80% today [170]. LN is a common and serious manifestation of SLE associated with significant morbidity and mortality. The incidence of LN ranges from 25% to 75% among SLE patients and up to 20% of these patients progress to renal failure. LN is regarded as a syndrome, including a variety of immunologic events [171].

3.4.2 Clinical Features - Diagnosis

Proteinuria is the dominant feature of LN and microscopic hematuria is almost always present. Hypertension is most prominent in patients with severe LN and about half will show a reduced glomerular filtration rate (GFR) and occasionally they will present with acute renal failure. Renal tubular function is occasionally disturbed. A rising anti-dsDNA titer and reduction of serum complement is observed in the proliferative forms of disease. Nephrotic syndrome can accompany active LN. Kidney biopsy is the mainstay of the diagnosis of LN. A diverse histopathology characterizes LN. The latest ISN/RPS (2003) classification of LN is currently in use (**Table 3.2**) [172].

In the following paragraphs we will focus on the current data regarding LN pathogenesis in particular. First implications regarding pathogenesis of LN come

from kidney biopsy histological findings. IC and complement deposition, interstitial and glomerular infiltration by inflammatory cells, mesangial cell proliferation, podocyte FP effacement are the initial findings. Matrix expansion and fibrosis occur at later stages.

Table 3.2 Synopsis of International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of LN

Class	Description
Class I Minimal mesangial LN	Minimal mesangial immune deposits
Class II Mesangial Proliferative LN	Mesangial hypercellularity - Normal glomeruli by light microscopy
Class III Focal LN	Segmental or global, endo- or extracapillary glomerulonephritis involving <50% of all glomeruli with or without messangial damage
Class III(A)	Active lesions
Class III (A/C)	Active + Chronic lesions
Class III (C)	Chronic lesions
Class IV Diffuse LN	Segmental or global endo- or extracapillary glomerulonephritis involving ≥50% of all glomeruli with subendothelial deposites /with or without messangial damage
Class IV S(A)	Segmental Active lesions
Class IV G(A)	Global Active lesions
Class IVS(A/C)	Segmental Active+Chronic lesions
Class IV G(A/C)	Global Active+Chronic lesions
Class IV S(C)	Segmental Chronic lesions
Class IV G(C)	Global Chronic lesions
Class V Membranous LN	Segmental or global subepithelial deposits /with or without mesangial damage /with or without presence of class III or class V LN / with or without chronic lesions
Class VI Advanced sclerosing LN	≥90% global sclerosis

Table 3.3 Working definitions in the management of LN

Disease severity	
	<u>Mild renal disease</u>
	Class III with chronicity index ≤3 / proteinuria <3g/24h (<3 creatinine/protein ratio*) and normal renal function
	Class V with proteinuria <3g/24h and normal renal function
	<u>Moderate severe renal disease</u>
	Mild disease with partial or no response to induction therapy
	Class III with chronicity index >3 or crescents or increase of serum creatinine
	Class IV with chronicity index ≤3 / proteinuria <3g/24h and normal renal function
	Class V with proteinuria ≥3g/24h and normal renal function
	<u>Severe renal disease</u>
	Moderately severe with partial or no response after 6–12 months of induction therapy
	Class IV, V with impaired renal function
	Class III, IV with fibrinoid necrosis or crescents, or chronicity index >4 or activity index >10
	Rapidly progressive glomerulonephritis

3.4.3 Pathogenesis of LN

3.4.3.a Autoimmunity disorder in LN

Loss of self tolerance results in a generalized autoimmunity with autoantibodies directed against a variety of self components plays a central role. Defect in the CD8 T cells and natural killer cells that suppress the activation of B cells that secret anti dsDNA IgG has been shown in patients with lupus [173]. At the same time a hyperactivity of Th cells is observed in human and murine lupus [174-175]. Defect in clearing of ICs has also been implicated. This is due ether to reduced numbers of CR1 receptors [176] or to inadequate phagocytosis and clearance of IgG2 and IgG3 containing complexes and of apoptotic materials predispose to LN [177-180]. Recently the expression of programmed death 1 (PD-1), of a negative T cell regulator and its ligand, in renal tissue suggests an important role in regulating peripheral T cell tolerance [181].

Autoantibodies in LN

LN is characterized by trapping of circulating autoantibodies against double stranded (ds) DNA, ribonucleoproteins, nucleosomes, α-actinin, laminin, vimentin, myosin, heparin sulphate, and anti-Sm antibodies, the last having significant association with LN. In situ formation also occurs via charge interaction between anti-DNA/histone antibodies and DNA/histones already deposited in the glomerulus [182]. The role of the autoantibodies in generating renal damage is unclear and conflicting data from murine studies raise contestation about the anti-DNA antibodies are sufficient to cause end stage renal failure [183]. Complement fixation may be essential for tissue damage.

Possible mechanisms by which autoantibodies cause LN are depicted in **Figure 3.17** (a) Some antibodies against dsDNA form complexes with DNA in the blood stream [184] and are passively trapped in the glomeruli and (b) others attach directly to

glomerular structures i.e. GBM [185]. Not all autoantibodies cause renal damage (nephritogenic antibodies). The capacity of autoantibodies to form immune deposits and their location are dependent on properties unique to individual Ig in LN [186]. IgG1 and IgG3 anti nuclear antibodies are considered more nephritogenic. Extracellular double stranded DNA occurs in the form of nucleosomes, which are fragments of chromatin that cells release when they undergo apoptosis. Positively charged chromatin fragments on GBM, as targets for autoantibodies [187] and high affinity antibodies to nucleosomal DNA mostly determines the pathogenic capacity of anti-DNA antibodies [188-190]. Their cross reactivity to alpha actinin, laminin, C1q and heparin sulphate attributes pathogenic properties [179, 191-193]. The antigen binding region is influential in this process [186].

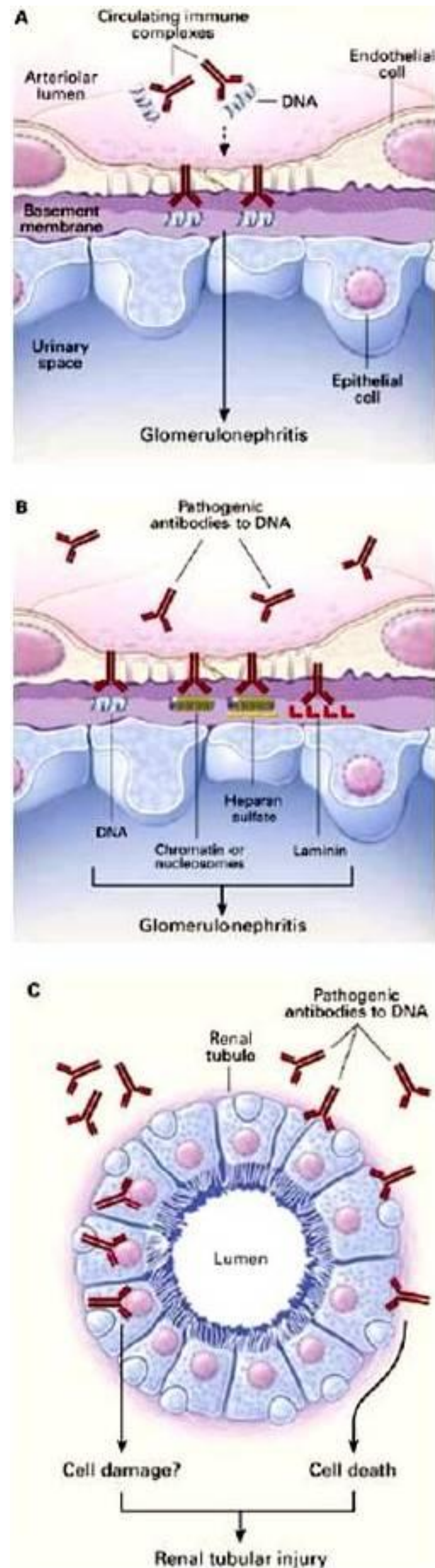

Figure 3.17 Pathogenesis of LN via autoantibody induced damage

Immune complexes–complement activation in LN

The MRL/lpr mice have widespread complement activation and ICs in the kidney. The ICs trapped in the GBM activate complement which initiates LN by generating chemotactic factors that attract leucocytes and macrophages. These cells release cytokines and clotting mediators [194-195]. The Fc receptor is crucial for the LN development. NZB/W F1 mice with defective Fc receptors generated and deposited IC and activated complement but were protected from severe LN [196]. Also lupus MN is characterized by subepithelial IC deposition and FP effacement without inflammation. The subsequent development of proteinuria is complement dependent and involves the formation of C5b-9 the same mechanism observed in idiopathic MN [197-198]. The CR1 loss from podocytes in human LN, which is due to reduced synthesis, might render podocytes highly sensitive to complement attack [199]. Local expression of complement genes C3, Factor B, C2 and C4 is increased in murine LN[200]. In MRL/*lpr* lupus mouse kidneys, C3aR and C5aR expression was up-regulated significantly at both the mRNA and protein levels. When C5a signaling or C3aR were blocked MRL/*lpr* mice as well as C5aR-deficient MRL/*lpr* mice displayed attenuated renal disease and prolonged viability [198, 201-202]. Dysfunction or down-regulation of DAF may contribute to autoimmune disease pathogenesis and manifestations in an LN model of MRL/*lpr* mice [203].

B and T cells – macrophages – renal dendritic cells

Breakdown of immunologic tolerance leads to the production of autoreactive B and T cells. The role of B cells is dominant in LN due to autoantibody production. BAFF (B cell activation factor belonging to the TNF family) is upregulated during inflammation promotes the formation of B memory cells resulting in persistence of autoantibodies [204]. Cell proliferation is dependent on the coordinated activation of specific cell-cycle regulatory proteins; cyclins and CDKs act as positive regulators [205]. APRIL (a proliferation-inducing ligand) promotes autoreactive B cell survival in some cases.

The role of T cells is help provision to B cells. However, there is a growing body of evidence for a crucial role of T cells. A local T cell response in kidney and regional lymph nodes exists early in the disease process [206]. Blocking T cell activation by CTLA4Ig in NZB/W F1 mice with LN could prevent disease progression [207]. The finding of Th1 and Th2 cytokines in the kidney underscore the T cell involvement [206]. Activated macrophages in the renal interstitium correspond with disease progression. Activation of renal dendritic cells occurs in murine and human LN, they induce T cell activation and are associated with expression of inflammatory cytokines [208].

Cytokines in LN

Proinflammatory cytokines are upregulated in LN. The local (renal) effects are different and more relevant to LN from the systemic effects [209]. TNF has been detected in macrophages infiltrating the glomerulus, as well as in glomerular podocytes, endothelial and mesangial cells and in the interstitium. The glomerular TNF is mainly produced locally by monocytes and macrophages but also by the mesangial cells. Its local production is induced by antibodies to dsDNA and its concentration in the kidney correlates with disease activity [210-211]. Since the circulating TNF levels are elevated in active SLE, and the renal cells express TNF receptors, kidney cells may bind circulating TNF too [212].

Increased IL-6 at the protein and mRNA level found in LN is associated with mesangial cell proliferation. However many LN samples do not express IL-6 [209]. IL-1 has been detected in infiltrating monocytes and macrophages and in some podocytes and mesangial cells in human LN [210]. IL-18 is overexpressed in nephritic kidneys of MRL/lpr mice, both in infiltrating monocytes and in tubular epithelial cells [213]. Upregulation of INFγ, the prototypical Th1 cytokine, produced by monocytes, has been shown in human and murine (MRL/lpr, NZM2328) LN [206, 214-215]. The finding that a relative defect in INFγ signaling can uncouple

autoantibody formation and IC deposition suggests that INFγ plays an essential local role in murine LN [216]. IL-4, the prototypical Th2 cytokine is found also in podocytes and in mesangial cells. IL-4 mRNA expression was associated with hypercellularity and extracellular matrix expansion in human LN [217]. IL-10 is found in intrarenal T cells and the increase of IL-10 is of pathogenic relevance in NZB/W mice, where the effect of IL-10 was opposite to the effect of TNF [209, 218].

3.4.3.b The endothelial cell

Inflammatory cell recruitment requires endothelial cell activation. Endothelial hypoxia results in cell death and further activation of innate immunity. Active human LN is characterized by upregulation of Tie2 antagonist. Tie2 maintains endothelial integrity [219].

3.4.3.c The mesangial cell

In MRL/lpr mice the IC deposition is followed by production of chemokines like MCP-1 and RANTES by the glomerular mesangial cells. The initial mesangial stimulation results in mesangial expansion and cellular infiltration. In a later stage a transition to fibroblast phenotype has been shown with an increase in expression of genes such as metalopeptidase, TGF-*β2*, insulin-like growth factor binding protein *2*. Activation of the TGF-*β2* signaling is the final common pathway leading to fibrosis, glomerulosclerosis and end stage renal failure [191, 220].

3.4.3.d The renal interstitial cells

Resident and migrating (e.g. macrophages) cells of the renal interstitium play a key role in the progression of renal damage to end stage renal disease in general and in LN in particular. Increased ICAM-1, Von Willebrand factor, soluble endothelial protein C receptor and decreased ADAMS-13 point to a diffuse vascular damage. Albuminuria induces ROS release by the proximal tubular cells, NF-kB activation,

endothelin-1 and TGF-*β1* upregulation. TGF-β1 enhances epithelial-to-mesenchymal transition and apoptosis. Albuminuria also enhances the MCP-1 expression. All these events can be counteracted by the hepatocyte growth factor (HGF) expressed in the tubular epithelial cells [221].

3.4.3.e The Podocyte

The podocyte is a target of anti dsDNA antibodies because the last cross-react with α-actinin, a protein that cross-links actin, critical for maintaining the function of podocytes. In murine model anti dsDNA antibodies that cross-react α-actinin are pathogenic but those that do not cross-react are not [192, 222]. Blockage of type 1 angiotensin receptors in the glomerulus reduced proteinuria, glomerular pathology and cytokine expression both in murine and in human LN [223-224]. Podocyte expression of B7-1 correlated with the severity of human LN [138].

3.4.3.f Toll-like receptors (TLRs)

TLR are microbe recognizing receptors expressed by B cells and by resident kidney cells and recognize self antigens thus contributing to autoimmune disease. TLR-9 is expressed in the tubulointerstitium and in the glomerulus in human LN. The ICs activate the renal cells through TLR to produce inflammatory mediators. TLR-9 protected against nephritis in the MRL/lpr mice as well as co-inhibition of the TLR-7 and TLR-9. Additionally TLR-9 ligand accelerated LN in MRL/lpr mice. [225-226].

3.4.4 The NZB/W F1 mice

Several features of NZB/W F1 mice model are consistent with lupus in humans. Females develop disease at a frequency 10 times that of males, and IC and complement deposition in glomeruli are observed. Significant proteinuria and severe GN is seen concomitant with the serological appearance of antibodies to DNA as well as ICs of the immunoglobulin G1 (IgG1), IgG2a, and IgG2b subclasses beginning at

4 months. Median survival is 6 months, with mortality resulting from renal failure. Several studies have demonstrated the essential role of B cells and autoantibodies in disease development [196, 227-229].

4. RESEARCH QUESTIONS AND AIM OF THE STUDY

Numerous data support the central role of the SD components in the pathogenesis of GN with proteinuria. Various components of the SD are affected in different renal diseases [230]; understanding the molecular mechanisms of the glomerular filtration pathology is essential for the development of targeted therapies.

Although recent data suggest a critical role for the podocyte SD and its components in the pathogenesis of immune-mediated GN, such as the Heymann model of membranous nephritis and experimental anti glomerular basement membrane disease, their contribution in LN remains to be defined [168-169]. The role of nephrin and podocin, of the main components of the SD has not been explored in a systematic and organized fashion. Specifically there is a paucity of data with regard to the glomerular expression of nephrin and podocin at both protein and mRNA levels. Moreover, there are no experimental data and previous studies in humans have not reported specifically on LN.

The aim of this study was to determine the ultra-structural alterations of the SD associated with altered glomerular expression of nephrin and podocin in the NZB/NZW (NZB/W) murine LN model. We investigated whether the expression of these molecules correlated with clinical and histological parameters of the renal disease. Finally we evaluated nephrin expression in the kidneys of patients with LN. We sought to test the hypothesis that podocytes and their SD components may be implicated in the pathogenesis of immune-mediated nephritis such as LN.

5. MATERIALS AND METHODS

5.1 Animal study

5.1.1 Animal experimental design

A total of 27 NZB X NZW F1 (briefly NZB/W) (Harlan, UK) and 24 C57BL/6 (FORTH, Greece) control female were studied. NZB/W nephritis of the mice morphologically and functionally resembles human LN. Various types of glomerulonephritis associated with IC deposition are developed [231]. C57BL6 were chosen as controls for normal protein excretion as spontaneous glomerulonephritis and impaired autoimmunity was not observed in such animals. Also 5 NZB and 4 NZW as parent strains of the NZB/W hybrid were studied. Little is known about renal characteristics of NZB and NZW. We sought to study females (n=3) NZB and males NZW (n=3) as the NZB/W are hybrids from a mating of a female NZB and a male NZW mouse. All animal procedures were conducted in accordance with the guidelines for the care and use of laboratory animals approved by the University of Crete, School of Medicine. Animals were housed in a constant temperature room, with a 12-hour dark light cycle. They had free access to tap water and standard mouse chow throughout the study and were euthanatized by CO_2. Because NZB/W nephritis occurs before the 20 weeks of age [232] mice were evaluated at 1, 3, 6, and 9 months of age in order to detect possible podocyte damage at the very early phase of LN when evident at the ultrastructural level.

5.1.2 Urine collection and assessment of proteinuria

Individual mice were placed in metabolic cages (Tecniplast) for 24h urine collections the day before they were sacrificed. Urine samples were aliquoted and stored at -80°C without any protease inhibitors until processed further. Urine samples were assayed for total protein using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories), using bovine serum albumin (BSA) as standard. Samples were read

on an Ultrospec 2100 Pro (Biochrom Ltd) spectrophotometer at 590nm. Baseline values for urinary protein excretion, as measured in 1-month old NZB/W mice and in C57BL6 controls were equal or less than 2mg/24h. Thus, abnormal proteinuria was defined as protein excretion above 2mg/24h, as previously described. NZB/W mice were categorized: a) non proteinuric: <2mg/24h; b) mild proteinuric: 2-10mg /24h and, c) severe proteinuric >10mg/24h.

5.1.3 Blood collection

Blood was collected by cardiac puncture, was left on ice for 20 minutes and then serum was separated by serial centrifugations at 3000 x *g*, 6000 x *g*, and 12000 x *g* at 4 ^{o}C for 10 minutes each centrifugation.

5.1.4 Determination of serum anti-ds DNA

Serum anti-dsDNA autoantibodies were detected by an enzyme-immunoassay (Mouse anti-ds DNA IgG (Total) ELISA kit; ADI V3-5100) according to the manufacturer's instruction. Briefly, blood serum samples, diluted 1:100 in assay diluent buffer (100μl/well), was plated in antigen coated, 96-well strip plate, then incubated for 30 minutes and subsequently washed three times with 1 x washing buffer. HRP labelled goat anti-mouse IgG conjugated antibody (1:100 in assay diluent buffer – 100μl/well) was added and incubated for 30min and then washed. Positive and negative control wells were also prepared. Finally 100 μl/well of TMB solution was used (15 minutes incubation in the dark) and the reaction was terminated by the addition of 100μl/well of stop solution. The plates were read at 450 nm in a Model 680 Microplate Reader, Bio-Rad Laboratories Ltd, UK. All incubations were performed at room temperature (RT).

5.1.5 Renal Histology

The left mouse kidney was processed for histological studies. Briefly, one part was fixed in 10% buffered formalin stored at room temperature and later dehydrated in alcohol, and embedded in paraffin and stored at RT for standard light microscopy staining techniques (hematoxyline - eosin, PAS, Jones, Masson's Trichrom). Another part of the kidney was snap frozen by liquid nitrogen in 22-oxycalcitriol (OCT) compound and stored at -80^oC for immunoflourescence (IgG, nephrin). A third part was fixed in 2.5% glutaraldehyde in phosphate buffer ($NaH_2PO_4H_2O$: 0.1 M + Na_2HPO_4: 0.1M pH: 7.2) stored at 4^oC for electron microscopy study.

5.1.5.a Light microscopy (LM)

Four μm sections of formalin-fixed paraffin-embedded tissue were examined in a Nikon THP Japan microscope. Kidney biopsies were evaluated for active lesions (mesangial expansion, endocapillary proliferation, glomerular deposits, extracapillary proliferation and interstitial infiltrates), as well as chronic lesions (tubular atrophy and interstitial fibrosis) of LN. At least 30 randomly selected glomeruli, visualized and photographed, from each animal were examined. All slides were scored by an experienced renal pathologist (L. N.) masked to the group. The 2003 International Society of Nephrology / Renal Pathology Society (ISN/RPS) classification of LN was adopted [233].

5.1.5.b Immunofluorescence

For immunofluorescence (IgG and nephrin) studies, kidney cryosections (5 μm) were fixed in acetone at -80°C and were blocked with 20% normal goat serum, before incubation with antibodies against IgG (1:50 for 1 hour at room temperature) and nephrin (1:100 overnight at 4^oC). Secondary species-appropriate fluroscein-isothyocyanate (FITC) conjugated antibody (1:20 for 1 hour at room temperature) was used and slides were examined in a Nikon eclipse 80i microscope. Pictures

were captured with a Nikon Digital Sightt using the W/S – Element F Software. Glomerular IgG staining was assessed as follows: 0, no signal; 1+, mild; 2+, moderate; 3+, strong signal intensity. At least 15 glomeruli per animal were studied. For distribution and quantification purposes, a semiquantitive score was assigned for glomerular nephrin expression according to the following patterns: 0, negative; 1+, weakly positive; 2+, strongly positive.

5.1.5.c Electron microscopy

For electron microscopy (EM) studies, specimens were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, post-fixed for 1 hour in 1% osmium tetroxide, dehydrated in a series of graded ethanols, cleared in propylene oxide and embedded in an Epon/Araldite mixture. Ultrathin sections were stained with uranyl acetate/lead citrate and examined in a JEM-1010 transmission electron microscope by the same pathologist (KK), in a blind manner.

Analysis was based on micrographs representing ≥20 glomeruli for each group of mice. The presence of mesangial, subendothelial and subepithelial electron dense deposits (EDD) was evaluated in a semiquantitive way according to the following pattern: 0, negative; 1+, frequent; 2+, extensive. The podocyte fusion was characterized as 0, negative; 1+, rare; 2+, extensive.

5.1.6 Glomerular isolation

The right kidney was processed for biochemical and molecular studies immediately after sacrifice. Glomeruli were enriched by differential sieving on ice as been described elsewhere [234]. One half of the kidney was minced in pasta like consistency, then passed through a 90 micrometer nylon sieve (Nitex 03-50/31B B B B, Sefar inc) and subsequently trapped the glomeruli on a 50 micrometer nylon sieve (Nitex 03-90/49B B B B, Sefar). Glomeruli from one half of the kidney

were lysed in 1ml RIPA buffer ((0.1 M HEPES, 0.15M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% glycerol, 0.1 M sodium fluoride, 10 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 1% Nonidet P-40, and protease inhibitors) [235]. The lysed glomeruli were centrifuged at 4°C at 14,000 x *g* for 15 minutes and the supernatant was aliquoted stored at -80°C until used for Western blot studies.

5.1.7 Western blot (WB) analysis

For Western blot analysis, 40μg of total protein from glomerular extract were diluted in Laemmli sample buffer with 5% 2-mercaptoethanol, boiled for 5 minutes and separated under reducing conditions by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN 3 Mini Vertical Electrophoresis System (BIO-RAD). Proteins were transferred to a nitrocellulose membrane (Protan BA85 - Schleicher & Schuell Bioscience), blocked for 1 h in 5% skim milk in TBS-T (0.05% Tween 20) at room temperature, and then incubated with the indicated primary antibody (1:500) diluted in 5% skim milk in TBS-T (0.05% Tween 20) at 4°C overnight. After three wash with TBS-T (0.05% Tween 20), membranes were incubated with the appropriate horseradish peroxidase (HRP) conjugated IgG diluted 1:3000 – 1:10000 according the manufacturer’s instruction at room temperature for 1 hour. Blots were developed using enhanced chemiluminescence (ECL) by Amersham (RNP2209) and exposed (SuperRX 12x18 – FUJIFILM). Nephrin and podocin were normalized to actin detected by a pan actin monoclonal antibody diluted 1:1000 in TBS-T (0.05% Tween 20) at room temperature for 1.5 hours. The density of the positive bands was quantitated by Tinascan.

5.1.8 RNA Isolation

Total RNA was isolated from kidneys by a single-step guanidinium isothiocyanate-phenol-chloroform extraction (Trizol Reagent Invitrogen) according to the

manufucturer's instructions. After tissue homogenation in Trizol reagent (50-100mg of tissue/1ml Trizol Reagent) at 4^0C, the nucleoprotein complex was completely dissociated by sample incubation at room temperature. The phase separation was performed by the incubation with 0.2ml of chloroform at room temperature for 3 minutes and centrifugation at 12000 x *g* at 4^0C for 15 minutes. The RNA precipitation from the resultant aqueous phase was done by mixing with 0.5ml isopropyl alcohol, incubation at room temperature for 10 minutes and centrifugation at 12000 x *g at* 4^0C for 10 minutes. One wash of the RNA pellet with 1ml of 75% ethanol followed and subsequently the RNA pellet was air-dried and finally redissolved in 30μl of RNAase-free water and stored at -80^0C.

5.1.9 cDNA preparation

One microgram of total RNA was used to prepare first strand cDNA in a total reaction volume of 20μl by AMV Reverse Transcriptase (Promega). The appropriate volume of RNAase free water and 0.5μg primer/μgRNA was added to the 1μg total RNA, heated at 65 ^{0}C for 5 minutes, chilled on ice for 5 minutes and centrifuged briefly. Then 4μl of 25mM $MgCl_2$, 2μl of Reverse Transcriptase Reaction 10x buffer, 2 μl of 10mM dNTPs, 0.5μl of 40u/ml RNasin and 0,75μl of 20U/ml RT enzyme (AMV) were added to the RNA solution up to the volume of 20μl. The mix then was sequentially incubated at 25 ^{0}C for 15 minutes, at 42 ^{0}C for 60 minutes, at 95 ^{0}C for 5 minutes and finally at 4 ^{0}C for 5 minutes. The cDNA was stored at -20 ^{0}C until used.

5.1.10 Real Time PCR reaction

A typical 20μl real time PCR reaction was performed in the ABI Prism 7700 Sequence Detection System using BioRad SYBR green Super mix 1x. Each reaction contained 10 μl of 1× SYBR green Super Mix, 0.6μM (1.2μl) of mouse nephrin or podocin or GAPDH primers, 5 μl of 1:5 dilution of the cDNA prepared as above, and water to 20 μl. The reactions were then followed by 40 cycles of 30 seconds at 95°C,

of 30 seconds at 55°C, and of 72 seconds at 72°C. All measurements were performed in duplicate. The primers used in this study were as follows: mouse GAPDH, forward 5´– AATGTGTCCGTCGTGGATCTGA-3´ and reverse 5´– GATGCCTGCTTCACCACCTTCT-3´, mouse nephrin forward 5´– ACACAAGAAGCTCCACGGTTAG-3´ and reverse 5´– TGGCGATATGACACCTCTTCC-3, mouse podocin forward 5' GTGTCCAAAGCCATCCAGTT 3' and reverse 5' GGCAACCTTTACATCTTGGG 3'. Nephrin and podocin mRNA quantity was normalized to GAPDH mRNA.

5.2 Human study

5.2.1 Patients with LN

Patients with biopsy-confirmed class II (*n*=5), class IV (*n*=4), and class V LN (*n*=6) were studied. Fresh kidney tissue was processed for immunofluorescence (immunoglobulin and complement) and tissue formalin-fixed and embedded in paraffin was used for standard LM and nephrin immunohistochemistry. Control tissue (n=2), was obtained from tumor nephrectomy samples.

5.2.2 Immunohistochemistry

For immunohistochemistry, an avidin-biotin technique was used (Vector Lab: Vectastain Elite ABC-Peroxidase Kit Standard VC-PK-6100). Briefly, antigen retrieval was performed with citrate buffer (pH 6) in a microwave oven at 750w for 20 minutes. Sections were incubated with blocking solution (normal horse serum: Vector 5200 diluted 1:5 in TBS) for 30 minutes at room temperature. After overnight incubation at 4°C with the primary antibody diluted in 1:100 in antibody diluent (Dako), the sections were incubated with biotinylated secondary antibody 1:80 for one hour, followed by 30-min incubation with Vectastain Elite ABC reagent (Vector Lab) at room tempreature. The same semiquantitive score was assigned as for nephrin immunofluorescence.

5.3 Antibodies

An anti mouse IgG (H+L) (BA 2000 Vector Laboratories) was used for IgG immunofluorescence. A FITC conjugated streptavidin (F72 Biomeda) was used as secondary for IgG immunofluorescence. A polyclonal rabbit anti-nephrin antibody directed against the cytoplasmic domain of mouse nephrin was a kind gift from LB Holzman [236] for the western blot and immunofluorescence studies. For immunohistochemistry purpose a primary goat anti nephrin antibody (N20 sc1900) was purchased from Santa Cruz. A FITC conjugated polyclonal Swine anti rabbit IgG (F0205 Dako) and a biotinylated anti goat IgG (BA9500 Vector Lab) were used as secondary for the nephrin immunofluorescence and immunohistochemistry respectively. Podocin was detected by a polyclonal rabbit anti-podocin antibody (ab50339 Abcam) and actin by MAB 1501 Chemicon pan actin monoclonal antibody. The HRP conjugated mouse anti rabbit and HRP conjugated mouse anti mouse with respective codes 7074 and 7076 were purchased from Cell Signalling and used.

5.4 Statistics

Data are expressed as the mean ± standard error of the mean (SEM) value. Comparisons were performed with the Mann-Whitney *U* test for numerical data and with the Fisher's exact test for categorical. Correlation analysis was done by Spearman's rho (ρ) test. All analysis were done with SPSS 16.0 (SPSS, Inc.) and *p*-values (two-tailed) <0.05 were considered as statistically significant.

6. RESULTS

6.1 Clinical picture and histology of NZB/W, C57BL/6, NZN and NZW mice

We first determined the clinical picture and histology of renal disease in our experimental animals.

NZB/W F1 characteristics

Fifteen NZB/W females were examined for the presence of serum anti ds DNA. All were positive for serum anti ds DNA from the age of three months. All (n=2) aged one month were anti ds DNA negative.

Twenty four hour urine protein excretion was determined in 23 NZB/W females. Eleven out of 12 NZB/W aged 3 months had mild and one no proteinuria. Three out of nine aged 6 months had sever and the remaining six severe proteinuria as well as all (n=2) aged 9 months. Severe proteinuria and associated anasarca was exclusively found in the NZBXW females at 6 months of age and older while it was significantly increased from the 3 months. Among the NZB/W population, a strong correlation of 24h proteinuria with age ($\rho=0.75$, $df=21$, $p<0.001$), with the severity of LN as expressed by light ($\rho=0.65$, $df=20$, $p=0.001$) and with the density of IgG deposits ($\rho=0.59$, $df=20$, $p<0.05$) was observed.

Twenty seven NZB/W females were evaluated by light microscopy. All (n=4) aged one month as well as 7 out of 12 aged 3 months had normal light microscopy findings and were encoded as (N). The remaining five showed mild segmental mesangial hyperplasia (MMLN). Five out of 9 aged 6 months and 1 out of 2 aged 9 months exhibited segmental or global, endo- or extracapillary glomerulonephritis involving <50% of all glomeruli (focal proliferative nephritis) and were encoded as FPLN. The remaining, 1 aged 6 months had global glomerulosclerosis, 3 (n=2 aged 6 months,

n=1 aged nine months), showed global extracapillary glomerulonephritis involving ≥50% of all glomeruli with active lesions, cellular crescents, pseudothrombi and subendothelial deposits and two aged 6 months with mainly chronic lesions – fibrocellular crescents. Those 4 were categorized as diffuse proliferative nephritis (DPLN).

IgG immunofluorescence was performed in 23 NZB/W females. All (n=2) aged one month and 9 out of 11 aged 3 months had no IgG deposits. One aged 3 months and 3 out of 9 aged 6 months exhibited a weak signal of IgG IF. Two aged 6 months had moderate amounts of IgG deposition. The remaining 4 as well as 2 aged 9 months exhibited large quantities of IgG (**Figure 6.1**). The findings on LM, IF, proteinuria, and serum anti-dsDNA levels of the NZB/W females are presented in **Table 6.1**.

Table 6.1 Histologic and laboratory findings in NZB/W F1 lupus mice

	Histology class (LM) [1]				
	Normal	MMLN	FPLN	DPLN	Total
Age (months, range)	1-3	1-3	6-9	6-9	
IgG IF staining					
<2+	8/8	5/5	2/6	1/4	16/23
≥2+	0/8	0/5	4/6	3/4	7/23
Proteinuria (mg/24-hr)	5.4 ± 0.5[2]	3.2 ± 1.0	36.9 ± 12.4	46.3 ± 6.7	19.4 ± 5.0
Positive serum anti-dsDNA	3/6	2/3	4/4	2/2	11/15

Abbreviations: LM, light microscopy, IF, immunofluorescence, Normal, normal LM findings, MMLN, mild mesangial hypercellularity, FPLN, focal proliferative lupus nephritis, DPLN, diffuse proliferative lupus nephritis

[1] Active lesions (cellular crescents, pseudothrombi, subendothelial deposits) were demonstrated in 3 out of 4 mice with DPLN. One mouse developed end stage renal disease at the age of 6 months.

[2] Data are expressed as mean ± SEM. Proteinuria correlated with age ($\rho = 0.75$, $p<0.001$), severity of LM findings ($\rho = 0.65$, p = 0.001), and density of IgG deposits ($\rho = 0.59$, p<0.05).

Thirteen NZB/W mice were examined in electron microscope (EM). Evidence of isolated sub-epithelial deposits in some capillary loops and rare sub-endothelial and mesangial deposits were seen in all mice at the age of 1 month. At the age of 3

moths a segmental increase of mesangial matrix was observed. Electron dense deposits were present in mesangial areas and in GBM, in both subendothelial (rare) and subepithelial deposits (more frequent). The sub-epithelial deposits were larger and more prominent. Food processes were relatively well preserved, but there was evidence of podocyte fusion first seen at this age in 2 out of 6. In all mice, at the age of 6 and 9 months there was a variable but at times extensive presence of electron dense deposits. Deposits were present in mesangial areas as well as within GBMs, also in both subendothelial and subepithelial locations. The expansion of the mesangium had caused occlusion of the capillary loops and fusion of podocytes that

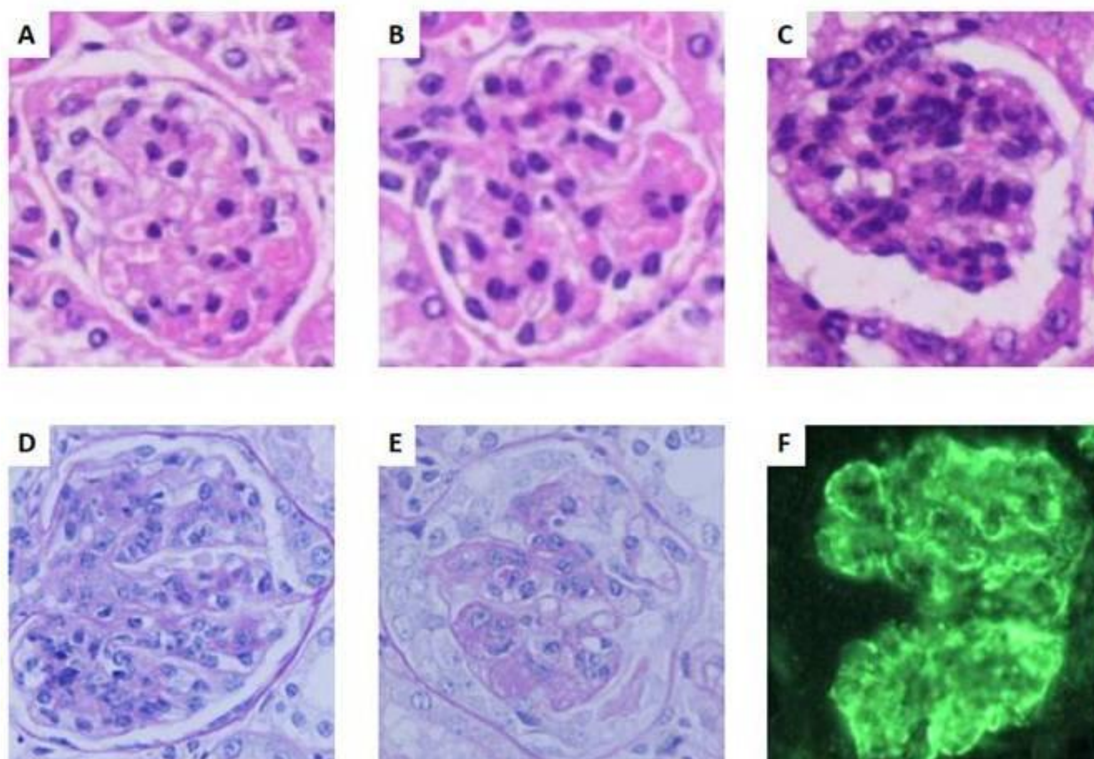

Figure 6.1 Light microscopy and IgG immunofluorescence findings in C57Bl/6 control and NZB/W lupus mice (A) Normal-light microscopy findings in C57BL/6 control and **(B)** 3-month-old NZB/W lupus mice. **(C)** Mild mesangial hyperplasia in 1-month-old NZB/W mouse (MMLN). **(D)** Mesangial proliferation and matrix expansion, narrowed glomerular capillaries, with extracapillary proliferation and adhesion from the glomerular basement membrane to the Bowman capsule in 6-month-old NZB/W mouse. Less than 50% of the glomeruli are affected (FPLN). **(E)** Cellular crescents in 6-month-old NZB/W mouse. More than 50% of the glomeruli are affected (DPLN). **(F)** Immunofluorescence staining in 6-month-old NZB/W mouse with DPLN with strong diffuse IgG staining and characterstic “wire-loop”. Original magnification ×400 in A-F.

was very extensive.

C57BL/6 characteristics

C57BL/6 females a widely used control which exhibit neither autoimmune disorder nor glomerulonephritis was also studied. Results from preliminary experiments confirmed that all C57BL/6 of different ages (1 to 9 months) had the same phenotype and did not differ significantly in between for all tested variables except in proteinuria which was generally within the normal range reaching the levels of mild proteinuria sporadically. Therefore after excluding the mild proteinuric C57BL/6 we considered the remaining 24 C57BL/6 females as the control group with which we have compared the NZB/W females (**Figure 6.2**). All C57BL/6 were anti ds DNA negative. Neither focal nor diffuse proliferative glomerulonephritis was observed in any of the C57BL/6. Large quantities of IgG deposits were absent in all tested C57BL/6 mice. Four out of 4, 8 out of 11, 2 out of 4 and 4 out of 5 aged 1, 3, 6, 9 months

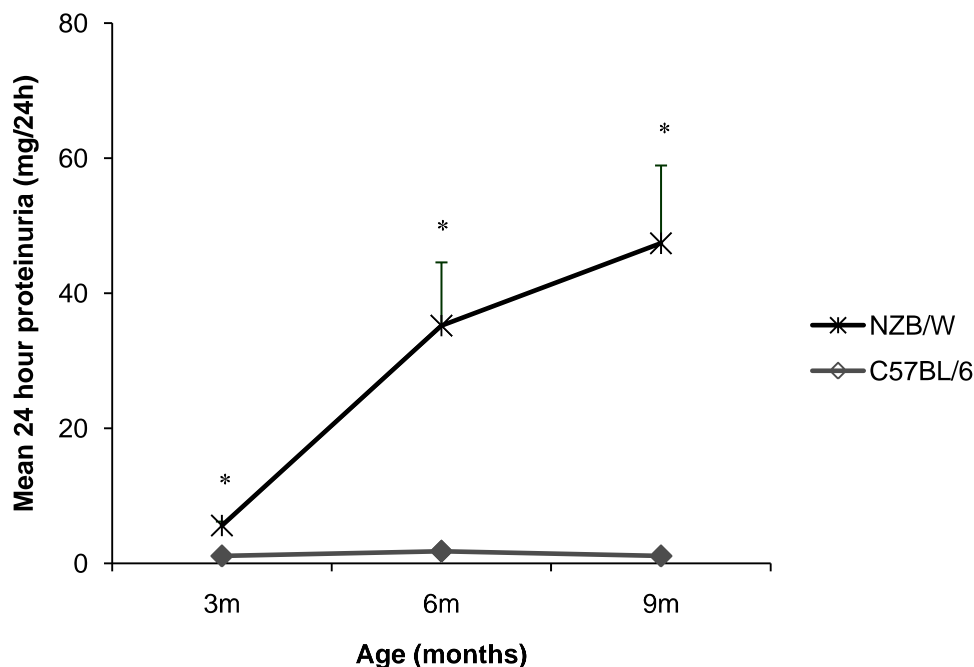

Figure 6.2 24-hr proteinuria of C57BL/6 and of NZB/W.
All C57BL/6 were no proteinuric. The NZB/W proteinuria was significantly higher in all age groups compared to the respective C57BL/6 (∗: $p < 0.05$).

respectively had normal LM findings (**Figure 6.1.A**). The remaining showed very rare segmental mesangial hyperplasia. Twenty one C57BL/6 were evaluated for IgG deposition. All mice, aged 1 month (n=2) and 6 months (n=3) as well as 6 out of 11 aged 3 months had negative IgG immunofluorescence. A weak IgG IF signal was observed in four mice aged 3 months. Moderate amounts of IgG deposits were found in one mouse aged 3 months and in all (n=5) aged 9 months. Electron microscopy confirmed a normal renal tissue with widely patent loops and occasional protrusions of the glomerular basement membrane (GBM) towards the subepithelial space without mesangial expansion and deposits (**Figure 6.4.A**).

<u>NZB and NZW characteristics</u>

The NZB and NZW characteristics are summarized in **table 6.2.** Three NZB mice (NZB2, NZB4, NZB3) and two NZW (NZW3, NZW4) were examined by electron microscope. The NZB exhibited extensive subendothelial and mesangial deposits

Table 6.2 Characteristics of NZB and NZW mice

Mouse code	**NZW1**	**NZW3**	**NZW4**	**NZB2**	**NZB4**	**NZB5**	**NZW2**	**NZB1**	**NZB3**
Age (m)	3	3	3	6	6	6	9	9	9
Sex	M	M	M	F	M	M		F	F
Serum anti ds DNA	pos	pos		pos	pos		pos	pos	pos
Proteinuria (mg/24h)	19,5	24,1	19,3	0,5	12,9	13,9	13,7	0,3	14,9
LM	N	N	N	N		mild mesangial expansion		N	focal proliferation
IgG IF	0	0	2	0	0	3	1	0	1

Abreviations – symbols: m, month, M, male, F, female, pos, positive, ↑, severe, LM, light microscopy, N, normal, IF, immunofluorescence

and showed more podocytes with effaced FPs compared to the NZW. Presence of isolated subendothelial and mesangial deposits and segmental fusion of podocytes were observed in the NZW (**Figure 6.3**)

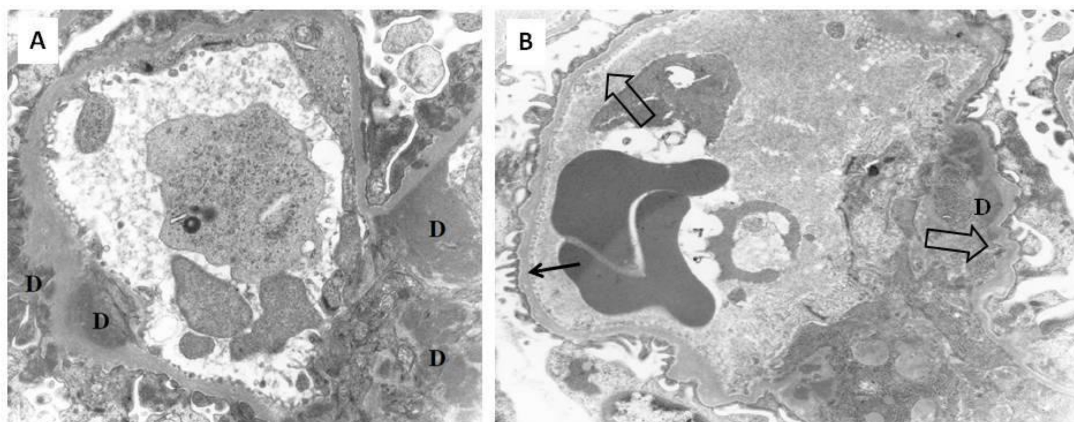

Figure 6.3. Electron microscopy in NZB and NZW mice
(A) Extensive mesangial and subendothelial EDD and diffuse FP effacement in a 9 month old NZB mice. **(B)** Subendothelial deposits and segmental FP fusion and basement membrane thickening (well preserved FP are depicted with the long arrow and fused FP with the two dimension arrows)

6.2 The SD alterations correlate with histology in NZB/W LN mice

EM revealed isolated subepithelial EDD in capillary loops and rare subendothelial and mesangial EDD in NZB/W mice with normal or MMLN LM findings. In these groups of mice podocyte FPs were relatively intact – even in areas overlaying the deposits – and SDs were well defined, with only two out of six mice showing rare FP effacements. These two animals also had more frequent mesangial EDD. On the contrary, the SDs were destructed in mice with LM findings of focal or diffuse proliferative LN (FPLN and DPLN, respectively). The mesangial, subendothelial, and subepithelial EDD were extensive and occlusion of capillary loops, subsequent fusion of podocytes, and distortion of SDs were found.

The severity of mesangial, subendothelial and subepithelial EDD, as well as FP effacement findings were significantly associated with 24-hr proteinuria ($\rho = 0.85$,

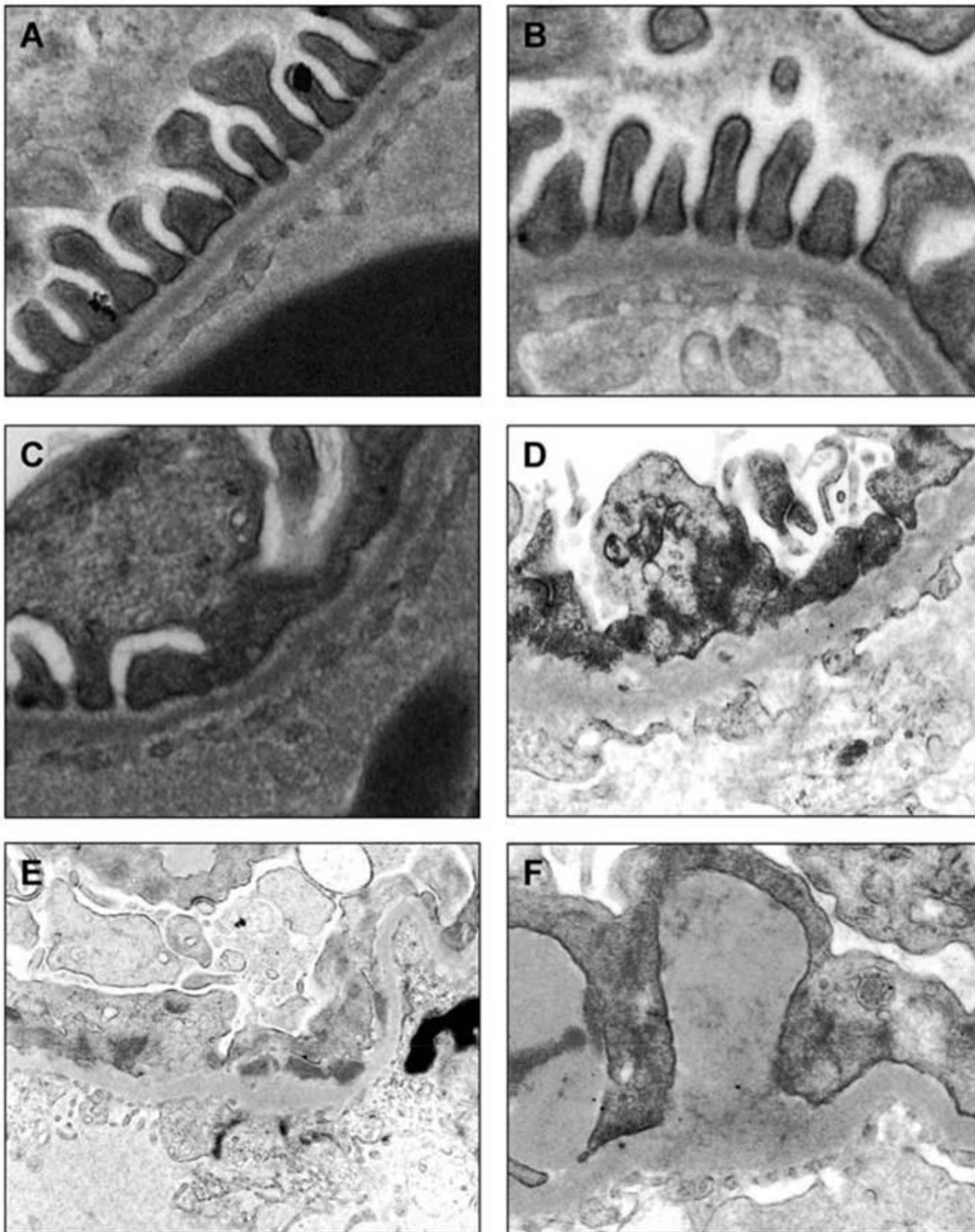

Figure 6.4 Electron microscopy findings in C57Bl/6 control and NZB/W LN mice
(A) Normal podocyte FPs in C57Bl/6 control and **(B)** NZB/W mice with normal LM findings. Slit diaphragms are intact and the glomerular basement membrane has normal thickness. No deposits are observed. **(C)** Rare subepithelial deposits and rare FP effacements in NZB/W mouse with MMLN. **(D)** Extensive subepithelial deposits, FP effacement, and thickening of the glomerular basement membrane in NZB/W with FPLN. **(E, F)** Very extensive FP effacement and thickening of the glomerular basement membrane in NZB/W with DPLN.

$p<0.003$) and IgG intensity ($\rho = 0.90$, $p<0.001$) (data not shown).

6.3 Reduced nephrin at the protein level in NZB/W mice with proliferative LN

In view of our findings of distorted SD structure in NZB/W mice with proliferative LN, we next assessed the glomerular expression of nephrin and podocin, the two major proteins involved in the formation of SD. In order to quantify the total glomerular nephrin and podocin we performed Western blot analysis in the glomerular extracts. We found that nephrin protein expression, was significantly reduced in mice with FPLN or DPLN as compared to C57Bl/6 control mice and NZB/W mice with normal LM findings ($p<0.05$ for all pair-wise comparisons) (**Figure 6.5 A, B**). There was also a trend to reduced nephrin expression in NZB/W mice with MMLN compared to normal mice, and in mice with DPLN compared to the FPLN mice but did not reach a significant level.

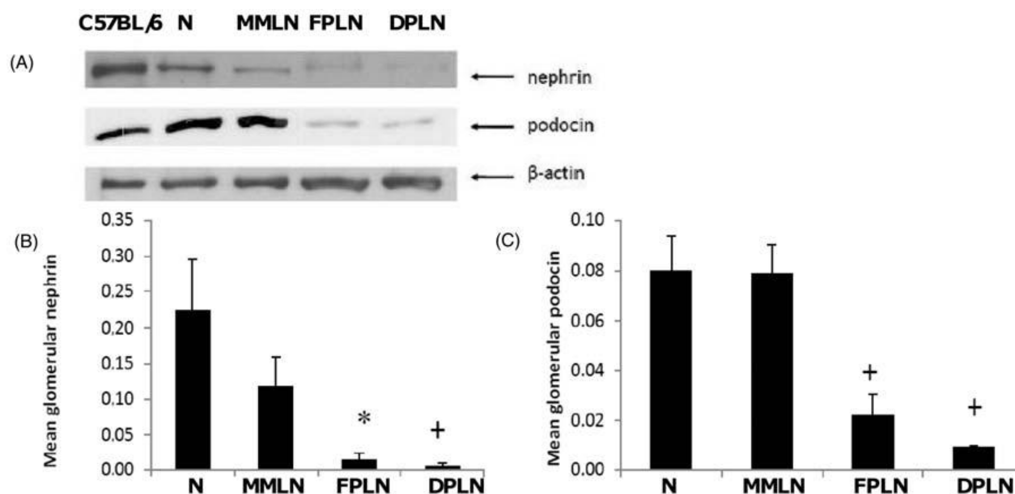

Figure 6.5 At the protein level, the slit diaphragm proteins, nephrin and podocin, are reduced in NZB/W LN mice

(A) Representative western blot analysis of nephrin, podocin, and *β*-actin (indicated by arrows) expression in glomerular extracts of C57Bl/6 control and NZB/W mice. Clear bands of approximately 180 kD and 42kD were visualized in the presence of nephrin and podocin respectively. Band density was measured and corrected with the correspondent *β*-actin band density**.** **(B)** Nephrin protein expression is reduced by almost 50% in NZB/W mice with MMLN, and becomes diminished in mice with FPLN and DPLN. **(C)** Podocin at the protein level is reduced in NZB/W mice with FPLN or DPLN.

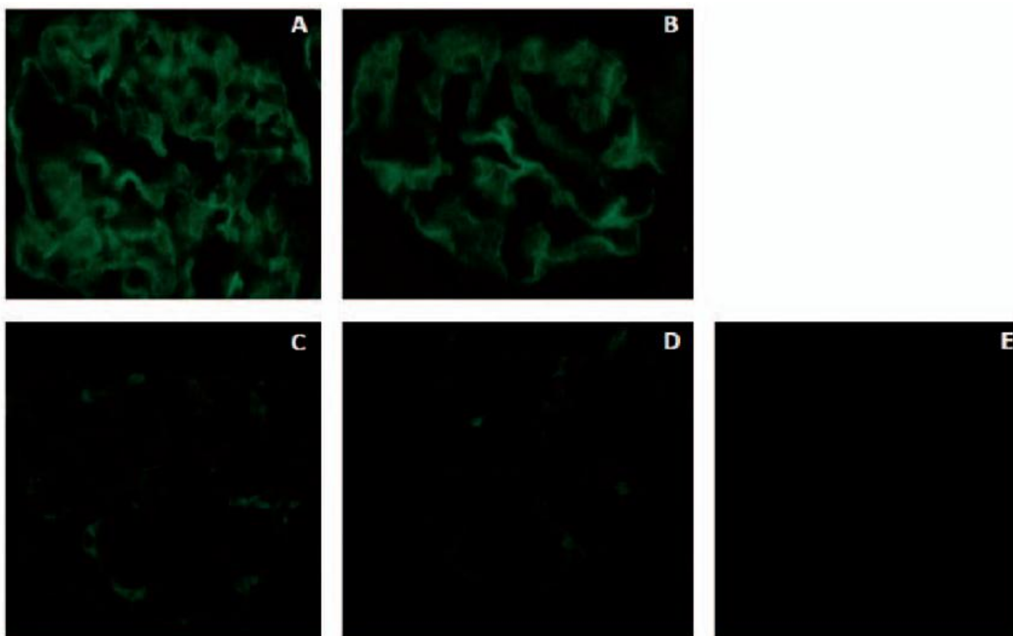

FIGURE 6.6 Immunofluorescence analysis reveals abnormal pattern of nephrin glomerular expression in NZB/W mice with proliferative LN
(A) Immunofluorescence study of C57Bl/6 kidney tissue shows intense linear nephrin staining along the glomerular capillary wall, in a glomerular basement membrane pattern. **(B)** A weaker, with finely granular pattern along the most part of the glomerular basement membrane is observed in NZB/W mice with MMLN. **(C)** Significantly attenuated and segmentally dispersed nephrin signal in NZB/W mice with FPLN. **(D)** Diffusely attenuated nephrin signal in NZB/W mice with DPLN. (**E)** Negative nephrin immunofluorescence in renal glomeruli of one NZB/W mouse with DPLN. Original magnification ×400 in panels A, C–E, ×250 in panel B.

We next evaluated the expression and distribution of nephrin in murine LN by IF. A strong nephrin signal was detected in renal glomeruli of control C57Bl/6 and NZB/W mice with normal LM (**Figure 6.6 A**). A less intense and sparser, yet linear pattern was observed in mice with MMLN (**Figure 6.6 B**). In contrast, mice with FPLN or DPLN had faint nephrin signal with segmental localization and granular appearance (**Figure 6.6 C-E**). Semiquantification of the staining intensity showed that nephrin was significantly decreased in FPLN/DPLN as compared to control C57Bl/6 mice and NZB/W mice with MMLN or normal LM (p<0.05 for both) (**Figure 6.7**)

6.4 Reduced podocin at the protein level in NZB/W mice with proliferative LN

Similar to nephrin, podocin protein expression was significantly decreased in renal glomeruli of NZB/W mice with FPLN and DPLN as compared to control C57Bl/6 mice and NZB/W mice with normal LM findings (p<0.05) (**Figure 6.5 A, C**). Glomerular podocin expression was inversely correlated to 24-hr proteinuria levels (ρ = –0.67,

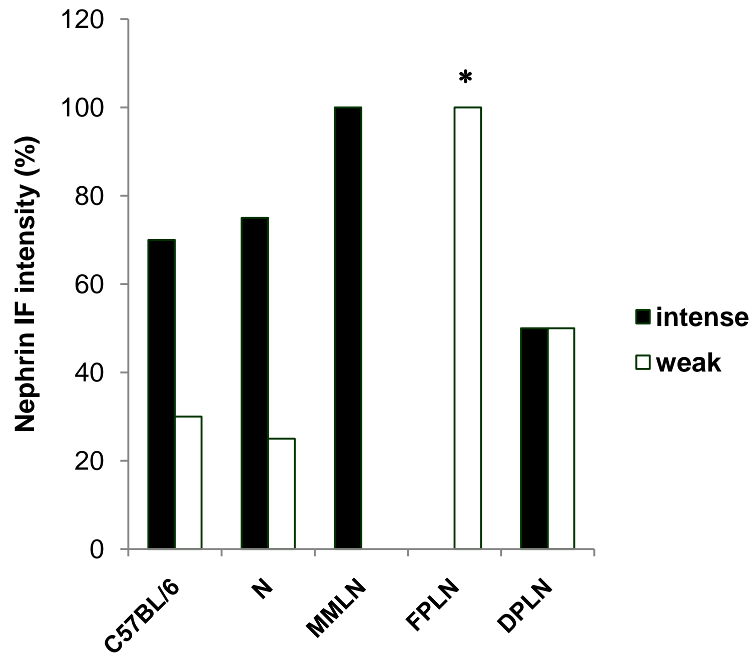

Figure 6.7 Nephrin IF signal is significantly weaker in FPLN compared to C57BL/6 and to MMLN (∗ p<0.05). When regarding FPLN and DPLN as one group of proliferative LN, nephrin is significantly reduced for all comparison (p<0.05)

p<0.003) (**Figure 6.8**).

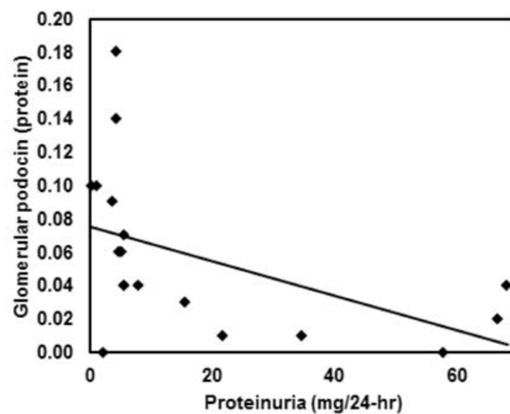

Figure 6.8 Inverse correlation between total glomerular podocin protein levels and 24-hr proteinuria in NZB/W mice (ρ=-0.67, p<0.003)

6.5 Glomerular expression of nephrin and podocin correlates with EM findings in NZB/W mice

We next examined glomerular nephrin and podocin expression in association with EM findings in

Table 6.3 Correlation between EM findings and glomerular nephrin and podocin protein expression

EM findings	Nephrin protein	Podocin protein
	Spearman's rho (ρ)	
Mesangial EDD	-0.79 (*p*=0.004)	-0.93 (*p*<0.001)
Subendothelial EDD	-0.81 (*p*=0.002)	-0.87 (*p*<0.001)
Subepithelial EDD	-0.79 (*p*=0.004)	-0.93 (*p*<0.001)
FP effacement	-0.85 (*p*<0.001)	-0.93 (*p*<0.001)

Abbreviations: EM, electron microscopy, EDD, electron dense deposis, FP, podocyte foot process.

Mesangial, subendothelial and subepithelial EDD were evaluated semi-quantitatively as follows: 0, negative; 1+, frequent; 2+, extensive. Presence of FP effacement was characterized as: 0, negative; 1+, rare; 2+, extensive.

NZB/W lupus mice. We observed a strong negative correlation between mesangial, subendothelial, and subepithelial EDD with nephrin ($\rho = -0.81$, $p<0.005$) and podocin ($\rho = -0.91$, $p<0.001$) at the protein level **(Table 6.3)**. Both proteins were significantly reduced in NZB/W mice with extensive FP effacements as compared to mice with no FP effacement ($p<0.05$ for both) (data not shown).

6.6 Nephrin and podocin mRNA levels are decreased in NZB/W mice with DPLN

To determine whether reduced protein expression of the SD proteins nephrin and podocin is due to altered transcription of the corresponding genes, we performed quantitative real-time PCR in kidney tissue from NZB/W LN and C57Bl/6 control mice. mRNA levels for nephrin and podocin were measured and corrected for the mRNA level of GAPDH. NZB/W nephrin mRNA level remained stable, not differing from control's in the various histological groups with a borderline significant ($P=0.05$) upregulation observed in the RMLN. Glomerular nephrin mRNA levels were significantly reduced in DPLN – but not in FPLN – compared to MMLN mice

(p=0.034) (**Figure 6.9 A**). Mice with DPLN had also significantly reduced podocin mRNA levels compared to other NZB/W histological groups (p<0.05 for all

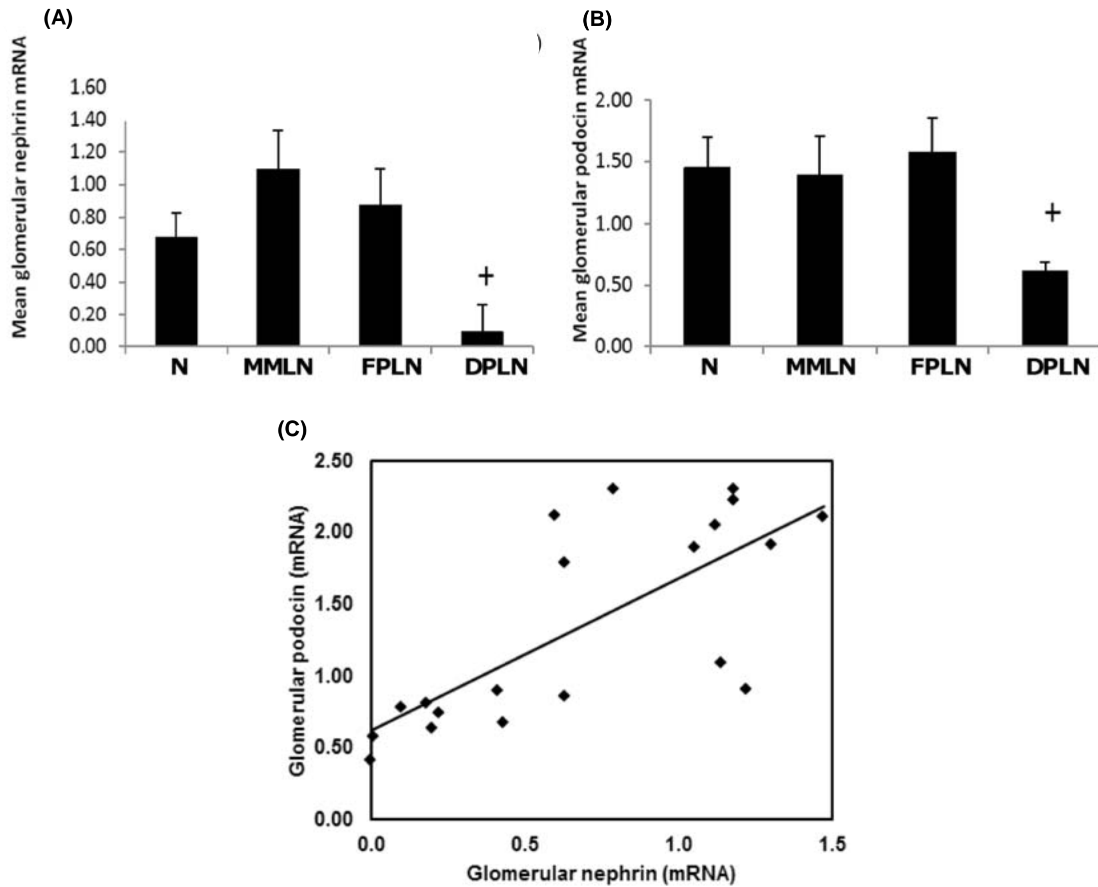

Figure 6.9 Corrected **(A)** Nephrin and **(B)** podocin glomerular mRNA levels are significantly reduced in NZB/W mice with DPLN. **(C)** Significant correlation between nephrin and podocin mRNA levels in NZB/W mice (ρ=0.77, p<0.001). *p<0.01; ^{+}p<0.05

comparisons) (**Figure 6.9 B**). We found a good correlation between glomerular nephrin and podocin mRNA levels in NZB/W mice (ρ = 0.77, p<0.001) **(Figure 6.9 C**).

6.7 Both NZB and NZW exhibit glomerular alterations

Based on our findings regarding (a) significant SD alterations in the NZB/W females with proliferative LN and (b) significant proteinuria of NZB and NZW mice we determined the total glomerular nephrin and podocin at the protein level by western blot and at the mRNA level by real time RT-PCR. We have also examined the renal

tissue with nephrin IF (**Figure 6.11**). Both western blot analysis and IF study revealed a reduced, however not significantly, glomerular nephrin protein in the NZB and in the NZW compared to the C57BL/6 and to the NZB/W mice with normal LM findings. Podocin protein was reduced in the NZB as well as in the NZW mice compared to NZB/W with normal LM findings (*p*= 0.024, *p*=0.030 respectively) and with MMLN (*p*= 0.034 for both NZB and NZW). Nephrin and podocin mRNA was found well preserved.

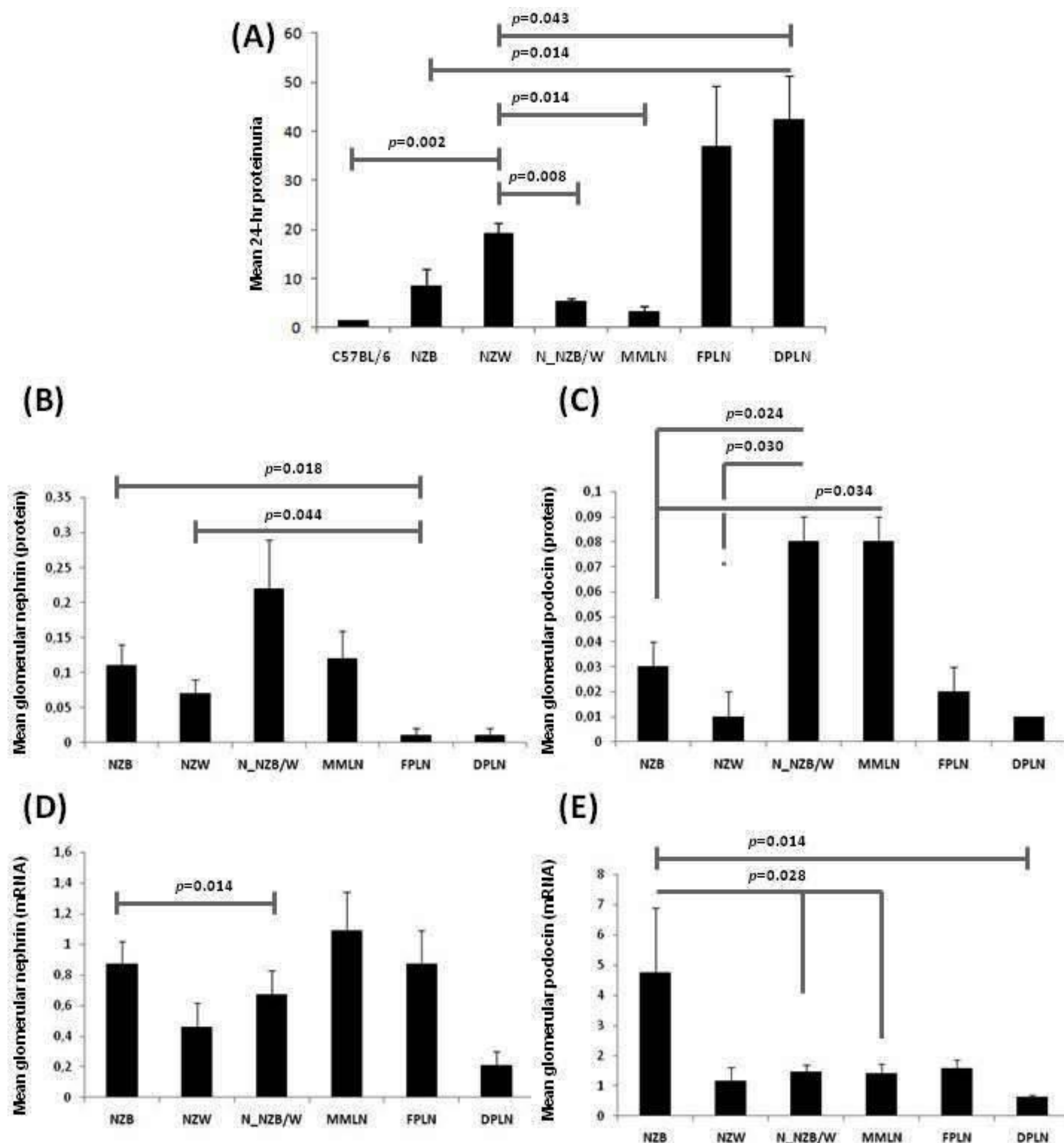

Figure 6.11 Mean **(A)** 24 hour proteinuria, **(B)** glomerular nephrin (protein), **(C)** glomerular podocin (protein), **(D)** glomerular nephrin (mRNA), **(E)** glomerular podocin (mRNA) of C57BL/6, NZB, NZW, NZB/W with normal light microscopy finding (N_NZB/W), MMLN, FPLN and DPLN.

6.8 Nephrin is reduced in patients with DPLN

We sought to verify our findings in murine LN by perfoming immunohistochemistry for nephrin expression in renal tissue from patients with various classes of LN (**Table 6.4**). As expected, 24 hour proteinuria was significantly higher in LN patients

Table 6.4 Clinical characteristics of patients with LN

	N (female)	Age (years)	Proteinuria (g/24-hr)
Class II	5 (4)	43 ± 6	0.9 ± 0.4
Class IV	4 (2)	36 ± 4	2.0 ± 1.9
Class V	7 (5)	46 ± 4	2.6 ± 0.5

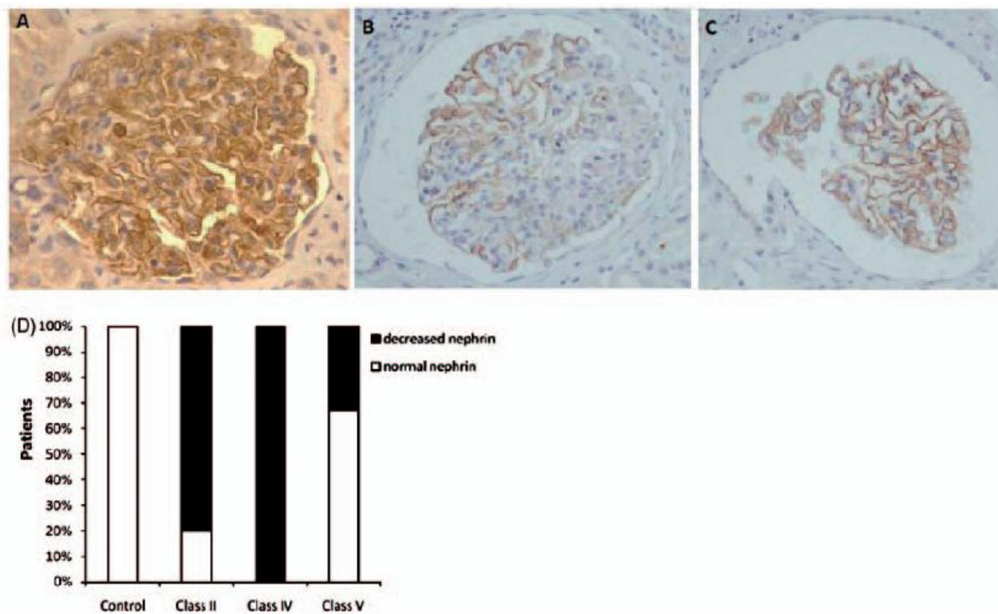

Figure 6.10 Reduced glomerular nephrin expression in patients with DPLN.
(A) Control kidney biopsy shows intense linear nephrin expression along the glomerular capillary wall. **(B)** Nephrin expression is significantly reduced in parts of the glomeruli with severe intra-capillary proliferative lesions in a patient with class IV LN. **(C)** Less intense but stil linear distribution of nephrin expression in a patient with class V LN. Original magnification ×300-400 in panels **A–C**. **(D)** Patients were categorized according to nephrin immunohistochemistry staining intensity into: a) reduced nephrin expression (0 or 1+ score in semi-quantitative analysis), and b) normal nephrin expression (2+ score). Significantly more patients with class IV LN had reduced nephrin expression, as compared to patients with class II or V LN (p<0.05).

compared to healthy controls, and in patients with class IV/V compared to those with class II LN (p<0.05 for both). In kidney biopsies from control patients (**Figure 6.10 A**) and patients with class II LN, nephrin showed an intense homogeneous and linear epithelial staining along the peripheral capillary loops of the glomeruli. Conversely, in patients with class IV LN, glomerular staining was faint and non-continuous, particularly in segments with severe intracapillary proliferation (**Figure 6.10 B**). A weak but homogeneous and linear staining along the peripheral capillary loops of the glomeruli was observed in patients with class V LN (**Figure 6.10 C**).

7 DISCUSSION

A wide spectrum of histologically and clinically heterogeneous renal diseases are included in LN. Proteinuria, a significant feature of LN, is present at varying degree and correlates with histological lesions. Alterations in SD components, such as nephrin and podocin, are implicated in the development of proteinuria in immune-mediated renal diseases, and SD expression pattern may differ depending upon the specific glomerular disease and the severity of proteinuria.[237-238].

Our study represents the first comprehensive analysis of podocin and nephrin expression at protein and mRNA level coupled with IF findings in murine and human LN.

We provide evidence for presence of podocytopathy occurring at early stages of murine LN. Glomerular nephrin protein levels were reduced by almost 50% in mild LN (MMLN) and became diminished at more advanced stages (FPLN/DPLN). Our IF study confirmed the WB results and also, indicated abnormal distribution pattern of nephrin in MMLN mice with reduced protein expression. Change in nephrin expression could precede ultra-structural changes in SD, since only two out of six NZB/W mice with normal or MMLN histology had rare FP effacements on EM. It is mentioned that podocin protein levels were reduced only at proliferative disease stages (FPLN/DPLN). This may be due to the fact that following podocyte injury and before proteinuria onset, podocin dissociates from nephrin, resulting in the urinary excretion of the latter[239].

Previous studies on nephrin and podocin expression in experimental immune-mediated nephritis have reached conflicting results. Luimula *et al.* [240] found no alterations in the late phase of active Heymann nephritis, a model of autologous IC

nephritis. Conversely, in PHN induced by rabbit anti-rat kidney antibodies, Clement *et al.*[241] reported decreased nephrin expression only during the autologous (late) proteinuric phase, whereas others [168, 239, 242] have reported decreased nephrin levels at early PHN (heterologous phase). Our findings agree with those of Hidaka *et al.*[169] demonstrating decreased nephrin expression at early stages of crescentic anti-GBM nephritis.

Much attention has recently been focused on the role of podocytes in human LN, and several studies have reported a correlation between proteinuria and diffuse podocyte FP effacement in cases of LN without evidence of immune deposits.[243-245] Limited data from human studies have indicated reduced nephrin expression assessed by IF in proteinuric LN,[246-250] and a single study has reported diminished nephrin levels in active LN patients [251]. However, the aforementioned studies included a small number of patients and did not examine associations with renal histology. We found significantly reduced nephrin expression in patients with class IV proliferative disease as compared to class V patients, despite both groups having comparable levels of proteinuria. A greater number of human biopsies are needed to determine possible alterations in nephrin expression in class II LN. These results confirm our findings in murine LN, suggesting that nephrin expression correlates better with the histological class than with the level of proteinuria. This is in line with previous reports in minimal change nephrotic syndrome (MCNS) and IgA nephropathy, [252-253] and in contrast with other studies which have demonstrated significant down-regulation of nephrin in patients with nephrotic syndrome[254-256]. Podocyte loss in the urine, which has been shown to correlate with LN activity and the degree of proteinuria, [257] might also explain the reduced expression of SD proteins.

Although small numbers of NZB and NZW mice have been studied, we give evidence that these mice exhibit immune mediated glomerular alterations early in their life with proteinuria being the prevalent clinical manifestation. The normal LM findings do not exclude the podocyte FP effacement, which were shown only under the electron microscope. NZB nephritis and the probability that NZW might be more susceptible to immune mediated renal damage has also been reported by others [227, 258-259]. Our findings regarding NZW nephritis are also in agreement with Hahn's *et al* [260] who demonstrated immune compromise nephritis in the previously regarded healthy NZW that was indistinguishable to NZB/W and NZB nephritis. The novel in our study is that the NZW nephritis is elucidated by the EM study, which reveals the FP effacement as the cause of the severe proteinuria. We give evidence that the ultrastructural alterations regarding reduced podocin in the NZB/W ancestors might is a co-factor contributing to NZB/W predisposition to renal disease. However we failed to demonstrate a significant alteration in the nephrin which might be due to the small number of animals studied or to the mild renal disease. The disparity of NZB and NZW nephritis as well as the inconsistency between the histological findings and the clinical signs remains to be clarified.

Although the exact pathogenesis of lupus-associated podocytopathy is unknown, there is evidence to support a role for kidney-infiltrating $CD8^+$ T cells, which secrete inflammatory cytokines and mediate podocyte cytotoxic effects [261]. Anti-dsDNA antibodies may cross-react with podocyte proteins like α-actinin-4 to induce podocyte injury independent of IC formation [262]. Moreover, using conditionally immortalized reporter podocytes, Takano *et al.*[263] demonstrated that bystander macrophages as well as macrophage-derived cytokines (IL-1b, TNF), which are known to be increased in proliferative LN, markedly suppressed nephrin gene promoter activity. This is in accordance with our findings of reduced nephrin and podocin mRNA expression only in the most severe forms of LN. In this context, podocytes may

possess immunological properties as they express cytokine and chemokine receptors, produce inflammatory mediators, and upregulate the costimulatory molecule B7-1 via TLR-4 signaling.[245] Recently, Machida *et al.*[251] showed induction of TLR-9 expression in renal podocytes in childhood-onset active class IV LN, associated with reduced nephrin and podocin expression. TLR-9 may respond to CpG-DNA thus providing a hypothetical mechanism for DNA-containing ICs to promote podocyte injury in proliferative LN. Finally, one cannot exclude the possibility that structures adjacent to podocytes may also be targets of immune attack in lupus.

In conclusion, we provide evidence for SD abnormalities starting at early stages of LN and becoming pronounced at more advanced stages of proliferative disease. Reduced nephrin and podocin expression correlates better with the histological class of LN than with proteinuria levels. These findings suggest a novel role for podocytes and their structures in the pathogenesis of immune-mediated nephritis, and point toward the potential development of targeted therapies that could preserve SD structure and podocyte function [264].

8. ACKNOWLEDGMENTS

We wish to thank Dr Symeon H Panagiotakis for critically reviewing the manuscript; Christiana Choulaki and Christina Kastrinaki for developing the sequences of nephrin and podocin mRNA primers; Maria Spiliotaki and Alexandra Voutsina for teaching the biochemical and molecular biology methods; Stauros Stamatakis, Ioannis Petrakis, Carmen Skembari and Savvas Mavides for technical support. We also acknowledge Lawrence B. Holzman and Rakesh Verma (University of Michigan Medical School, Ann Arbor) for providing the anti nephrin antibody.

9. REFERENCES

1. Briggs J.P, K.W., Schnermann J.B, ed. *Overview of renal function and structure*. third ed. Primer on Kidney Diseases, ed. G. A. 2001, Academic Press: san Diego, California.
2. Glassock, R.J.C.A.H., ed. *Normal vascular and glomerular anatomy*. Atlas of diseases of the kidney, ed. S. RW. Vol. 2. 1999, Blackwell Science: Philladelphia.
3. Tryggvason, K. and J. Wartiovaara, *How does the kidney filter plasma?* Physiology (Bethesda), 2005. **20**: p. 96-101.
4. Chadban, S.J. and R.C. Atkins, *Glomerulonephritis.* Lancet, 2005. **365**(9473): p. 1797-806.
5. Couser, W.G., *Pathogenesis of glomerulonephritis.* Kidney Int Suppl, 1993. **42**: p. S19-26.
6. Couser, W.G., *Research opportunities and future directions in glomerular disease.* Semin Nephrol, 1993. **13**(5): p. 457-71.
7. Wolf, G., *Molecular mechanisms of renal hypertrophy: role of p27Kip1.* Kidney Int, 1999. **56**(4): p. 1262-5.
8. Kashtan, C., *Autotopes and allotopes.* J Am Soc Nephrol, 2005. **16**(12): p. 3455-7.
9. Couser, W.G. and D.J. Salant, *In situ immune complex formation and glomerular injury.* Kidney Int, 1980. **17**(1): p. 1-13.
10. Nangaku, M. and W.G. Couser, *Mechanisms of immune-deposit formation and the mediation of immune renal injury.* Clin Exp Nephrol, 2005. **9**(3): p. 183-91.
11. Hiki, Y., et al., *Mass spectrometry proves under-O-glycosylation of glomerular IgA1 in IgA nephropathy.* Kidney Int, 2001. **59**(3): p. 1077-85.
12. Allen, A.C., et al., *Mesangial IgA1 in IgA nephropathy exhibits aberrant O-glycosylation: observations in three patients.* Kidney Int, 2001. **60**(3): p. 969-73.
13. Smith, A.C., et al., *O-glycosylation of serum IgA1 antibodies against mucosal and systemic antigens in IgA nephropathy.* J Am Soc Nephrol, 2006. **17**(12): p. 3520-8.
14. Giannakakis, K., et al., *Aberrantly glycosylated IgA1 in glomerular immune deposits of IgA nephropathy.* J Am Soc Nephrol, 2007. **18**(12): p. 3139-46.
15. McDonald, K.J., et al., *Expression of Fc alpha/mu receptor by human mesangial cells: a candidate receptor for immune complex deposition in IgA nephropathy.* Biochem Biophys Res Commun, 2002. **290**(1): p. 438-42.
16. Haddad, E., et al., *Enhanced expression of the CD71 mesangial IgA1 receptor in Berger disease and Henoch-Schonlein nephritis: association between CD71 expression and IgA deposits.* J Am Soc Nephrol, 2003. **14**(2): p. 327-37.
17. Moura, I.C., et al., *The glomerular response to IgA deposition in IgA nephropathy.* Semin Nephrol, 2008. **28**(1): p. 88-95.
18. Gomez-Guerrero, C., N. Duque, and J. Egido, *Mesangial cells possess an asialoglycoprotein receptor with affinity for human immunoglobulin A.* J Am Soc Nephrol, 1998. **9**(4): p. 568-76.
19. Barratt, J., et al., *Identification of a novel Fcalpha receptor expressed by human mesangial cells.* Kidney Int, 2000. **57**(5): p. 1936-48.
20. Moura, I.C., et al., *Identification of the transferrin receptor as a novel immunoglobulin (Ig)A1 receptor and its enhanced expression on mesangial cells in IgA nephropathy.* J Exp Med, 2001. **194**(4): p. 417-25.

21. Moura, I.C., et al., *Engagement of transferrin receptor by polymeric IgA1: evidence for a positive feedback loop involving increased receptor expression and mesangial cell proliferation in IgA nephropathy.* J Am Soc Nephrol, 2005. **16**(9): p. 2667-76.
22. Xiao, H., et al., *Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies.* Am J Pathol, 2007. **170**(1): p. 52-64.
23. Jennette, J.C., H. Xiao, and R.J. Falk, *Pathogenesis of vascular inflammation by anti-neutrophil cytoplasmic antibodies.* J Am Soc Nephrol, 2006. **17**(5): p. 1235-42.
24. Shlomchik, M.J. and M.P. Madaio, *The role of antibodies and B cells in the pathogenesis of lupus nephritis.* Springer Semin Immunopathol, 2003. **24**(4): p. 363-75.
25. Horl, W.H. and D. Kerjaschki, *Membranous glomerulonephritis (MGN).* J Nephrol, 2000. **13**(4): p. 291-316.
26. Ronco, P.M., *Paraneoplastic glomerulopathies: new insights into an old entity.* Kidney Int, 1999. **56**(1): p. 355-77.
27. Debiec, H., et al., *Antenatal membranous glomerulonephritis with vascular injury induced by anti-neutral endopeptidase antibodies: toward new concepts in the pathogenesis of glomerular diseases.* J Am Soc Nephrol, 2003. **14 Suppl 1**: p. S27-32.
28. Kerjaschki, D., *Pathomechanisms and molecular basis of membranous glomerulopathy.* Lancet, 2004. **364**(9441): p. 1194-6.
29. Kerjaschki, D. and M.G. Farquhar, *The pathogenic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border.* Proc Natl Acad Sci U S A, 1982. **79**(18): p. 5557-61.
30. Van Damme, B.J., et al., *Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex glomerulonephritis.* Lab Invest, 1978. **38**(4): p. 502-10.
31. Couser, W.G., et al., *Experimental glomerulonephritis in the isolated perfused rat kidney.* J Clin Invest, 1978. **62**(6): p. 1275-87.
32. Beck, L.H., Jr., et al., *M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy.* N Engl J Med, 2009. **361**(1): p. 11-21.
33. Glassock, R.J., *Human idiopathic membranous nephropathy--a mystery solved?* N Engl J Med, 2009. **361**(1): p. 81-3.
34. Hudson, B.G., S.T. Reeders, and K. Tryggvason, *Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis.* J Biol Chem, 1993. **268**(35): p. 26033-6.
35. Kalluri, R., et al., *Specificity of circulating and tissue-bound autoantibodies in Goodpasture syndrome.* Proc Assoc Am Physicians, 1996. **108**(2): p. 134-9.
36. Leinonen, A., et al., *Goodpasture antigen: expression of the full-length alpha3(IV) chain of collagen IV and localization of epitopes exclusively to the noncollagenous domain.* Kidney Int, 1999. **55**(3): p. 926-35.
37. Nangaku, M., *Complement regulatory proteins in glomerular diseases.* Kidney Int, 1998. **54**(5): p. 1419-28.
38. Hansch, G.M., et al., *Immune damage to the mesangium: antibody- and complement-mediated stimulation and destruction of mesangial cells.* J Am Soc Nephrol, 1992. **2**(10 Suppl): p. S139-43.
39. Pippin, J.W., et al., *DNA damage is a novel response to sublytic complement C5b-9-induced injury in podocytes.* J Clin Invest, 2003. **111**(6): p. 877-85.
40. Endoh, M., et al., *Immunohistochemical demonstration of membrane cofactor protein (MCP) of complement in normal and diseased kidney tissues.* Clin Exp Immunol, 1993. **94**(1): p. 182-8.

41. Cosio, F.G., et al., *Localization of decay accelerating factor in normal and diseased kidneys.* Kidney Int, 1989. **36**(1): p. 100-7.
42. Puri, T.S. and R.J. Quigg, *The many effects of complement C3- and C5-binding proteins in renal injury.* Semin Nephrol, 2007. **27**(3): p. 321-37.
43. Schiller, B., et al., *Inhibition of complement regulation is key to the pathogenesis of active Heymann nephritis.* J Exp Med, 1998. **188**(7): p. 1353-8.
44. Nishikage, H., et al., *The role of a complement regulatory protein in rat mesangial glomerulonephritis.* J Am Soc Nephrol, 1995. **6**(2): p. 234-41.
45. Ikezumi, Y., et al., *Adoptive transfer studies demonstrate that macrophages can induce proteinuria and mesangial cell proliferation.* Kidney Int, 2003. **63**(1): p. 83-95.
46. Okada, H., et al., *Tubular osteopontin expression in human glomerulonephritis and renal vasculitis.* Am J Kidney Dis, 2000. **36**(3): p. 498-506.
47. Nikolic-Paterson, D.J. and R.C. Atkins, *The role of macrophages in glomerulonephritis.* Nephrol Dial Transplant, 2001. **16 Suppl 5**: p. 3-7.
48. Lan, H.Y., et al., *The pathogenic role of macrophage migration inhibitory factor in immunologically induced kidney disease in the rat.* J Exp Med, 1997. **185**(8): p. 1455-65.
49. Timoshanko, J.R., et al., *Granulocyte macrophage colony-stimulating factor expression by both renal parenchymal and immune cells mediates murine crescentic glomerulonephritis.* J Am Soc Nephrol, 2005. **16**(9): p. 2646-56.
50. Shimizu, H., et al., *Anti-monocyte chemoattractant protein-1 gene therapy attenuates renal injury induced by protein-overload proteinuria.* J Am Soc Nephrol, 2003. **14**(6): p. 1496-505.
51. Lan, H.Y., et al., *Local macrophage proliferation in the progression of glomerular and tubulointerstitial injury in rat anti-GBM glomerulonephritis.* Kidney Int, 1995. **48**(3): p. 753-60.
52. Yang, N., et al., *Local macrophage proliferation in human glomerulonephritis.* Kidney Int, 1998. **54**(1): p. 143-51.
53. Isbel, N.M., et al., *Local macrophage proliferation correlates with increased renal M-CSF expression in human glomerulonephritis.* Nephrol Dial Transplant, 2001. **16**(8): p. 1638-47.
54. Kaneko, Y., et al., *Macrophage metalloelastase as a major factor for glomerular injury in anti-glomerular basement membrane nephritis.* J Immunol, 2003. **170**(6): p. 3377-85.
55. Hewitson, T.D. and G.J. Becker, *Interstitial myofibroblasts in IgA glomerulonephritis.* Am J Nephrol, 1995. **15**(2): p. 111-7.
56. Johnson, R.J., et al., *The activated mesangial cell: a glomerular "myofibroblast"?* J Am Soc Nephrol, 1992. **2**(10 Suppl): p. S190-7.
57. Border, W.A., et al., *Suppression of experimental glomerulonephritis by antiserum against transforming growth factor beta 1.* Nature, 1990. **346**(6282): p. 371-4.
58. Quiza, C.G., P.L. Leenaerts, and B.M. Hall, *The role of T cells in the mediation of glomerular injury in Heymann's nephritis in the rat.* Int Immunol, 1992. **4**(4): p. 423-32.
59. Penny, M.J., R.A. Boyd, and B.M. Hall, *Permanent CD8(+) T cell depletion prevents proteinuria in active Heymann nephritis.* J Exp Med, 1998. **188**(10): p. 1775-84.
60. Bolton, W.K., et al., *Transfer of experimental glomerulonephritis in chickens by mononuclear cells.* Kidney Int, 1988. **34**(5): p. 598-610.
61. Tipping, P.G., T.J. Neale, and S.R. Holdsworth, *T lymphocyte participation in antibody-induced experimental glomerulonephritis.* Kidney Int, 1985. **27**(3): p. 530-7.

62. Baker, A.J., et al., *Mesangial cell apoptosis: the major mechanism for resolution of glomerular hypercellularity in experimental mesangial proliferative nephritis.* J Clin Invest, 1994. **94**(5): p. 2105-16.
63. Koyama, A., et al., *A glomerular permeability factor produced by human T cell hybridomas.* Kidney Int, 1991. **40**(3): p. 453-60.
64. Sellier-Leclerc, A.L., et al., *A humanized mouse model of idiopathic nephrotic syndrome suggests a pathogenic role for immature cells.* J Am Soc Nephrol, 2007. **18**(10): p. 2732-9.
65. Dantal, J., et al., *Effect of plasma protein adsorption on protein excretion in kidney-transplant recipients with recurrent nephrotic syndrome.* N Engl J Med, 1994. **330**(1): p. 7-14.
66. Kitching, A.R., et al., *The requirement for granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in leukocyte-mediated immune glomerular injury.* J Am Soc Nephrol, 2002. **13**(2): p. 350-8.
67. Xiao, H., et al., *The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies.* Am J Pathol, 2005. **167**(1): p. 39-45.
68. Segerer, S. and D. Schlondorff, *Role of chemokines for the localization of leukocyte subsets in the kidney.* Semin Nephrol, 2007. **27**(3): p. 260-74.
69. Segerer, S., P.J. Nelson, and D. Schlondorff, *Chemokines, chemokine receptors, and renal disease: from basic science to pathophysiologic and therapeutic studies.* J Am Soc Nephrol, 2000. **11**(1): p. 152-76.
70. De Vriese, A.S., et al., *The role of selectins in glomerular leukocyte recruitment in rat anti-glomerular basement membrane glomerulonephritis.* J Am Soc Nephrol, 1999. **10**(12): p. 2510-7.
71. Johnson, R.J., et al., *New mechanism for glomerular injury. Myeloperoxidase-hydrogen peroxide-halide system.* J Clin Invest, 1987. **79**(5): p. 1379-87.
72. Jennette, J.C. and R.J. Falk, *New insight into the pathogenesis of vasculitis associated with antineutrophil cytoplasmic autoantibodies.* Curr Opin Rheumatol, 2008. **20**(1): p. 55-60.
73. Mundel, P. and S.J. Shankland, *Podocyte biology and response to injury.* J Am Soc Nephrol, 2002. **13**(12): p. 3005-15.
74. Shankland, S.J., *The podocyte's response to injury: Role in proteinuria and glomerulosclerosis.* Kidney International, 2006. **69**.
75. Drenckhahn, D. and R.P. Franke, *Ultrastructural organization of contractile and cytoskeletal proteins in glomerular podocytes of chicken, rat, and man.* Lab Invest, 1988. **59**(5): p. 673-82.
76. Machuca, E., G. Benoit, and C. Antignac, *Genetics of nephrotic syndrome: connecting molecular genetics to podocyte physiology.* Hum Mol Genet, 2009. **18**(R2): p. R185-94.
77. K, M., *Podoplanin, a novel 43-kDa membrane protein, controls the shape of podocytes.* Nephrology Dialysis and Transplantation, 1999. **14 (Suppl 1)**: p. 9.
78. Schnabel, E., et al., *Biogenesis of podocalyxin--the major glomerular sialoglycoprotein--in the newborn rat kidney.* Eur J Cell Biol, 1989. **48**(2): p. 313-26.
79. Pagtalunan, M.E., et al., *Podocyte loss and progressive glomerular injury in type II diabetes.* J Clin Invest, 1997. **99**(2): p. 342-8.
80. Kurihara, H., et al., *The altered glomerular filtration slits seen in puromycin aminonucleoside nephrosis and protamine sulfate-treated rats contain the tight junction protein ZO-1.* Am J Pathol, 1992. **141**(4): p. 805-16.
81. Takeda, T., et al., *Loss of glomerular foot processes is associated with uncoupling of podocalyxin from the actin cytoskeleton.* J Clin Invest, 2001. **108**(2): p. 289-301.
82. Nielsen, J.S. and K.M. McNagny, *The role of podocalyxin in health and disease.* J Am Soc Nephrol, 2009. **20**(8): p. 1669-76.

83. Endlich, N., et al., *Palladin is a dynamic actin-associated protein in podocytes.* Kidney Int, 2009. **75**(2): p. 214-26.
84. V, E., *Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases.* Journal of Clinical Investigation, 2003. **111**: p. 707.
85. Eremina, V., et al., *VEGF inhibition and renal thrombotic microangiopathy.* N Engl J Med, 2008. **358**(11): p. 1129-36.
86. Vaughan, M.R., et al., *ATRA induces podocyte differentiation and alters nephrin and podocin expression in vitro and in vivo.* Kidney Int, 2005. **68**(1): p. 133-44.
87. Liu, Y., *New insights into epithelial-mesenchymal transition in kidney fibrosis.* J Am Soc Nephrol, 2010. **21**(2): p. 212-22.
88. Wiggins, J.E., et al., *Podocyte hypertrophy, "adaptation," and "decompensation" associated with glomerular enlargement and glomerulosclerosis in the aging rat: prevention by calorie restriction.* J Am Soc Nephrol, 2005. **16**(10): p. 2953-66.
89. Petermann, A.T., et al., *Mechanical stretch induces podocyte hypertrophy in vitro.* Kidney Int, 2005. **67**(1): p. 157-66.
90. Moeller, M.J., et al., *Podocytes populate cellular crescents in a murine model of inflammatory glomerulonephritis.* J Am Soc Nephrol, 2004. **15**(1): p. 61-7.
91. Chuang, P.Y. and J.C. He, *Signaling in regulation of podocyte phenotypes.* Nephron Physiol, 2009. **111**(2): p. p9-15.
92. He, J.C., et al., *Nef stimulates proliferation of glomerular podocytes through activation of Src-dependent Stat3 and MAPK1,2 pathways.* J Clin Invest, 2004. **114**(5): p. 643-51.
93. Yamaguchi, Y., et al., *Epithelial-mesenchymal transition as a potential explanation for podocyte depletion in diabetic nephropathy.* Am J Kidney Dis, 2009. **54**(4): p. 653-64.
94. Schiffer, M., et al., *Apoptosis in podocytes induced by TGF-beta and Smad7.* J Clin Invest, 2001. **108**(6): p. 807-16.
95. Ding, G., et al., *Angiotensin II induces apoptosis in rat glomerular epithelial cells.* Am J Physiol Renal Physiol, 2002. **283**(1): p. F173-80.
96. Durvasula, R.V., et al., *Activation of a local tissue angiotensin system in podocytes by mechanical strain.* Kidney Int, 2004. **65**(1): p. 30-9.
97. Sanwal, V., et al., *Puromycin aminonucleoside induces glomerular epithelial cell apoptosis.* Exp Mol Pathol, 2001. **70**(1): p. 54-64.
98. Nangaku, M., S.J. Shankland, and W.G. Couser, *Cellular response to injury in membranous nephropathy.* J Am Soc Nephrol, 2005. **16**(5): p. 1195-204.
99. Huber, T.B., et al., *Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling.* Mol Cell Biol, 2003. **23**(14): p. 4917-28.
100. Niranjan, T., et al., *The Notch pathway in podocytes plays a role in the development of glomerular disease.* Nat Med, 2008. **14**(3): p. 290-8.
101. Lemley, K.V., et al., *Podocytopenia and disease severity in IgA nephropathy.* Kidney Int, 2002. **61**(4): p. 1475-85.
102. Kim, Y.H., et al., *Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat.* Kidney Int, 2001. **60**(3): p. 957-68.
103. Petermann, A.T., et al., *Podocytes that detach in experimental membranous nephropathy are viable.* Kidney Int, 2003. **64**(4): p. 1222-31.
104. Petermann, A.T., et al., *Viable podocytes detach in experimental diabetic nephropathy: potential mechanism underlying glomerulosclerosis.* Nephron Exp Nephrol, 2004. **98**(4): p. e114-23.
105. Chen, H.C., et al., *Altering expression of alpha3beta1 integrin on podocytes of human and rats with diabetes.* Life Sci, 2000. **67**(19): p. 2345-53.

106. Regoli, M. and M. Bendayan, *Alterations in the expression of the alpha 3 beta 1 integrin in certain membrane domains of the glomerular epithelial cells (podocytes) in diabetes mellitus.* Diabetologia, 1997. **40**(1): p. 15-22.
107. Shankland, S.J., et al., *Cyclin kinase inhibitors are increased during experimental membranous nephropathy: potential role in limiting glomerular epithelial cell proliferation in vivo.* Kidney Int, 1997. **52**(2): p. 404-13.
108. Ophascharoensuk, V., et al., *The cyclin-dependent kinase inhibitor p27Kip1 safeguards against inflammatory injury.* Nat Med, 1998. **4**(5): p. 575-80.
109. Kim, Y.G., et al., *The cyclin kinase inhibitor p21CIP1/WAF1 limits glomerular epithelial cell proliferation in experimental glomerulonephritis.* Kidney Int, 1999. **55**(6): p. 2349-61.
110. Shankland, S.J., et al., *Differential expression of cyclin-dependent kinase inhibitors in human glomerular disease: role in podocyte proliferation and maturation.* Kidney Int, 2000. **58**(2): p. 674-83.
111. Floege, J., et al., *Markers of complement-dependent and complement-independent glomerular visceral epithelial cell injury in vivo. Expression of antiadhesive proteins and cytoskeletal changes.* Lab Invest, 1992. **67**(4): p. 486-97.
112. Simons, M., et al., *Involvement of lipid rafts in nephrin phosphorylation and organization of the glomerular slit diaphragm.* Am J Pathol, 2001. **159**(3): p. 1069-77.
113. Regele, H.M., et al., *Glomerular expression of dystroglycans is reduced in minimal change nephrosis but not in focal segmental glomerulosclerosis.* J Am Soc Nephrol, 2000. **11**(3): p. 403-12.
114. Miner, J.H. and J.R. Sanes, *Molecular and functional defects in kidneys of mice lacking collagen alpha 3(IV): implications for Alport syndrome.* J Cell Biol, 1996. **135**(5): p. 1403-13.
115. Kreidberg, J.A., et al., *Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis.* Development, 1996. **122**(11): p. 3537-47.
116. Ronco, P., *Proteinuria: is it all in the foot?* J Clin Invest, 2007. **117**(8): p. 2079-82.
117. Binder, C.J., et al., *Glomerular overproduction of oxygen radicals in Mpv17 gene-inactivated mice causes podocyte foot process flattening and proteinuria: A model of steroid-resistant nephrosis sensitive to radical scavenger therapy.* Am J Pathol, 1999. **154**(4): p. 1067-75.
118. Neale, T.J., et al., *Proteinuria in passive Heymann nephritis is associated with lipid peroxidation and formation of adducts on type IV collagen.* J Clin Invest, 1994. **94**(4): p. 1577-84.
119. McMillan, J.I., et al., *Characterization of a glomerular epithelial cell metalloproteinase as matrix metalloproteinase-9 with enhanced expression in a model of membranous nephropathy.* J Clin Invest, 1996. **97**(4): p. 1094-101.
120. Asanuma, K., et al., *Selective modulation of the secretion of proteinases and their inhibitors by growth factors in cultured differentiated podocytes.* Kidney Int, 2002. **62**(3): p. 822-31.
121. Torbohm, I., et al., *C5b-8 and C5b-9 modulate the collagen release of human glomerular epithelial cells.* Kidney Int, 1990. **37**(4): p. 1098-104.
122. Shankland, S.J., et al., *Differential expression of transforming growth factor-beta isoforms and receptors in experimental membranous nephropathy.* Kidney Int, 1996. **50**(1): p. 116-24.
123. Kaplan, J.M., et al., *Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis.* Nat Genet, 2000. **24**(3): p. 251-6.
124. Orlando, R.A., et al., *The glomerular epithelial cell anti-adhesin podocalyxin associates with the actin cytoskeleton through interactions with ezrin.* J Am Soc Nephrol, 2001. **12**(8): p. 1589-98.

125. Harrison-Bernard, L.M., et al., *Immunohistochemical localization of ANG II AT1 receptor in adult rat kidney using a monoclonal antibody.* Am J Physiol, 1997. **273**(1 Pt 2): p. F170-7.
126. Sharma, M., et al., *Documentation of angiotensin II receptors in glomerular epithelial cells.* Am J Physiol, 1998. **274**(3 Pt 2): p. F623-7.
127. Hoffmann, S., et al., *Angiotensin II type 1 receptor overexpression in podocytes induces glomerulosclerosis in transgenic rats.* J Am Soc Nephrol, 2004. **15**(6): p. 1475-87.
128. Fornoni, A., *Proteinuria, the podocyte, and insulin resistance.* N Engl J Med, 2010. **363**(21): p. 2068-9.
129. Welsh, G.I., et al., *Insulin signaling to the glomerular podocyte is critical for normal kidney function.* Cell Metab, 2010. **12**(4): p. 329-40.
130. Comper, W.D., et al., *Disease-dependent mechanisms of albuminuria.* Am J Physiol Renal Physiol, 2008. **295**(6): p. F1589-600.
131. Huber, T.B., et al., *Expression of functional CCR and CXCR chemokine receptors in podocytes.* J Immunol, 2002. **168**(12): p. 6244-52.
132. Niemir, Z.I., et al., *Podocytes are the major source of IL-1 alpha and IL-1 beta in human glomerulonephritides.* Kidney Int, 1997. **52**(2): p. 393-403.
133. Sacks, S.H., et al., *Complement C3 gene expression and regulation in human glomerular epithelial cells.* Immunology, 1993. **79**(3): p. 348-54.
134. Kazatchkine, M.D., et al., *Immunohistochemical study of the human glomerular C3b receptor in normal kidney and in seventy-five cases of renal diseases: loss of C3b receptor antigen in focal hyalinosis and in proliferative nephritis of systemic lupus erythematosus.* J Clin Invest, 1982. **69**(4): p. 900-12.
135. Banas, M.C., et al., *TLR4 links podocytes with the innate immune system to mediate glomerular injury.* J Am Soc Nephrol, 2008. **19**(4): p. 704-13.
136. Alexander, J.J., et al., *Mouse podocyte complement factor H: the functional analog to human complement receptor 1.* J Am Soc Nephrol, 2007. **18**(4): p. 1157-66.
137. Bao, L., et al., *Decay-accelerating factor expression in the rat kidney is restricted to the apical surface of podocytes.* Kidney Int, 2002. **62**(6): p. 2010-21.
138. Reiser, J., et al., *Induction of B7-1 in podocytes is associated with nephrotic syndrome.* J Clin Invest, 2004. **113**(10): p. 1390-7.
139. Reiser, J. and P. Mundel, *Danger signaling by glomerular podocytes defines a novel function of inducible B7-1 in the pathogenesis of nephrotic syndrome.* J Am Soc Nephrol, 2004. **15**(9): p. 2246-8.
140. Sharma, K., et al., *Adiponectin regulates albuminuria and podocyte function in mice.* J Clin Invest, 2008. **118**(5): p. 1645-56.
141. Karnovsky, M.J. and S.K. Ainsworth, *The structural basis of glomerular filtration.* Adv Nephrol Necker Hosp, 1972. **2**: p. 35-60.
142. Johnstone, D.B. and L.B. Holzman, *Clinical impact of research on the podocyte slit diaphragm.* Nat Clin Pract Nephrol, 2006. **2**(5): p. 271-82.
143. Fukasawa, H., et al., *Slit diaphragms contain tight junction proteins.* J Am Soc Nephrol, 2009. **20**(7): p. 1491-503.
144. Hirose, T., et al., *An essential role of the universal polarity protein, aPKClambda, on the maintenance of podocyte slit diaphragms.* PLoS One, 2009. **4**(1): p. e4194.
145. Hartleben, B., et al., *Neph-Nephrin proteins bind the Par3-Par6-atypical protein kinase C (aPKC) complex to regulate podocyte cell polarity.* J Biol Chem, 2008. **283**(34): p. 23033-8.
146. Huber, T.B., et al., *Loss of podocyte aPKClambda/iota causes polarity defects and nephrotic syndrome.* J Am Soc Nephrol, 2009. **20**(4): p. 798-806.

147. Verma, R., et al., *Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization.* J Clin Invest, 2006. **116**(5): p. 1346-59.
148. Jones, N., et al., *Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes.* Nature, 2006. **440**(7085): p. 818-23.
149. Ahola, H., et al., *A novel protein, densin, expressed by glomerular podocytes.* J Am Soc Nephrol, 2003. **14**(7): p. 1731-7.
150. Hussain, S., et al., *Nephrin deficiency activates NF-kappaB and promotes glomerular injury.* J Am Soc Nephrol, 2009. **20**(8): p. 1733-43.
151. Duner, F., et al., *Dendrin expression in glomerulogenesis and in human minimal change nephrotic syndrome.* Nephrol Dial Transplant, 2008. **23**(8): p. 2504-11.
152. Kestila, M., et al., *Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome.* Mol Cell, 1998. **1**(4): p. 575-82.
153. Ruotsalainen, V., et al., *Nephrin is specifically located at the slit diaphragm of glomerular podocytes.* Proc Natl Acad Sci U S A, 1999. **96**(14): p. 7962-7.
154. Tryggvason, K., *Unraveling the mechanisms of glomerular ultrafiltration: nephrin, a key component of the slit diaphragm.* J Am Soc Nephrol, 1999. **10**(11): p. 2440-5.
155. Huber, T.B., et al., *The carboxyl terminus of Neph family members binds to the PDZ domain protein zonula occludens-1.* J Biol Chem, 2003. **278**(15): p. 13417-21.
156. Lehtonen, S., et al., *Cell junction-associated proteins IQGAP1, MAGI-2, CASK, spectrins, and alpha-actinin are components of the nephrin multiprotein complex.* Proc Natl Acad Sci U S A, 2005. **102**(28): p. 9814-9.
157. Liu, X.L., et al., *Characterization of the interactions of the nephrin intracellular domain.* FEBS J, 2005. **272**(1): p. 228-43.
158. Verma, R., et al., *Fyn binds to and phosphorylates the kidney slit diaphragm component Nephrin.* J Biol Chem, 2003. **278**(23): p. 20716-23.
159. Lahdenpera, J., et al., *Clustering-induced tyrosine phosphorylation of nephrin by Src family kinases.* Kidney Int, 2003. **64**(2): p. 404-13.
160. Schiffer, M., et al., *A novel role for the adaptor molecule CD2-associated protein in transforming growth factor-beta-induced apoptosis.* J Biol Chem, 2004. **279**(35): p. 37004-12.
161. Shen, K., R.D. Fetter, and C.I. Bargmann, *Synaptic specificity is generated by the synaptic guidepost protein SYG-2 and its receptor, SYG-1.* Cell, 2004. **116**(6): p. 869-81.
162. Boute, N., et al., *NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome.* Nat Genet, 2000. **24**(4): p. 349-54.
163. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 31-9.
164. Schwarz, K., et al., *Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin.* J Clin Invest, 2001. **108**(11): p. 1621-9.
165. Luimula, P., et al., *Podocyte-associated molecules in puromycin aminonucleoside nephrosis of the rat.* Lab Invest, 2002. **82**(6): p. 713-8.
166. Forbes, J.M., et al., *Modulation of nephrin in the diabetic kidney: association with systemic hypertension and increasing albuminuria.* J Hypertens, 2002. **20**(5): p. 985-92.
167. Patrakka, J., et al., *The number of podocyte slit diaphragms is decreased in minimal change nephrotic syndrome.* Pediatr Res, 2002. **52**(3): p. 349-55.

168. Saran, A.M., et al., *Complement mediates nephrin redistribution and actin dissociation in experimental membranous nephropathy.* Kidney Int, 2003. **64**(6): p. 2072-8.
169. Hidaka, T., et al., *Amelioration of crescentic glomerulonephritis by RhoA kinase inhibitor, Fasudil, through podocyte protection and prevention of leukocyte migration.* Am J Pathol, 2008. **172**(3): p. 603-14.
170. Rahman, A. and D.A. Isenberg, *Systemic lupus erythematosus.* N Engl J Med, 2008. **358**(9): p. 929-39.
171. Cameron, J.S., *Lupus nephritis.* J Am Soc Nephrol, 1999. **10**(2): p. 413-24.
172. Boumpas, D.T., P. Sidiropoulos, and G. Bertsias, *Optimum therapeutic approaches for lupus nephritis: what therapy and for whom?* Nat Clin Pract Rheumatol, 2005. **1**(1): p. 22-30.
173. Linker-Israeli, M., F.P. Quismorio, Jr., and D.A. Horwitz, *CD8+ lymphocytes from patients with systemic lupus erythematosus sustain, rather than suppress, spontaneous polyclonal IgG production and synergize with CD4+ cells to support autoantibody synthesis.* Arthritis Rheum, 1990. **33**(8): p. 1216-25.
174. Williams, W.M., et al., *Human T cell responses to autoantibody variable region peptides.* Lupus, 1995. **4**(6): p. 464-71.
175. Singh, R.R., et al., *Immune tolerance to autoantibody-derived peptides delays development of autoimmunity in murine lupus.* J Clin Invest, 1995. **96**(6): p. 2990-6.
176. Krych, M., J.P. Atkinson, and V.M. Holers, *Complement receptors.* Curr Opin Immunol, 1992. **4**(1): p. 8-13.
177. Imai, H., et al., *IgG subclasses in patients with membranoproliferative glomerulonephritis, membranous nephropathy, and lupus nephritis.* Kidney Int, 1997. **51**(1): p. 270-6.
178. Salmon, J.E., et al., *Fc gamma RIIA alleles are heritable risk factors for lupus nephritis in African Americans.* J Clin Invest, 1996. **97**(5): p. 1348-54.
179. Hahn, B.H., *Antibodies to DNA.* N Engl J Med, 1998. **338**(19): p. 1359-68.
180. Zykova, S.N., et al., *Reduced fragmentation of apoptotic chromatin is associated with nephritis in lupus-prone (NZB x NZW)F(1) mice.* Arthritis Rheum, 2008. **58**(3): p. 813-25.
181. Bertsias, G.K., et al., *Genetic, immunologic, and immunohistochemical analysis of the programmed death 1/programmed death ligand 1 pathway in human systemic lupus erythematosus.* Arthritis Rheum, 2009. **60**(1): p. 207-18.
182. Morioka, T., et al., *Histone mediates glomerular deposition of small size DNA anti-DNA complex.* Kidney Int, 1994. **45**(4): p. 991-7.
183. Waters, S.T., et al., *Breaking tolerance to double stranded DNA, nucleosome, and other nuclear antigens is not required for the pathogenesis of lupus glomerulonephritis.* J Exp Med, 2004. **199**(2): p. 255-64.
184. Berden, J.H., et al., *Role of nucleosomes for induction and glomerular binding of autoantibodies in lupus nephritis.* Curr Opin Nephrol Hypertens, 1999. **8**(3): p. 299-306.
185. Lefkowith, J.B., et al., *Heterogeneity and clinical significance of glomerular-binding antibodies in systemic lupus erythematosus.* J Clin Invest, 1996. **98**(6): p. 1373-80.
186. Vlahakos, D.V., et al., *Anti-DNA antibodies form immune deposits at distinct glomerular and vascular sites.* Kidney Int, 1992. **41**(6): p. 1690-700.
187. Mortensen, E.S., K.A. Fenton, and O.P. Rekvig, *Lupus nephritis: the central role of nucleosomes revealed.* Am J Pathol, 2008. **172**(2): p. 275-83.
188. Kalaaji, M., et al., *Nephritogenic lupus antibodies recognize glomerular basement membrane-associated chromatin fragments released from apoptotic intraglomerular cells.* Am J Pathol, 2006. **168**(6): p. 1779-92.

189. Suzuki, N., et al., *Possible pathogenic role of cationic anti-DNA autoantibodies in the development of nephritis in patients with systemic lupus erythematosus.* J Immunol, 1993. **151**(2): p. 1128-36.
190. Winfield, J.B., I. Faiferman, and D. Koffler, *Avidity of anti-DNA antibodies in serum and IgG glomerular eluates from patients with systemic lupus erythematosus. Association of high avidity antinative DNA antibody with glomerulonephritis.* J Clin Invest, 1977. **59**(1): p. 90-6.
191. Bagavant, H. and S.M. Fu, *Pathogenesis of kidney disease in systemic lupus erythematosus.* Curr Opin Rheumatol, 2009. **21**(5): p. 489-94.
192. Deocharan, B., et al., *Alpha-actinin is a cross-reactive renal target for pathogenic anti-DNA antibodies.* J Immunol, 2002. **168**(6): p. 3072-8.
193. Brinkman, K., et al., *Anti-DNA antibodies and lupus nephritis: the complexity of crossreactivity.* Immunol Today, 1990. **11**(7): p. 232-4.
194. Kramers, C., et al., *Anti-nucleosome antibodies complexed to nucleosomal antigens show anti-DNA reactivity and bind to rat glomerular basement membrane in vivo.* J Clin Invest, 1994. **94**(2): p. 568-77.
195. van Bruggen, M.C., et al., *Antigen specificity of anti-nuclear antibodies complexed to nucleosomes determines glomerular basement membrane binding in vivo.* Eur J Immunol, 1997. **27**(6): p. 1564-9.
196. Clynes, R., C. Dumitru, and J.V. Ravetch, *Uncoupling of immune complex formation and kidney damage in autoimmune glomerulonephritis.* Science, 1998. **279**(5353): p. 1052-4.
197. Austin, H.A. and G.G. Illei, *Membranous lupus nephritis.* Lupus, 2005. **14**(1): p. 65-71.
198. Bao, L., et al., *C5a promotes development of experimental lupus nephritis which can be blocked with a specific receptor antagonist.* Eur J Immunol, 2005. **35**(8): p. 2496-506.
199. Moll, S., et al., *No complement receptor 1 stumps on podocytes in human glomerulopathies.* Kidney Int, 2001. **59**(1): p. 160-8.
200. Passwell, J., et al., *Local extrahepatic expression of complement genes C3, factor B, C2, and C4 is increased in murine lupus nephritis.* J Clin Invest, 1988. **82**(5): p. 1676-84.
201. Bao, L., et al., *Signaling through up-regulated C3a receptor is key to the development of experimental lupus nephritis.* J Immunol, 2005. **175**(3): p. 1947-55.
202. Wenderfer, S.E., et al., *C5a receptor deficiency attenuates T cell function and renal disease in MRLlpr mice.* J Am Soc Nephrol, 2005. **16**(12): p. 3572-82.
203. Miwa, T., et al., *Deletion of decay-accelerating factor (CD55) exacerbates autoimmune disease development in MRL/lpr mice.* Am J Pathol, 2002. **161**(3): p. 1077-86.
204. Ramanujam, M. and A. Davidson, *BAFF blockade for systemic lupus erythematosus: will the promise be fulfilled?* Immunol Rev, 2008. **223**: p. 156-74.
205. Zoja, C., et al., *Cyclin-dependent kinase inhibition limits glomerulonephritis and extends lifespan of mice with systemic lupus.* Arthritis Rheum, 2007. **56**(5): p. 1629-37.
206. Bagavant, H., et al., *Role for nephritogenic T cells in lupus glomerulonephritis: progression to renal failure is accompanied by T cell activation and expansion in regional lymph nodes.* J Immunol, 2006. **177**(11): p. 8258-65.
207. Schiffer, L., et al., *Short term administration of costimulatory blockade and cyclophosphamide induces remission of systemic lupus erythematosus nephritis in NZB/W F1 mice by a mechanism downstream of renal immune complex deposition.* J Immunol, 2003. **171**(1): p. 489-97.
208. Davidson, A. and C. Aranow, *Lupus nephritis: lessons from murine models.* Nat Rev Rheumatol, 2010. **6**(1): p. 13-20.

209. Aringer, M. and J.S. Smolen, *Cytokine expression in lupus kidneys.* Lupus, 2005. **14**(1): p. 13-8.
210. Takemura, T., et al., *Cellular localization of inflammatory cytokines in human glomerulonephritis.* Virchows Arch, 1994. **424**(5): p. 459-64.
211. Herrera-Esparza, R., et al., *Renal expression of IL-6 and TNFalpha genes in lupus nephritis.* Lupus, 1998. **7**(3): p. 154-8.
212. Schlondorff, D., *Roles of the mesangium in glomerular function.* Kidney Int, 1996. **49**(6): p. 1583-5.
213. Faust, J., et al., *Correlation of renal tubular epithelial cell-derived interleukin-18 up-regulation with disease activity in MRL-Faslpr mice with autoimmune lupus nephritis.* Arthritis Rheum, 2002. **46**(11): p. 3083-95.
214. Uhm, W.S., et al., *Cytokine balance in kidney tissue from lupus nephritis patients.* Rheumatology (Oxford), 2003. **42**(8): p. 935-8.
215. Masutani, K., et al., *Predominance of Th1 immune response in diffuse proliferative lupus nephritis.* Arthritis Rheum, 2001. **44**(9): p. 2097-106.
216. Carvalho-Pinto, C.E., et al., *Autocrine production of IFN-gamma by macrophages controls their recruitment to kidney and the development of glomerulonephritis in MRL/lpr mice.* J Immunol, 2002. **169**(2): p. 1058-67.
217. Furusu, A., et al., *Involvement of IL-4 in human glomerulonephritis: an in situ hybridization study of IL-4 mRNA and IL-4 receptor mRNA.* J Am Soc Nephrol, 1997. **8**(5): p. 730-41.
218. Kalechman, Y., et al., *Delay in the onset of systemic lupus erythematosus following treatment with the immunomodulator AS101: association with IL-10 inhibition and increase in TNF-alpha levels.* J Immunol, 1997. **159**(6): p. 2658-67.
219. Kumpers, P., et al., *The Tie2 receptor antagonist angiopoietin 2 facilitates vascular inflammation in systemic lupus erythematosus.* Ann Rheum Dis, 2009. **68**(10): p. 1638-43.
220. De Albuquerque, D.A., et al., *An ACE inhibitor reduces Th2 cytokines and TGF-beta1 and TGF-beta2 isoforms in murine lupus nephritis.* Kidney Int, 2004. **65**(3): p. 846-59.
221. Ferraccioli, G. and G. Romano, *Renal interstitial cells, proteinuria and progression of lupus nephritis: new frontiers for old factors.* Lupus, 2008. **17**(6): p. 533-40.
222. Mostoslavsky, G., et al., *Lupus anti-DNA autoantibodies cross-react with a glomerular structural protein: a case for tissue injury by molecular mimicry.* Eur J Immunol, 2001. **31**(4): p. 1221-7.
223. Duran-Barragan, S., et al., *Angiotensin-converting enzyme inhibitors delay the occurrence of renal involvement and are associated with a decreased risk of disease activity in patients with systemic lupus erythematosus--results from LUMINA (LIX): a multiethnic US cohort.* Rheumatology (Oxford), 2008. **47**(7): p. 1093-6.
224. Crowley, S.D., et al., *Glomerular type 1 angiotensin receptors augment kidney injury and inflammation in murine autoimmune nephritis.* J Clin Invest, 2009. **119**(4): p. 943-53.
225. Papadimitraki, E.D., et al., *Glomerular expression of toll-like receptor-9 in lupus nephritis but not in normal kidneys: implications for the amplification of the inflammatory response.* Lupus, 2009. **18**(9): p. 831-5.
226. Anders, H.J. and D. Schlondorff, *Toll-like receptors: emerging concepts in kidney disease.* Curr Opin Nephrol Hypertens, 2007. **16**(3): p. 177-83.
227. Andrews, B.S., et al., *Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains.* J Exp Med, 1978. **148**(5): p. 1198-215.
228. Theofilopoulos, A.N. and F.J. Dixon, *Murine models of systemic lupus erythematosus.* Adv Immunol, 1985. **37**: p. 269-390.

229. Reininger, L., et al., *Development of autoimmune disease in SCID mice populated with long-term "in vitro" proliferating (NZB x NZW)F1 pre-B cells.* J Exp Med, 1992. **176**(5): p. 1343-53.
230. Barker, D.F., et al., *Identification of mutations in the COL4A5 collagen gene in Alport syndrome.* Science, 1990. **248**(4960): p. 1224-7.
231. Morgan, A.G. and M.W. Steward, *Macrophage clearance function and immune complex disease in New Zealand Black/White F1 hybrid mice.* Clin Exp Immunol, 1976. **26**(1): p. 133-6.
232. BH, H., ed. *Animal models of systemic lupus erythematosus.* . 5th ed. Dubois' Lupus Erythematosus, ed. W.D.H. BH. 1997, Williams and Wilkins: Baltimore. 339–380.
233. Weening, J.J., et al., *The classification of glomerulonephritis in systemic lupus erythematosus revisited.* Kidney Int, 2004. **65**(2): p. 521-30.
234. Salant, D.J. and A.V. Cybulsky, *Experimental glomerulonephritis.* Methods Enzymol, 1988. **162**: p. 421-61.
235. Barletta, G.M., et al., *Nephrin and Neph1 co-localize at the podocyte foot process intercellular junction and form cis hetero-oligomers.* J Biol Chem, 2003. **278**(21): p. 19266-71.
236. Holzman, L.B., et al., *Nephrin localizes to the slit pore of the glomerular epithelial cell.* Kidney Int, 1999. **56**(4): p. 1481-91.
237. Horinouchi, I., et al., *In situ evaluation of podocin in normal and glomerular diseases.* Kidney Int, 2003. **64**(6): p. 2092-9.
238. Kawachi, H., et al., *Slit diaphragm dysfunction in proteinuric states: identification of novel therapeutic targets for nephrotic syndrome.* Clin Exp Nephrol, 2009. **13**(4): p. 275-80.
239. Nakatsue, T., et al., *Nephrin and podocin dissociate at the onset of proteinuria in experimental membranous nephropathy.* Kidney Int, 2005. **67**(6): p. 2239-53.
240. Luimula, P., et al., *Nephrin in experimental glomerular disease.* Kidney Int, 2000. **58**(4): p. 1461-8.
241. Clement, L.C., et al., *Early changes in gene expression that influence the course of primary glomerular disease.* Kidney Int, 2007. **72**(3): p. 337-47.
242. Yuan, H., et al., *Nephrin dissociates from actin, and its expression is reduced in early experimental membranous nephropathy.* J Am Soc Nephrol, 2002. **13**(4): p. 946-56.
243. Kraft, S.W., et al., *Glomerular podocytopathy in patients with systemic lupus erythematosus.* J Am Soc Nephrol, 2005. **16**(1): p. 175-9.
244. Han, T.S., M.M. Schwartz, and E.J. Lewis, *Association of glomerular podocytopathy and nephrotic proteinuria in mesangial lupus nephritis.* Lupus, 2006. **15**(2): p. 71-5.
245. Trivedi, S., M. Zeier, and J. Reiser, *Role of podocytes in lupus nephritis.* Nephrol Dial Transplant, 2009. **24**(12): p. 3607-12.
246. Huh, W., et al., *Expression of nephrin in acquired human glomerular disease.* Nephrol Dial Transplant, 2002. **17**(3): p. 478-84.
247. Koop, K., et al., *Expression of podocyte-associated molecules in acquired human kidney diseases.* J Am Soc Nephrol, 2003. **14**(8): p. 2063-71.
248. Meyer-Schwesinger, C., et al., *A new role for the neuronal ubiquitin C-terminal hydrolase-L1 (UCH-L1) in podocyte process formation and podocyte injury in human glomerulopathies.* J Pathol, 2009. **217**(3): p. 452-64.
249. Patrakka, J., et al., *Expression of nephrin in pediatric kidney diseases.* J Am Soc Nephrol, 2001. **12**(2): p. 289-96.
250. Wang, S.X., et al., *Patterns of nephrin and a new proteinuria-associated protein expression in human renal diseases.* Kidney Int, 2002. **61**(1): p. 141-7.

251. Machida, H., et al., *Expression of Toll-like receptor 9 in renal podocytes in childhood-onset active and inactive lupus nephritis.* Nephrol Dial Transplant, 2010. **25**(8): p. 2530-537.
252. Gagliardini, E., et al., *Targeted downregulation of extracellular nephrin in human IgA nephropathy.* Am J Nephrol, 2003. **23**(4): p. 277-86.
253. van den Berg, J.G., et al., *Podocyte foot process effacement is not correlated with the level of proteinuria in human glomerulopathies.* Kidney Int, 2004. **66**(5): p. 1901-6.
254. Lai, K.N., et al., *Podocyte injury induced by mesangial-derived cytokines in IgA nephropathy.* Nephrol Dial Transplant, 2009. **24**(1): p. 62-72.
255. Mao, J., et al., *Expression profile of nephrin, podocin, and CD2AP in Chinese children with MCNS and IgA nephropathy.* Pediatr Nephrol, 2006. **21**(11): p. 1666-75.
256. Doublier, S., et al., *Nephrin redistribution on podocytes is a potential mechanism for proteinuria in patients with primary acquired nephrotic syndrome.* Am J Pathol, 2001. **158**(5): p. 1723-31.
257. Wang, G., et al., *Messenger RNA expression of podocyte-associated molecules in urinary sediment of patients with lupus nephritis.* J Rheumatol, 2007. **34**(12): p. 2358-64.
258. Yoshida, H., et al., *Genetic studies of autoimmunity in New Zealand mice. III. Associations among anti-DNA antibodies, NTA, and renal disease in (NZB x NZW)F1 x NZW backcross mice.* J Immunol, 1981. **127**(2): p. 433-7.
259. Wu, T. and C. Mohan, *Three pathogenic determinants in immune nephritis--anti-glomerular antibody specificity, innate triggers and host genetics.* Front Biosci, 2007. **12**: p. 2207-11.
260. Hahn, B.H. and L.E. Shulman, *Autoantibodies and nephritis in the white strain (NZW) of New Zealand mice.* Arthritis Rheum, 1969. **12**(4): p. 355-64.
261. Couzi, L., et al., *Predominance of CD8+ T lymphocytes among periglomerular infiltrating cells and link to the prognosis of class III and class IV lupus nephritis.* Arthritis Rheum, 2007. **56**(7): p. 2362-70.
262. Mason, L.J., et al., *Is alpha-actinin a target for pathogenic anti-DNA antibodies in lupus nephritis?* Arthritis Rheum, 2004. **50**(3): p. 866-70.
263. Takano, Y., et al., *Transcriptional suppression of nephrin in podocytes by macrophages: roles of inflammatory cytokines and involvement of the PI3K/Akt pathway.* FEBS Lett, 2007. **581**(3): p. 421-6.
264. Marasa, M. and J.B. Kopp, *Monoclonal antibodies for podocytopathies: rationale and clinical responses.* Nat Rev Nephrol, 2009. **5**(6): p. 337-48.