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Zusammenfassung des wissenschaftlichen Inhalts

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Das Alport Syndrom ist eine erbliche Typ IV Kollagen-Erkrankung, die in den meisten Fällen X-chromosomal übertragen wird (ein kleinerer Anteil wird autosomal vererbt). Homo- bzw. hemizygote Mutationen führen schon im frühen Erwachsenenalter zum vollständigen Nierenversagen. Auch bei heterozygoten Anlageträgern liegt das Risiko bei bis zu 30%, im späteren Erwachsenenalter (mit 40-60 Jahren) dialysepflichtig zu werden. Aufgrund des zumeist X-chromosomalen Erbgangs sind fast alle Mütter von betroffenen Jungen heterozygote Genträgerinnen. Die Prävalenz von heterozygoten Mutationsträgern insgesamt beträgt ca. 1% der Bevölkerung und hat somit eine hohe gesundheitsökonomische Relevanz.

Wir haben mit Hilfe des Europäischen Alport-Registers erstmals weltweit eine 4-jährige prospektive Verlaufsbeobachtung dieser Mütter und der heterozygoten Patienten durchgeführt. Zuvor konnten wir in einer retrospektiven Registerauswertung 2010 zeigen, dass eine RAAS-Blockade (Therapie mit einem ACE-Hemmer oder AT1-Antagonisten) das Nierenversagen bei Patienten mit Alport Syndrom und den heterozygoten Mutationsträgern verzögern kann.

Unsere aktuellen Ergebnisse untermauern diese Daten und zeigen erstmals in einer prospektiven Studie, dass heterozygote Alport-Mutationsträger besonders nachdrücklich von einer frühen RAAS-Hemmung profitieren: Dies wird daran deutlich, dass keiner der 52 Patienten während des 4-jährigen Beobachtungszeitraums dialysepflichtig wurde; einige Patienten (10,3%) zeigten sogar eine Verbesserung der Nierenfunktion.

Die amerikanische Fachgesellschaft für Nephrologie misst unserer Präventions-Studie eine hohe Bedeutung zu und hat ihre Mitglieder eigens in einem Newsletter per email auf unsere Ergebnisse hingewiesen. Die hohe Anzahl der Patienten, die eine Therapie erhielten, ist als großer Erfolg der Informationsarbeit der letzten Jahre zu werten, zumal die Krankheit im Anfangsstadium bei heterozygoten Trägern oftmals nicht einfach zu diagnostizieren ist. Die teilweise lange Dauer bis zur Diagnosestellung (im Mittel 8 Jahre) zeigt nach wie vor einen hohen Informationsbedarf, um möglichst alle heterozygoten Alport-Patienten mit Hilfe einer frühzeitigen Therapie vor der Dialyse zu bewahren.

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Prospective study on the potential of RAAS blockade to halt renal disease in Alport syndrome patients with heterozygous mutations

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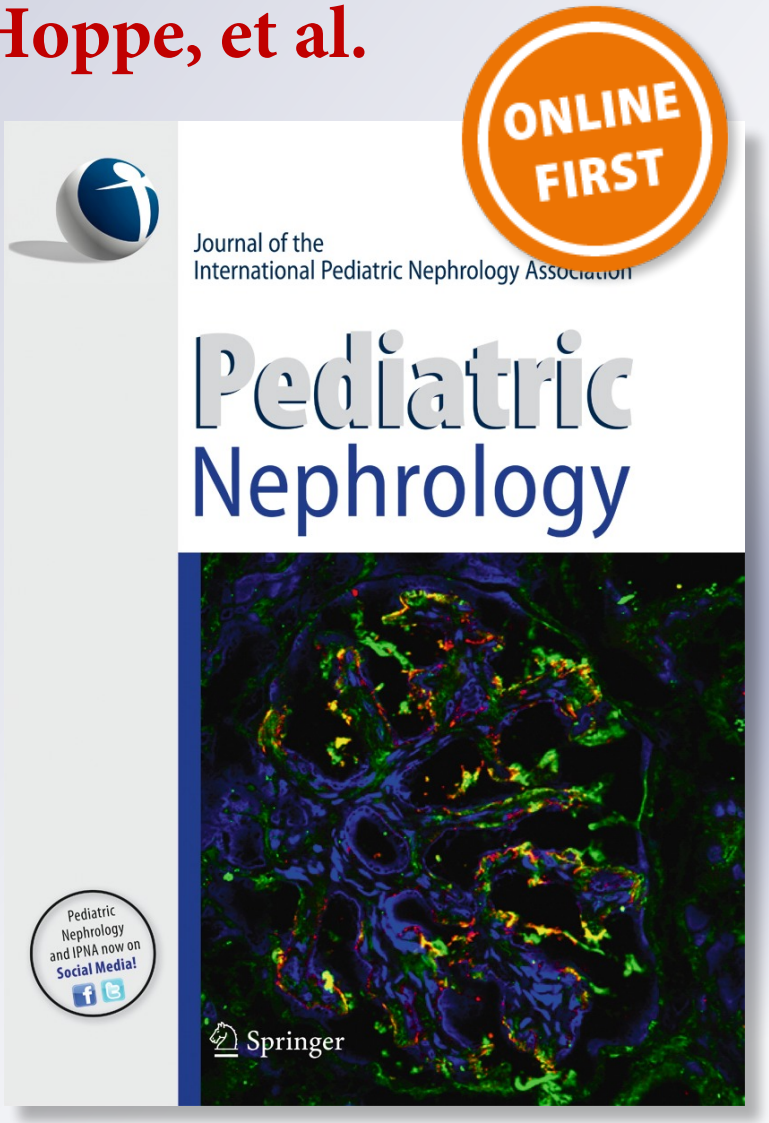

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ORIGINAL ARTICLE

Prospective study on the potential of RAAS blockade to halt renal disease in Alport syndrome patients with heterozygous mutations

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Abstract
Background Patients with autosomal or X-linked Alport syndrome (AS) with heterozygous mutations in type IV collagen genes have a 1–20 % risk of progressing to end-stage renal disease during their lifetime. We evaluated the long-term renal outcome of patients at risk of progressive disease (chronic kidney disease stages 1–4) with/without nephroprotective therapy.
Methods This was a prospective, non-interventional, observational study which included data from a 4-year follow-up of AS patients with heterozygous mutations whose datasets had been included in an analysis of the 2010 database of the European Alport Registry. Using Kaplan–Meier estimates and logrank tests, we prospectively analyzed the updated datasets of 52 of these patients and 13 new datasets (patients added to the Registry after 2011). The effects of therapy, extrarenal symptoms and inheritance pattern on renal outcome were analyzed.
Results The mean prospective follow-up was 46 ± 10 months, and the mean time on therapy was 8.4 ± 4.4 (median 7; range 2–18) years. The time from the appearance of the first symptom to diagnosis was 8.1 ± 14.2 (range 0–52) years. At the time of starting therapy, 5.4 % of patients had an estimated glomerular filtration rate of <60 ml/min, 67.6 % had proteinuria and 27.0 % had microalbuminuria. Therapeutic strategies included angiotensin-converting enzymer inhibitors (97.1 %), angiotensin receptor antagonists (1 patient), dual therapy (11.8 %) and statins (8.8 %). Among patients included in the prospective dataset, prevented the need for dialysis. Among new patients, no patient at risk for renal failure progressed to the next disease stage after 4 years follow-up; three patients even regressed to a lower stage during therapy.
Conclusions Treatment with blockers of the renin–angiotensin–aldosterone system prevents progressive renal failure in AS patients with heterozygous mutations in the genes causing AS. Considerable numbers of aging AS patients on dialysis may have heterozygous mutations in these genes (present in 1 % of total population) as underlying disease. Hence, greater alertness towards timely diagnosis and therapy has the potential to prevent progressive renal failure in most—if not all—AS patients with heterozygous mutations in the causal genes.

Keywords Chronic kidney disease · Renal insufficiency · Fibrosis · Collagen · Alport syndrome · Thin basement membrane disease · Familial benign hematuria

Trial Registration ClinicalTrials.gov NCT02378805; EudraCTnumber 2014-003533-25.

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Introduction

Alport syndrome (AS) is a hereditary kidney and basement membrane disease caused by mutations in type IV collagen genes (*COL4*) [1, 2]. X-linked AS (XLAS) is caused by mutations of the *COL4A5* gene encoding the α5-chain of type IV collagen, and mutations of the *COL4A4/COL4A3* genes encoding the α3/α4 (IV) chains induce the autosomal recessive form (ARAS) of AS [3]. Males with XLAS and patients of both gender with homozygous (or compound heterozygous) ARAS mutations develop the complete severe pattern of the disease with end-stage renal disease (ESRD) during adolescence or early adulthood, including hearing loss and ocular lesions in most cases [4, 5]. The course of AS has been well studied in these patients, including their genotype–phenotype correlations [4–7] and the beneficial effect of timely therapy on renal failure and life expectancy [8–11].

Analysis of female patients who are heterozygous for XLAS has revealed a large variability in the clinical course, with a lifetime risk of ESRD of 12 and 30 % (at the age of 40 and 60 years, respectively) [12]. These data show that the diagnosis of heterozygous *COL4A5* mutations is not associated with a benign course of disease. There is a large inter- and intra-familial variability in clinical manifestations in female patients who are heterozygous for XLAS, at least in part due to random X-chromosome inactivation [13].

Patients who are heterozygous for ARAS show the phenotype "familial benign hematuria" or "thin basement membrane nephropathy" (TBMN) [14–18]. Familial benign hematuria is not a benign condition. Heterozygous ARAS mutations cause progressive renal disease in mice [19], and in humans they are associated with a 1–5 % lifetime risk of severe renal impairment [14–18]. TBMN is not a rare disease, as at least 1 % of the population is affected [14, 18]. A retrospective study of the European Alport Registry showed that patients heterozygous for ARAS and XLAS both benefit from renin–angiotensin–aldosterone system (RAAS) blockade and that the risk of renal failure can be reduced with such therapy [20]. As a consequence, early diagnosis of heterozygous ARAS and XLAS mutations, even in minors, is recommended, as well as RAAS blockade in those patients with proteinuria [9, 10]. Due to the retrospective collection of data in 2010 [20], the results of the European Alport Registry did not address the question of whether consequent and timely RAAS blockade can halt renal disease or even prevent ESRD.

Therefore, we report here the first prospective evaluation of the long-term renal outcome of patients who are heterozygous for ARAS and XLAS and at risk of progressive disease and compared those who received nephroprotective therapy with those who did not. The results of this 4-year prospective study raise the hope that timely intervention can prevent progressive renal failure in most, if not all patients with heterozygous mutations in the genes causing AS. These data are very relevant to the clinical practice of pediatric nephrologists since many of these patients are first identified during childhood.

Materials and methods

Study population, inclusion and exclusion criteria

Diagnosis of heterozygous status was based on a genetic consultation regarding inheritance of XLAS or ARAS (including a conclusive genealogic tree and, if available, linkage analysis) and either mutation analysis or kidney biopsy [see Electronic Supplementary Material (ESM) Table 1]. Patients were excluded if they were affected males with XLAS or patients with genetically proven homozygous ARAS. Patients were also excluded if they did not provide informed consent, if the diagnosis was suspected but not confirmed or if they had donated a kidney (living donor to affected family member) [21].

Prospective 4-year follow-up

The Registry retraces data over several decades in three generations of AS patients, hindering a prospective study design at the primary data collection in 2010 [20]. For the prospective follow-up, data were updated via telephone interviews, email or facsimile or by personal contact with both physicians and patients. For ethical and data safety reasons, we only recontacted patients from German-speaking countries and only patients who had contacted us previously by email, facsimile or personally. We also included in the analysis all patients from our Alport outpatient clinic in Göttingen, Germany. Data were hosted centrally on a non-open access computer and were pseudonymized at the University Medical Center Göttingen. Questionnaires included questions regarding demographic data and clinical and laboratory data as described previously [20]. The updated version also included new questions on "time from first symptom to diagnosis", "initial diagnosis on renal biopsy", "extrarenal symptoms" and risk factors such as "smoking" and "hypertension". The registry and data storage, in conformity with Good Clinical Practice guidelines, were approved by the Ethics Committee of the University Medical Center Göttingen (AZ 10/11/06; renewed version in 2014; ClinicalTrials.gov Identifier NCT 02378805; EudraCT number 2014-003533-25). Data were collected from Germany, Austria and Switzerland.

Intervention and outcome measures

The observational, non-interventional study explored the treatment effects of angiotensin-converting enzyme inhibitors (ACEis) and angiotensin receptor blockers (ARBs); the control intervention was no RAAS blockade. The most

commonly used ACEi was ramipril (2.5–10 mg in adults). The study end-points were "renal replacement therapy" (RRT) or "age at onset of RRT", "impaired renal function", "proteinuria", "micro-albuminuria" and "hematuria" [see ESM Fig. 1]. Impaired creatinine clearance was defined as <60 ml/min in a 24-h urine collection, if available, or the estimated glomerular filtration rate (eGFR). Proteinuria was defined as >300 mg protein per day, micro-albuminuria as 30–300 mg protein per day in a 24-h urine collection and hematuria as >4 erythrocytes per high power field (400-fold magnification).

Statistical analysis

Median event times are reported with 95 % confidence intervals (CI) which are based on log–log-transformed confidence intervals of the event probabilities. If the confidence intervals of the event probabilities are too wide across all observed times due to the small sample size, the confidence limits for the median cannot be determined. All analyses reported here are of an exploratory nature and therefore no correction for multiple testing was applied. All reported p values are two-sided, and p values of <0.05 were considered to be statistically significant. All inferential analyses were carried out using STATISTICA version 12.0 (StatSoft Europe GmbH, Hamburg, Germany). Kaplan–Meier estimates were calculated using R version 3.1.2 (The R Foundation for Statistical Computing, Vienna, Austria). Distributions of continuous variables were summarized by means, whereas frequencies and percentages were used for categorical (including binary) variables. The efficacy endpoint "age at onset of RRT" was censored in some patients since not all patients included in the analyses had started RRT (or had died). Therefore, appropriate statistical methods for censored time-to-event data were used, including the Kaplan–Meier estimator and the log rank test [22].

Results

The European Alport Registry retraces clinical and therapeutic data over more than two decades in three generations of families with AS across Europe [8, 20]. For the analysis reported here, for the first time we have combined these retrospective data from our last update in 2010 with prospective 4-year follow-up data.

Clinical characteristics and genotype–phenotype correlation

The prospective 4-year analysis included 52 updated datasets plus 13 new patients (included into the Registry after 2011). The datasets from 24 untreated patients and 41 patients who received RAAS blockade (ACEi and/or ARB) were combined in the data analysis. Four prospective patients had been started on RAAS blockade between 2011 and 2014. Among the 52 prospective patients (7 males, 45 females), 72.6 % (37/51) had mutations for XLAS, and 27.4 % (14/51) had mutations for ARAS (one patient had an uncertain inheritance). Mean age at follow-up was 38.1± 20.3 (median 38, range 6–81) years. The underlying cause was "in frame" mutations in 17/22 (77 %) patients, deletions in 5/22 (22 %) patients (5/22) and a large rearrangement (XLAS with leiomyomatosis) in one (1/22) patient. Only 8 % of patients (2/24) showed ocular changes; in contrast, 59 % (17/29) showed hearing impairment. Mean time from the first symptom to diagnosis of ARAS or XLAS due to heterozygous mutation was 8.1 ± 14.2 years (n = 16; range 0–52 years). Of 11 kidney biopsies, light microscopy showed no pathological findings in 45 % (5/11) and focal segmental glomerulosclerosis (FSGS) in the remaining 55 % (6/11). Three patients on RRT received a kidney transplant and still have a functioning transplant (9–24 years after transplantation). Hearing impairment was a negative predictor for ESRD (Fig. 1a): none (0/17) of the patients without hearing loss proceeded to RRT, but 25 % (3/12) of patients with hearing loss did require RRT (p = 0.046 in Mann–Whitney U test; 95 % CI 5–57 %). In contrast, mode of inheritance was not a significant predictor of renal outcome (Fig. 1b), which was not surprising as we only selected patients with progressive renal disease from the Registry.

RAAS blockade prevents new events of ESRD in the prospective cohort of patients with heterozygous AS

The duration of the prospective follow-up of our patients was 46 ± 10 months. Mean time on therapy was 8.4 ± 4.4 (median 7; range 2–18) years. Mean age at onset of therapy was 29.5 ± 18 (median 31.5; range 4–62) years.

Therapy included ACEis in 97.1 % (33/34) of patients, and ARBs in the remaining 2.9 % (1/34); 11.8 % (4/34) and 8.8 % (3/34) of patients had dual therapy statins (added to RAAS blockade), respectively. Compared to our 2011 cohort, the risk of ESRD did not change significantly in our prospective cohort. The mean age at onset of RRT in untreated patients was 42.9 ± 17.7 (95 % CI 26.5–59.2) years in 2011 and 2014 (Fig. 2a, b). When we considered only the most affected heterozygous patients, by the age of 60 years, the mean risk for ESRD in untreated patients was 62 % in 2011 and 60 % in 2014. When the 13 new patients were included in the analysis of data in 2014 (Fig. 2c), 37 % (24/65) patients did not receive therapy, 5.1 % (2/39) started therapy in stage III (impaired renal function), 66.7 % (26/39) started therapy in stage II (proteinuria) and 28.2 % (10/39) started therapy in stage I (microalbuminuria). When these 13 new patients were

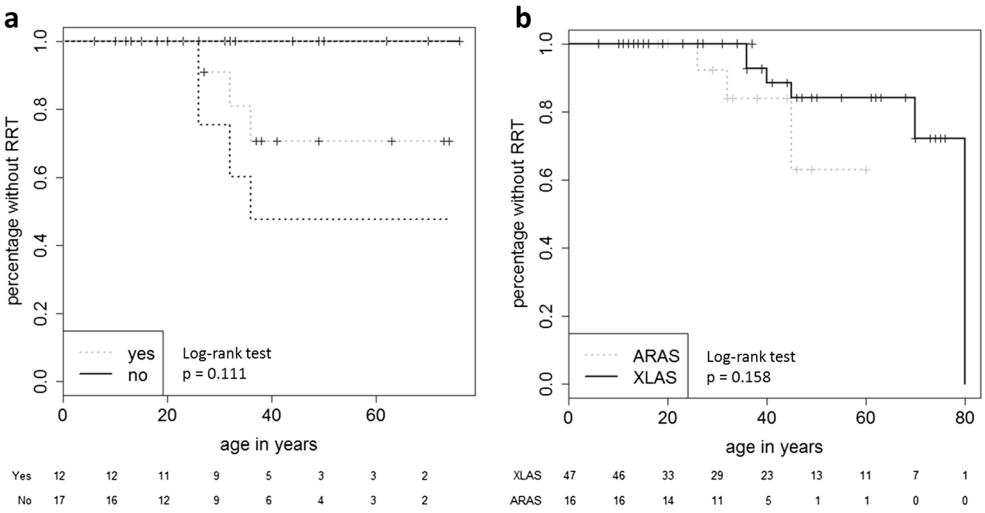

Fig. 1 Genotype–phenotype correlation in patients with Alport Syndrome (AS) with heterozygous mutations in type IV collagen genes. **a** Hearing impairment was a negative predictor for end-stage renal disease . *Dotted lines* 95 % confidence interval (CI). **b** Mode of inheritance was not a significant predictor of renal outcome. *RRT* Renal replacement therapy, *XLAS* X-linked AS, *ARAS* autosomal recessive AS

excluded from the analysis, 5.4 % (2/37) started therapy with a GFR of <60 ml/min, 67.6 % (25/37) started therapy with proteinuria and 27.0 % (10/37) started therapy with microalbuminuria. Mean age at onset of RRT was 42.9 ± 17.7 years in untreated patients (range 26–80 years; 95 % CI 26.5–59.2 years), while none of the treated patients progressed to ESRD.

Therapy halts progression of renal disease in the prospective cohort of patients with heterozygous AS

The update was able to generate prospective 4-year follow-up data in 52 patients with heterozygous forms of AS. In 2011, 13.5 % (7/52) of these patients were on RRT, and this percentage did not change in 2014 as none of the treated patients had progressed to ESRD within the 4-year treatment period (Fig. 3). In 2011, 31.0 % (9/29) of the treated patients were in stage III; with continuing therapy this percentage even decreased to 24 % (7/29) in the 4-year follow-up as renal function improved in 10.3 % (3/29) of the patients with heterozygous AS: 3.4 % (1/29) improved from stage III to II, 3.4 % (1/29) from stage III to I and 3.4 % (1/29) from stage II to 0. In parallel, 55 % (16/29) of treated patients were in stage II in 2011, and this percentage remained unchanged in 2014 (55 %, 16/29). In 2011, 10 % (3/29) of treated patients were in stage I. This percentage increased to 14 % (4/29; mean age 15 years) in 2014 as one (1/29; 3.4 %) of the patients in stage II regressed to stage 0 (1/29; 3.4 %). None of the patients progressed to the next stage of disease during the 4-year follow-up. The patient in stage 0 (3.4 %; 1/29) in 2011 remained in stage 0 during the 4-year follow-up in 2014 (plus one additional patient in stage 0, who improved from stage II to stage 0).

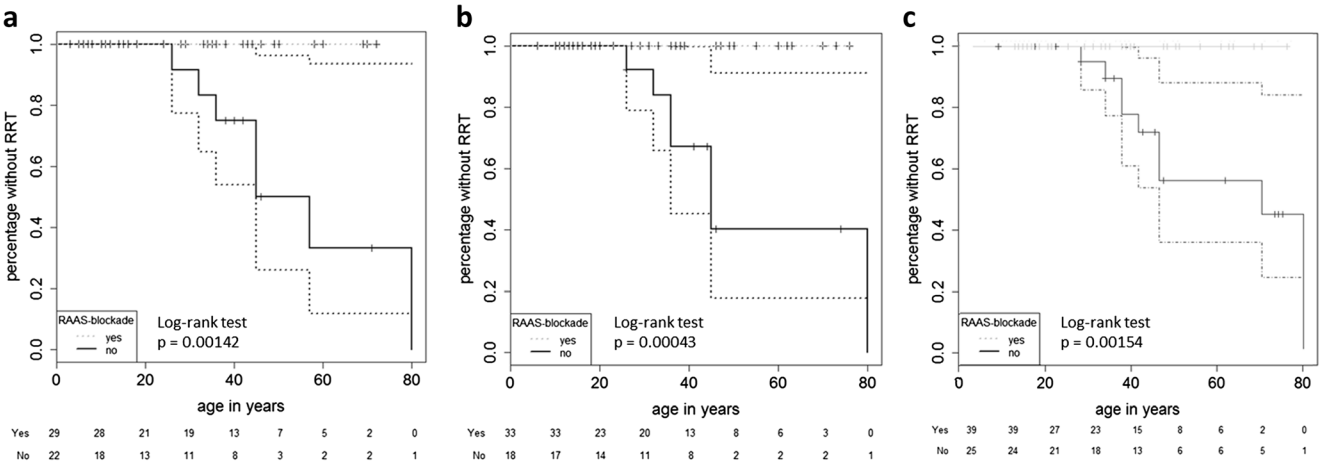

Fig. 2 Renin–angiotensin–aldosterone system (*RAAS*) blockade prevents new events of end-stage renal disease in the prospective cohort of Alport Syndrome (AS) patients with heterozygous mutations. **a** Percentage of patients on renal replacement theraphy (RRT) with therapy vs. those without therapy in 2011 (retrospective data). *Dotted lines* 95 % confidence interval (CI). **b** Percentage of patients on RRT with therapy vs. those without therapy (prospective data). *Dotted lines* 95 % CI. **c** Percentage of patients on RRT with therapy vs. those without therapy (prospective data + new patients). *Dotted lines* 95 % CI. (one patient could not be included from the "number at risk" table in Fig. 2a–c because of missing date of birth)

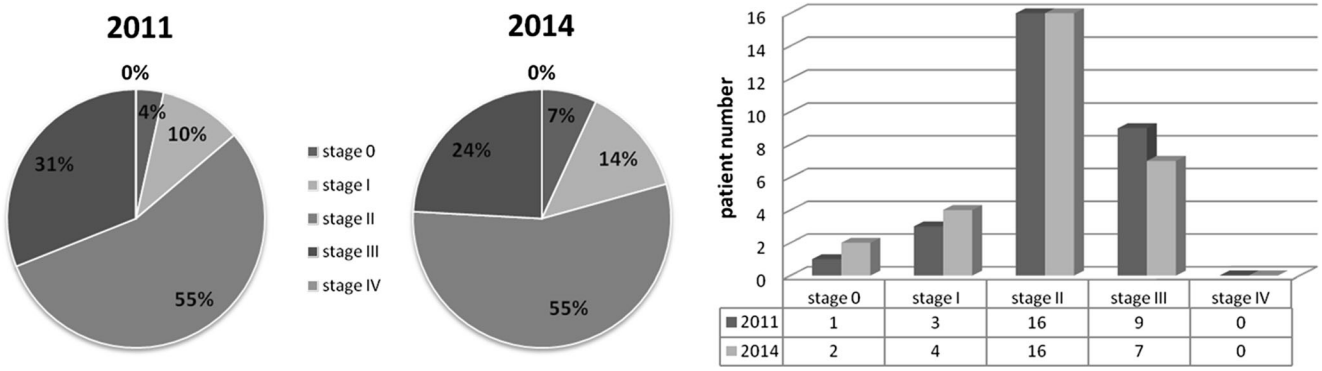

Fig. 3 Therapy can halt progression of renal disease in the prospective cohort of patients with heterozygous mutations for Alport Syndrome (AS). *Left* Percentage of treated patients in different stages of Alport disease in 2011 vs. the percentage in the prospective follow-up period. *Right* Number of treated patients in different stages of Alport disease in 2011 vs. that in the prospective follow-up

Discussion

The aim of this study was to increase the scientific evidence for the nephroprotective effect of RAAS blockade in AS patients with heterozygous ARAS and XLAS. Our previous study on Registry data from 2010 was limited to a retrospective information analysis with a risk of selection bias [20]. In contrast, in the present study reported here we used a prospective design with 4-year follow-up data of patients at risk for renal failure [chronic kidney disease (CKD) stages 1–4] who had received—or not— therapy. Legal regulatory and ethical factors limited the prospective analysis to patients who had contacted us previously by email and facsimile or by personal contact, and to our own outpatients. To our knowledge, this study is the largest prospective analysis of patients with heterozygous forms of AS.

In a previous study by Jais and coworkers, the risk of ESRD was about 30 % at the age of 60 years (12 % at the age of 40 years) in 288 XLAS carriers [12]. In our study we may have overestimateed the risk of ESRD in patients heterozygous for XLAS as we deliberately followed only the symptomatic patients with CKD. Interestingly, 59 % of our "at risk" patients had hearing impairment, and hearing impairment was a predictor for progressive renal disease in untreated patients.

FSGS was the most common diagnosis (55 % of patients) on light microscopy examination. These data are in agreement with results reported in other studies on AS patients with heterozygous mutations [23–26]. In our patients, the average time-span from first symptom to diagnosis was more than 8 years. This delay in establishing a diagnosis (and initiating adequate therapy in a subset of patients) was unexpected and—as the disease is treatable—not acceptable from a preventive point of view. Patients with both heterozygous XLAS and ARAS mutations show an impaired life expectancy when reaching RRT [20]. Preventive RAAS blockade might prolong life and improve the quality of life of these patients. As 1 % of the entire population represent AS patients who are "at risk", future educational efforts should focus on early diagnosis and preventive therapy in those patients (see also review by Rheault [25]).

Possible additional risk factors, such as hypertension, nephrotoxic medication and/or kidney donation [21], might play an important role in the aggravation of renal disease. We excluded age differences between treated and untreated groups as a potential confounder (as older patients show a higher incidence of renal damage caused by other reasons, such as diabetes, hypertension, atherosclerosis etc.). Further studies in animal models for TBMN should focus on these risk factors in order to identify new nephroprotective strategies [19]. Interestingly, almost all of our treated patients started RAAS blockade at early stages of disease (GFR of >60 ml/min), and more than 25 % of patients started therapy even before the onset of proteinuria. Evidence supporting the initiation of RAAS blockade in very early stages of disease (isolated hematuria or microalbuminuria) is limited, and RAAS blockade is currently not recommended at these very early stages in AS patients with heterozygous disease [9, 10]. In our patients, we had three different settings that could explain the very early start of therapy:

1) Pregnancy: Women previously undiagnosed for heterozygous forms of AS became symptomatic (proteinuric) during pregnancy. Proteinuria regressed to microalbuminuria after delivery; however, heterozygous form of AS was diagnosed and therapy started.
2) High blood pressure: Previously diagnosed heterozygous patients with microalbuminuria became hypertensive and therapy with RAAS blockade was started.
3) Parental distress: One heterozygous XLAS female with microalbuminuria developed proteinuria during a viral infection. Her affected father had developed ESRD, leading the parents to urge initiation of therapy in their daughter.

Our study design and selection of patients at risk of progressive renal disease may have led to an overestimation of the

risk of ESRD in heterozygous XLAS and ARAS patients. Patients who had not been recognized as having a heterozygous form of the disease were not included in any analysis. However, our observation every single AS patient with (preventable) ESRD due to a heterozygous mutation still presents a very significant finding. Early diagnosis by family history, kidney biopsy, or genetic testing is a prerequisite to initiating preemptive therapy. However, one can expect a high rate of patients with heterozygous ARAS (TBMN) to remain undiscovered because their ongoing renal disease is unrecognized (or otherwise explained by "unknown disease", hypertension, atherosclerosis, or abuse of analgetics). Over the upcoming years, the use of genetic mutation screening will become more common, with the result that patients with heterozygous XLAS and ARAS will be more easily identified, leading to the potential to start nephroprotective therapy earlier.

According to our study, wide-spread preemptive RAAS-blockade in patients with heterozygous ARAS and XLAS-mutations with microalbuminuria, proteinuria or high blood pressure could prevent a large number of severe impairments of renal function and even dialysis in most cases.

The limitations of this study were the rather small number of patients and its non-interventional design. Both limitations were caused by financial and legal obligations, limiting us to patients from non-German speaking countries which we were able to re-contact in order to follow them prospectively and to initiate or harmonize therapeutic intervention. In addition, in our patients with several features of heterozygous AS (hearing loss and ocular changes), we need to acknowledge the up to 10 % possibility of missed mutations in the autosomal (or X-chromosomal) genes causing AS. Further, in contrast to the interventional EARLY PRO-TECT Alport trial [27, 28], our non-randomized, non-interventional observational design only allowed us to increase the evidence base for a causative association betweem a better renal outcome in patients with heterozygous forms of AS and therapy with RAAS blockade. We tried to limit the shortcomings of a non-interventional study by our prospective 4-year follow-up design. Current and future observational studies, such as ASTOR (ClinicalTrials.gov NCT00481130) and the ATHENA study (NCT02136862), might provide additional evidence supporting nephroprotective therapy in patients with heterozygous AS.

In conclusion, our data demonstrate that treatment with RAAS blockers, such as ACEis and/or ARBs, can halt renal disease in AS patients with heterozygous mutations. The relatively high risk of ESRD and the impaired life-expectancy of AS patients with heterozygous mutations on RRT should lead to a greater alertness regarding patients with TBMN presenting the non-benign symptoms of familial benign hematuria. Due to the high frequency of heterozygous mutations in the genes causing AS in the general population, patients with heterozygous ARAS and XLAS are very relevant to nephrologists. Yearly follow-up by a nephrologist and early-on nephroprotective therapy with RAAS blockade should be advised for all newly diagnosed patients, as this strategy may prevent ESRD in most—if not all—patients with heterozygous ARAS and XLAS.

In this context, the data presented here build on previous publications of retrospective data on RAAS blockade in AS [8, 20]. In the future, worldwide efforts with an evidence-based synthesis of international retrospective and prospective data will hopefully further improve knowledge on the early genetic diagnosis and the optimal starting-point and therapeutic regimen in patients with AS [29].

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Authors' contributions The first and the last author JS and OG had full access to all the data in the study and take responsibility for the content of the manuscript, including the data and analysis. JS and OG contributed to the conception and design of the study, analysis and interpretation of data, drafting the article, revising the paper and final approval of the manuscript. All other authors contributed to the acquisition of data, revision of the manuscript and final approval. All authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethics statement The registry and data storage, in conformity with GCP guidelines, were approved by the Ethics Committee of the University Medical Center Göttingen. For ethical and data safety reasons, we only re-contacted patients from German-speaking countries and only patients who contacted us previously by email, facsimile or personally. Informed consent was obtained for all participants.

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