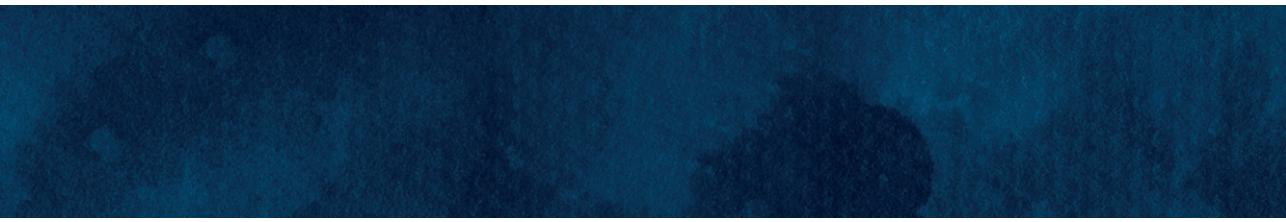
Pathways to proteinuria

Khalil, R.



CHAPTER 4

Mutations in the heparan sulfate backbone elongating enzymes EXT1 and EXT2 have no major effect on endothelial glycocalyx and the glomerular filtration barrier

Molecular Genetics and Genomics 2022, 297, 397–405

Ramzi Khalil[1], Margien G.S. Boels[2], PALGA Group[3], Bernard M. van den Berg[2], Jan A. Bruijn[1], Ton J. Rabelink[2], Pancras C.W. Hogendoorn[1], Hans J. Baelde[1]

[1] *Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands*
[2] *The Einthoven Laboratory for Vascular and Regenerative Medicine, Department of Internal Medicine, Division of Nephrology, Leiden University Medical Center, Leiden, The Netherlands,*
[3] *PALGA Group*

Abstract

In this study, the effect of heterozygous germline mutations in the heparan sulfate (HS) glycosaminoglycan chain co-polymerases EXT1 and EXT2 on glomerular barrier function and the endothelial glycocalyx in humans is investigated.

Heparan sulfate (HS) glycosaminoglycans are deemed essential to the glomerular filtration barrier, including the glomerular endothelial glycocalyx. Animal studies have shown that loss of HS results in a thinner glycocalyx. Also, decreased glomerular HS expression is observed in various proteinuric renal disease in humans. A case report of a patient with an *EXT1* mutation indicated that this could result in a specific renal phenotype. This patient suffered from multiple osteochondromas, an autosomal dominant disease caused by mono-allelic germline mutations in the *EXT1* or *EXT2* gene. These studies imply that HS is indeed essential to the glomerular filtration barrier. However, loss of HS did not lead to proteinuria in various animal models.

We demonstrate that multiple osteochondroma patients do not have more microalbuminuria or altered glycocalyx properties compared to age-matched controls (n = 19). A search for all Dutch patients registered with both osteochondroma and kidney biopsy (n = 39), showed that an *EXT1* or *EXT2* mutation does not necessarily lead to specific glomerular morphological phenotypic changes.

In conclusion, this study shows that a heterozygous mutation in the HS backbone elongating enzymes EXT1 and EXT2 in humans does not result in (micro)albuminuria, a specific renal phenotype or changes to the endothelial glycocalyx, adding to the growing knowledge on the role of EXT1 and EXT2 genes in pathophysiology.

Background

The effect of heterozygous germline mutations in *EXT1* or *EXT2* on the glomerular filtration barrier, with special attention to the endothelial glycocalyx, is investigated in this study. We hypothesized that patients with a mutation in *EXT1* or *EXT2* have a perturbed endothelial glycocalyx and glomerular basement membrane that results in changes in renal morphology and altered glomerular permeability. Therefore, we performed a cross-sectional observational study in a cohort of patients with *EXT1* and *EXT2* mutations to investigate endothelial glycocalyx health and the glomerular barrier function in humans. In addition, we identified a historic cohort of renal biopsy or autopsy tissue from patients with osteochondromas in which we examined whether a specific renal morphological phenotype is present. HS is made up of repeating disaccharide units of *N*-acetyl glucosamine and glucuronic acid, covalently attached to a core protein, such as syndecans and glypicans.(9) Biosynthesis of HS comprises three steps: chain initiation, chain elongation, (requiring the EXT1 and EXT2 co-polymerase)and chain modification. Depending on the modification state of HS, various sulfate groups are attached to the chain and thus infer negative charge, anti-coagulatory properties, and the capability to bind various cytokines and chemokines (52).

HS has been studied extensively in the glomerular filtration barrier (GFB).(7, 8) The GFB consists of fenestrated endothelial cells covered by glycocalyx, the glomerular basement membrane, and specialized visceral epithelial cells (podocytes) with interdigitating foot processes. There has a been a longstanding discussion on whether HS is essential to glomerular permeability (53). Glomeruli of patients with lupus nephritis, membranous glomerulonephritis, minimal change disease, and diabetic nephropathy showed a decreased staining of heparan sulfate.(22) Degradation of HS in the glycocalyx by heparanase increases glomerular permeability to albumin, results in increased accessibility to autoantibodies, and loss of HS was observed in various proteinuric renal diseases (22, 29, 37). On the other hand, a genetic HS deficiency has been shown not to result in significant proteinuria in various experimental animal models. (23, 25, 27, 54, 55)

Gleadle *et al.* reported on a patient with a monoallelic germline mutation in an HS polymerizing gene, *EXT1*, that presented with a nephrotic syndrome. Histology from a renal biopsy of this patient showed specific changes on EM of fibrillar deposition in the GBM and mesangium (56). Based on this case, the renal disease 'glomerulopathy of hereditary multiple exostoses' is recognised as a separate disease entity (57). *EXT1* or *EXT2* loss of function mutations result in the autosomal dominant disease multiple

osteochondromas (MO, OMIM no. 133700 and 133701), previously also known as hereditary multiple exostoses (HME) and multiple hereditary multiple exostoses (MHE) (58). MO is defined as a disease 'characterized by development of two or more cartilage capped bony outgrowths (osteochondromas) of the long bones.' (59, 60). More than 85% of MO patients have a germline mutation in the *EXT1* or *EXT2* gene.(59) The *EXT1* and *EXT2* gene products form a co-polymerase that initiates chain elongation in heparan sulfate glycosaminoglycan synthesis.(9) Existence of another exostosin gene, *EXT3,* has been suggested but not proven.(61) However, as stated above, the diagnosis is often based on radiological and clinical assessment. Therefore, in many patients, genetic testing is regarded as optional in establishing the diagnosis.(62)

In MO patients, a decrease in HS levels has been described in the circulatory system and in exostosis growth plates (63, 64). However, the effect of heterozygous *EXT1* and *EXT2* mutations on the endothelial glycocalyx have not yet been described. Moreover, it is currently unknown whether a loss of HS as a result of perturbed HS assembly results in an increase in glomerular permeability in humans.

HS is also a major structural component of the glycocalyx, a coat of sugars, proteins, and lipids, lining the luminal side of the endothelium (65). Endothelial dysfunction is an important process in both cardiovascular and renal disease (66, 67). Recent technological advances have now made it possible to assess the red blood cell (RBC) count accessibility to the endothelial surface glycocalyx, in which a healthy glycocalyx is reflected by a low perfused boundary region (PBR) and a glycocalyx at risk is reflected by a high PBR (5, 68).

Methods

Ethics

Ethical approval for this study was obtained through the LUMC Medical Ethical Committee under registration number P15.106. Anonymized patient material from the PALGA search was handled in accordance with institutional guidelines, Good Research Practice, and the Code of conduct for responsible use.

MO patient study population

Patients were approached through the Dutch HME-MO Association (www.hme-mo.nl). Controls were obtained from the general population and matched for age and gender. All participants were required to be capacitated and over 18 years of age. After obtaining informed consent, participants were handed a digital questionnaire. Data on mutation status, use of medication, smoking, hypertension, and relevant medical history was

collected. Participants were asked to perform one site visit, during which a glycocalyx measurement was performed and urine samples were collected.

Glycocalyx measurements

Sublingual microvasculature was visualised non-invasively using a sidestream–darkfield MicroScan Video Microscope (MicroVision Medical, Inc., Wallingford, PA), connected to Glycocheck™ acquisition and analysis software (Microvascular Health Solutions Inc., Salt Lake City, UT, USA) to automatically acquire microvascular video recordings after predefined image quality criteria (motion, intensity, and focus) are fulfilled. Each complete measurement consists of at least ten 2-second videos (40 frames/video), containing a total of about 3000 vascular segments of 10μm length each. During the measurement, the camera is moved to different positions under the tongue to obtain different sublingual microvascular sites. The vascular segments are automatically subjected to a quality check by the GlycoCheck™ software, discarding invalid segments. Next, the software obtains up to 840 radial intensity profiles for each valid vascular segment and, based on the RBC column width (RBCW) it automatically groups vessels from 4 to 25 μm diameter in 22 separate diameter classes (1μm each). Data from two complete measurements were extracted, analyzed and averaged offline to avoid sampling error and to counterbalance spatial-temporal heterogeneity of the sublingual microcirculation. These analyses resulted in the following parameters: perfused capillary density; capillary blood volume; dynamic perfused boundary region (PBR). PBR is a measure for glycocalyx quality by measuring dynamic lateral RBC movement into the glycocalyx: a larger PBR reflects a disturbance of the glycocalyx (5, 68).

Detailed information about the video acquisition and software used is described elsewhere (68).

Urinary albumin / protein excretion

Spot urine samples were collected during the site visit and analysed for albumin, total protein, and creatinine at the LUMC department of Clinical Chemistry. Albumin-creatinin ratio (ACR) and protein-creatinin ratio (PCR) were used to identify the presence of (micro)albuminuria and (micro)proteinuria according to the KDIGO CKD guideline (2).

Historic PALGA cohort selection

The Dutch pathology national automated archive (PALGA) was searched for cases containing both the search terms 'kidney and biopsy' and 'osteochondroma and multiple osteochondromas', to identify potential MO patients who have undergone a renal biopsy

or autopsy in which renal tissue was collected between 1978 and 2014. The slides and EM material of the identified cases were requested from the corresponding hospitals. Sections were assessed for specific light microscopic changes and, if available, irregularities on EM.

Statistical analyses

Baseline characteristics are expressed as mean (± standard deviation [SD]), or as percentage. Groups were matched for age and gender. Difference between groups in sublingual measures and albuminuria was determined using unpaired t-tests. ANOVA was used for side-by-side comparison of PBR in various vessel sizes. Pearson's correlation coefficient was calculated to assess correlations. Graphpad Prism (version 8.4.2 (GraphPad Software, San Diego, California USA) was used for statistical analyses. $p < 0.05$ was considered statistically significant.

Results

Patient Characteristics

20 MO patients responded to the call for our study. 1 patient retracted from the study before performing the site visit. Of the 19 remaining patients, 6 had a confirmed *EXT1* (4) or *EXT2* (2) mutation and 13 had a clinical diagnosis of MO without mutational confirmation. (62) 27 Controls, of which 19 are age and sex matched, were selected from the general population. No differences were observed in gender, age or body mass index (Table 1).

Table 1. Patient characteristics

	MO patients	**Healthy controls**
Subjects	19	19
Age, mean (SD), y	45 (±15.6)	45 (±16.0)
Men, n (%)	2 (10.5)	2 (10.5)
BMI (SD)	26,0 (± 6.5)	23,4 (± 2.8)
Smoking, n (%)	1 (5.3)	1 (5.3)
Hypertension, n (%)	4 (21.1)	0
ACE inhibitor, n (%)	2 (10.5)	0
ARB, n (%)	0	0
NSAID use, n (&)	7 (33.3)	3 (14.3)
Diabetes, n (%)	2 (10.5)	0
Type 1, n (%)	0	0
Type 2 n (%)	2 (10.5)	0
EXT1 mutation, n (%)	4 (21.1)	N/A
EXT2 mutation, n (%)	2 (10.5)	N/A
Mutation unknown, n (%)	13 (68.4)	N/A

BMI: Body Mass Index, ACE: angiotensin converting enzyme, ARB: angiotensin 2 receptor blocker, NSAID: non-steroidal anti inflammatory drugs, EXT: exostosin

EXT1/EXT2 mutations do not result in changed microvascular endothelial glycocalyx properties

Since mutations in one of the HS elongation enzymes *EXT1* and *EXT2* could result in compositional changes of the endothelial glycocalyx we investigated possible perturbations in glycocalyx properties. Sublingual measurements of the microcirculation of control and MO patients did not reveal a difference in perfused vascular density (352.69±102.16 vs. 322.25±68.17 μm/mm^2, respectively; p>0.99, Figure 1A) nor in changes in the perfused boundary regions (2.05±0.29 vs. 2.15±0.21 μm, respectively; p>0.99, Figure 1B). Subanalysis of PBR in vessels 5 to 9 μm, 10 to 19 μm, and 20 to 25 μm also showed no difference between MO patients and controls (p>0.99 in all instances Figure 1C through E). No correlation was found between increasing age and any of the used outcome parameters, which were density, PBR 5-25, PBR 5-9, PBR 10-19, and PBR 20-25. This was the case in the overall group (r = 0.20, 0.21, -0.13, 0.20, and 0.24 with p = 0.19, 0.18, 0.42, 0,21, and 0,13 respectively) as well as when analysing the MO patient (r = 0.28, -0.12, -0.08, -0.25, and 0.06 with p = 0.27, 0.66, 0.77, 0.34, and 0.82 respectively) and control group (r = 0.18, 0.35, -0.17, 0.38, and 0.33 with p = 0.37, 0.08, 0.43, 0.06, and 0.11 respectively) separately.

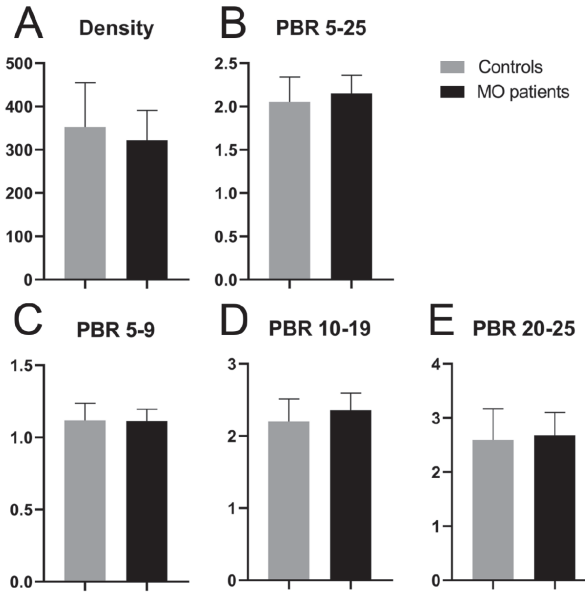


Figure 1. MO patients have normal vascular density and perfused boundary region
(**A** through **E**) Summary of vascular density (**A**) and perfused boundary region (**B** through **E**). No difference is seen in both vascular density and PBR between controls and MO patients. Subanalysis of PBR in vessels of 5 to 9 μm (**C**), 10 to 19 μm (**D**), and 20 to 25 μm (**E**) also shows no difference between controls and MO patients. *: p > 0.99

EXT1/EXT2 mutations do not directly induce proteinuria

Analysis of the collected urine samples was performed by matching MO patients to controls based on age and gender. Analysis revealed no difference in ACR nor PCR of patients compared to controls (p=0.13, Figure 2). All MO patients in this study were found to be normoalbuminuric. Five control cases were microalbuminuric and one control case was albuminuric according to the KDIGO CKD guideline (2). No correlation was found between age and ACR or PCR in both patients (r = -0.09 and 0.10 with p = 0.72 and 0.69*)* and controls (r = -0.24 and -0.19 with p = 0.33 and 0.43 respectively).

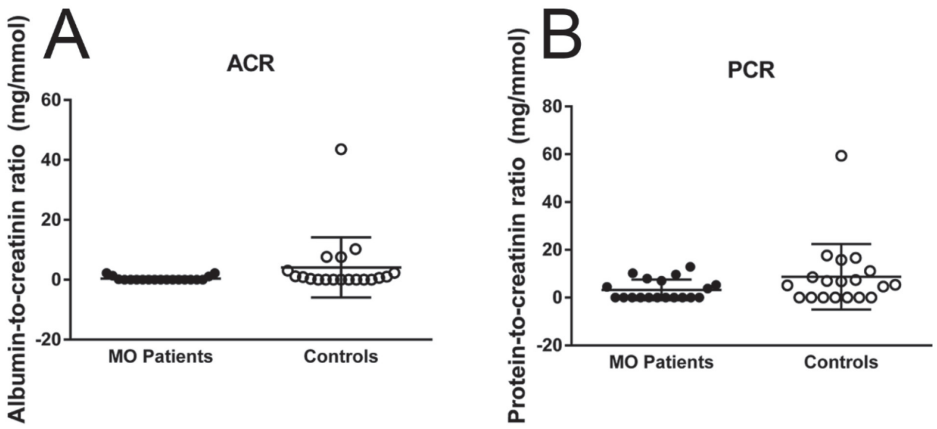


Figure 2. MO patients are normoalbuminuric
(**A** and **B**) MO patients do not have a higher albumin-to-creatinin (**A**) nor a higher protein-to-creatinin ratio (B). p = 0.1251 and p = 0.1282 respectively.

Renal morphological change in patients with osteochondromas

Searching the Dutch pathological anatomy national automated archive (PALGA) for cases in which both 'osteochondroma' and 'kidney' are mentioned, resulted in 178 reports from 63 patients. After excluding cases in which no renal tissue was present, slides and EM material from 77 reports out of 39 cases were requested from the corresponding hospitals. We received material from 24 cases. Out of these cases, at least 4 can be classified as having MO based on clinical criteria available in the pathology reports. Genetic data of this cohort is unknown.

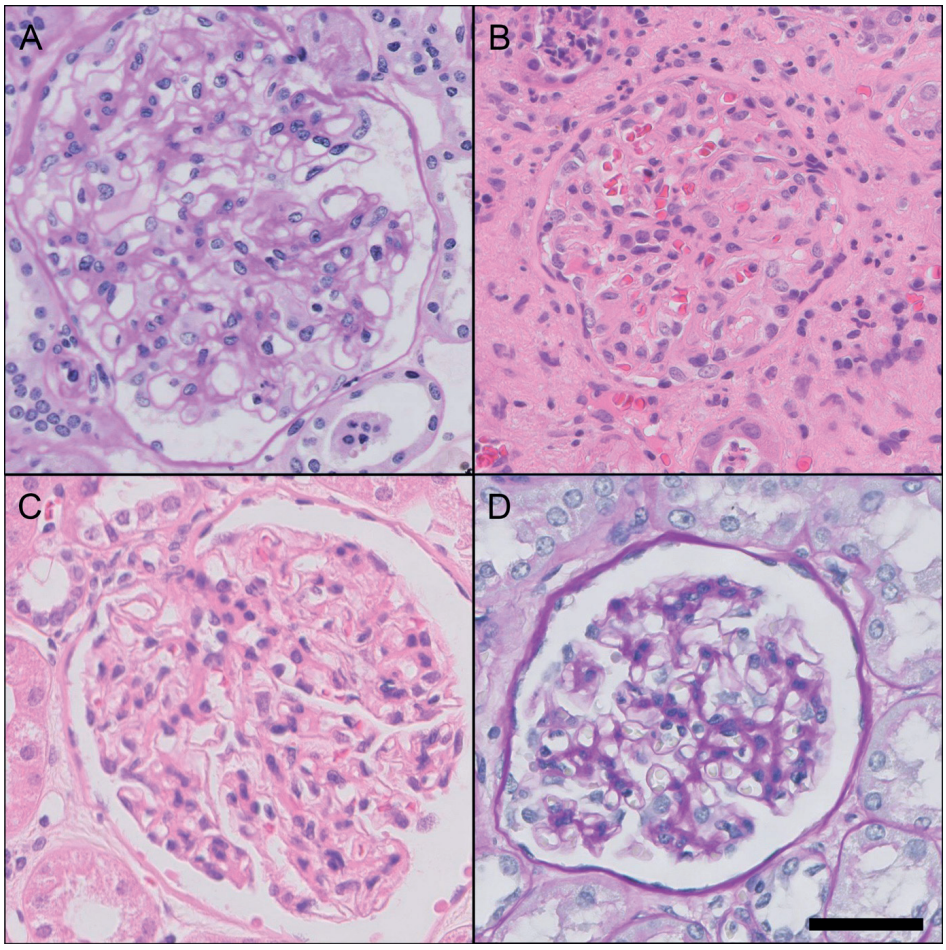


Figure 3. Light microscopy of glomeruli from MO patients shows relatively normal morphology
Snapshots of glomeruli from MO patients are shown. No specific lesions other than those related to the primary disease were observed. The examples are derived from patients with IgA nephropathy (**A**, PAS), glomerulonephritis after transplantation (**B**, H&E), and neoplastic diseases (**C**, H&E and D, PASD). Original magnification 40x. Scale bar = 50 µm

Evaluation of the renal sections by light microscopy did not reveal notable changes that could be specific for MO (56). A selection of representative images is shown in figure 3. However, EM analysis revealed a highly aberrant phenotype in one case with a noteworthy clinical history. This patient had received a kidney transplantation after suffering from a renal insufficiency. The primary disease was noted to have 'membrane anomalies reminiscent of a hereditary nephritis. We re-examined the EM grids of this patient, and found segmental fibrillar deposition that seemed similar to that reported by Roberts *et al.*

(56). Cross-striated fibrils with a diameter of 140 nm and regular periodicity were found in the mesangial area and the laminae rarae of the glomerular basement membrane of the native kidney (Figure 4). This was the only patient in the cohort for whom EM data was available. Genetic data of this patient was not available.

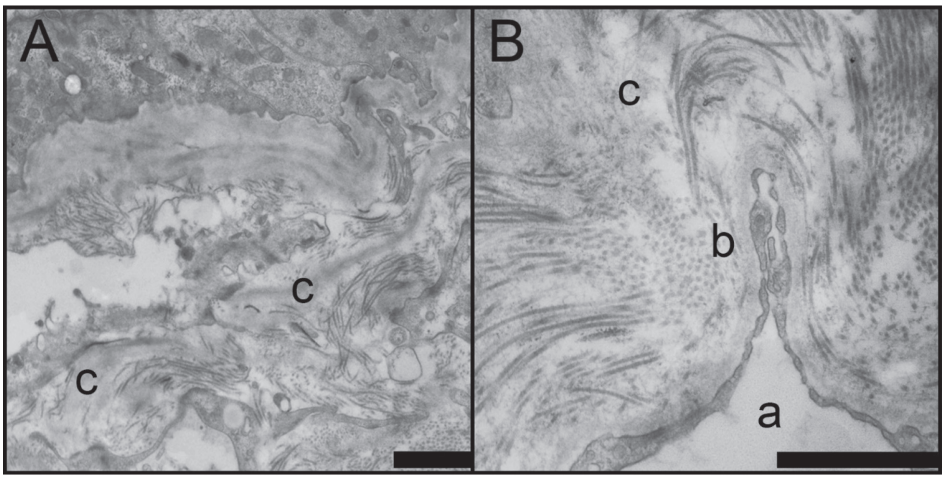


Figure 4. Glomerular fibril deposition HME-MO glomerulopathy
(**A** and **B**) Deposition of large fibrils with a diameter of 140 nm was seen in the glomerulus of an MO patient. The fibril depositions are seen in the laminae rarae of the GBM and in the mesangium. (c) General morphology is disrupted due to the fibril deposition. In figure B, endothelial lumen (a) and fenestrated endothelial cells can be seen (b). The fibrils in the GBM appear to be collagenous in nature. (c) Scale bar = 5 μm.

Discussion

In this study, we investigated the effect of heterozygous *EXT1* or *EXT2* germline mutations on glomerular permeability, glomerular morphology, and the endothelial glycocalyx. No (micro)albuminuria was observed in these patients, despite also having a higher prevalence of other risk factors for developing proteinuria with more hypertension, diabetes and NSAID use, as shown in Table 1. Furthermore, no specific glomerular morphological changes were observed in biopsies from a historic cohort of MO patients without clinical symptoms of renal damage. However, one of MO patient with renal pathology showed a glomerular phenotype resembling MO glomerulopathy. No significant difference was found in PBR thickness or vascular density between MO patients and controls.

This study shows that heterozygous *EXT1* or *EXT2* germline mutations do not significantly influence glomerular permeability or the endothelial glycocalyx. Glomerular permeability was not affected by these mutations, as no difference was found in albuminuria between

patients and controls. We found no difference in PBR and vascular density between patients with an *EXT1* or *EXT2* mutation and controls, indicating that these mutations do not significantly alter the endothelial glycocalyx. Also, we found no specific glomerular phenotype in patients with osteochondromas. These results contribute to the widely discussed topic of the role of HS in the GFB.(25, 55)

Interestingly, one patient in this group showed a complicated medical history with an unexplained renal insufficiency that resulted in renal replacement therapy. Our EM analysis of this patient showed glomerular deposition of large collagen fibrils in the mesangium and GBM, similar to those found by Roberts *et al.* (56).

Previously, circulating HS derived from MO patients was found to be structurally normal, but quantitatively diminished compared to healthy controls (63). Whether MO patients have structurally aberrant HS in the GFB and glycocalyx is currently unknown. Our results could imply that the mono-allelic germline mutation in *EXT1* or *EXT2* in MO does not lead to these structural changes. Potentially, one functional allele is sufficient to prevent a phenotype from developing. Because most MO patients showed a normal glomerular morphology, we hypothesize that a second trigger is required to develop glomerular pathology specific to the *EXT1* or *EXT2* gene defect. In MO pathogenesis, germline heterozygous mutations in EXT1 or EXT2 are not sufficient to cause osteochondroma formation. Loss of heterozygosity is needed for osteochondroma to develop.(69, 70) Analogous to this tenet, our data supports the notion that a germline heterozygous mutation in the EXT1 or EXT2 gene alone is not enough to cause a pathologic phenotype in the glomerulus or endothelial glycocalyx. Cases such as those described by Roberts *et al.* and here, are likely to have a distinct, local loss of heterozygosity and as such, loss of HS, leading to a specific phenotype. Potentially, factors other than HS could also play a role in these rare cases. Our group has previously shown that a bi-allelic germline mutation of EXT in zebrafish also does not lead to a renal phenotype, although it does result in a specific cartilaginous and dental phenotype.(31, 33, 55, 71) Overall, based on the results from the current study and others, MO patients do not seem to have an increased risk of developing clinically significant microvascular complications. However, rare pathology exists in this disease and the current study might be underpowered to fully assess that risk.

A limitation of this study is that both glycocalyx measurements and urine samples were not collected multiple times. Glycocalyx measurements are of a dynamic nature with a small variability, and multiple measurements, as we have performed, increase reproducibility.(72)

Although multiple urine samples over a period of time would result in a more representative result of a patient's typical albumin excretion, the absence of proteinuria, especially in the MO patient group, strongly suggests that indeed, no clinically significant pathology is present.

In this study, 6 out of 19 patients had a confirmed *EXT1* or *EXT2* mutation status. As stated in the introduction, the clinical diagnosis of the autosomal dominant disease of MO is often made without genetic testing, as in previous studies, a germline mutation in the *EXT1* or *EXT2* gene was found in almost 90% of cases.(59, 62) All MO patients in this study were included through the Dutch Multiple Hereditary Exostoses/Multiple Osteochondromas Association. In this study, outcome parameters were similar in subjects with and without a confirmed genetically confirmed diagnosis.

Most patients from the historic PALGA cohort did not have a confirmed *EXT1* or *EXT2* mutation. At least four patients from this cohort can be classified as having MO based on clinical criteria. The only clinical data we had available on these patients, were the pathology reports. So this is most likely an underestimation of the total amount of MO patients within this cohort. We deduce that if a specific morphological phenotype with pathophysiological consequences would exist due to these mutations, our method results in an adequate appreciation of this topic. Besides the single patient described, the fact that no changes were found implies that *EXT1* or *EXT2* mutations do not necessarily cause changes to glomerular morphology. However, these results could be misinterpreted due to a lack of power.

In conclusion, this study shows that in humans, mono-allelic germline *EXT1* and *EXT2* mutations do not result in proteinuria or significant changes to the endothelial glycocalyx. Loss of heterozygosity could be an explanation for the very rare case of MO glomerulopathy described previously.(56) Based on the current study, MO patients do not appear to be at an increased risk for clinically significant pathology of the glycocalyx or glomerular filtration barrier. Future studies might further elucidate the loss of heterozygosity theory and potentially identify other factors that might lead to the rare specific renal phenotype seen in only a very small subset of MO patients. This could assist in understanding not only the MO phenotype, but the pathophysiology of the glomerular filtration barrier and endothelial glycocalyx.

List of abbreviations

GAG: glycosaminoglycan
GBM: glomerular basement membrane
GFB: glomerular filtration barrier
HME: hereditary multiple exostoses
HS: Heparan sulfate
MO: multiple osteochondromas
PBR: perfused boundary region
RBC(w): red blood cell (width)

Ethics approval and consent to participate

Ethical approval for this study was obtained through the LUMC Medical Ethical Committee under registration number P15.106. Patient material from the PALGA search was handled in accordance with institutional guidelines, Good Research Practice, and the Code of conduct for responsible use.

Consent for publication

Not applicable

Competing interests

No potential conflicts of interest relevant to this article were reported.

Funding

No external funding was used in this study.

Authors' contributions

RK designed and performed experiments, analyzed and interpreted data, and wrote the paper. MGSB designed and performed experiments, and analyzed and interpreted data. PALGA group, consisting of those mentioned in the acknowledgements section, provided clinical data and material from the Dutch pathological anatomy national automated archive. JAB and TJR made substantial contributions to the conception and design of the work. BB, PCWH, and HJB made substantial contributions to the conception and design of the work, interpretation of data, and revisions of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors extend their gratitude to the Dutch Multiple Hereditary Exostoses/Multiple Osteochondromas Association for their involvement in this project.

We thank Hans Vink (Glycocheck) for the use of the Glycocheck software.

We also thank the PALGA foundation and all participating laboratories and researchers: A. Bezuijen, J.E. Boers, P.C. de Bruin, M.A.A.M. van Dijk, P. Drillenburg, A.F. Hamel, H.M. Hazelbag, G.N. Jonges, R.E. Kibbelaar, K.H. Lam, H. van der Linden, J. van Marsdijk, C. Meijer, I.D. Nagtegaal, J.J. Oudejans, J.J.T.H. Roelofs, L. Rozendaal, S.H. Sastrowijoto, M.M. Smits, J. Stavast

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