Amniotic fluid peptides predict postnatal renal survival in developmental renal disease

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Running headline: amniotic fluid peptides for CAKUT outcome prediction

Abstract

Although a rare disease, bilateral congenital anomalies of the kidney and urinary tract (CAKUT) are the first cause of end stage renal disease (ESRD) in children. Ultrasound-based prenatal prediction of postnatal renal survival in CAKUT pregnancies is far from accurate. In a prospective multicenter study spanning 178 CAKUT patients, we applied amniotic fluid (AF) peptidome analysis to improve on this prediction. Using a training cohort of 53 CAKUT pregnancies, we identified a signature of 98 endogenous AF peptides, composed for the major part of fragments from extracellular matrix proteins and thymosin-β4, predicting postnatal renal outcome with an AUC of 0.96 in a hold-out validation set of 51 CAKUT patients. The signature was validated in a geographically different setting (AUC 1.00) and displayed high specificity (82% to 94%) in non-CAKUT settings. Modification of AF thymosin-β4 abundance was confirmed with ELISA. Invalidation of thymosin-β4 in zebrafish altered proximal and distal tubule pronephros development. The introduction of this AF peptide signature in the diagnostic workup of prenatally detected CAKUT fetuses can provide a long-sought evidence base for accurate management of the CAKUT disorder that is currently unavailable.

Keywords: Congenital anomalies of the kidney and the urinary tract, peptides, amniotic fluid, prediction, termination of pregnancy, management, infants.

Introduction

Although a rare disease, congenital anomalies of the kidney and the urinary tract (CAKUT) represent 20-30% of all inborn malformations[1]. Whereas prognosis is generally favorable in unilateral disease, bilateral CAKUT are the predominant causes of chronic kidney disease (CKD) in childhood[2] and account for ~50% of pediatric and young adult end stage renal disease (ESRD) cases[3]. Bilateral CAKUT display a wide spectrum of outcomes ranging from death *in utero* to normal renal function after birth. Current routine ultrasound-based prenatal clinical advice on the postnatal renal outcome to parents expecting a child with CAKUT is far from accurate[4,5]. Except in the extreme cases of CAKUT (e.g. anamnios or bilateral renal agenesis), the underlying CAKUT etiology obtained by ultrasound (e.g. the presence of hypoplasia, hyperechogenicity, dysplasia or urinary tract anomalies) is not associated to the prognosis of the disease. Alternatively, invasive testing such as assessing fetal serum β2-microglobulin[6] has been used, but is controversial due to the absence of clear cutoff values and the fact that only measurements at advanced gestational age are prognostic [7,8]. This prognostic uncertainty of disease progression has particularly serious implications for prenatal counseling of the parents confronted with the decision of continuation or elective termination of pregnancy. Such uncertainty has led to documented situations where half of the cases of severe bilateral CAKUT for whom termination of pregnancy was considered but not performed had normal postnatal renal function[9]. In the case parents decide to continue the pregnancy, knowledge of the precise outcome would allow anticipating dialysis, transplantation or palliative care.

The absence of a clear link between the ultrasound defined CAKUT etiology and postnatal renal survival suggests that the disease should be approached at a molecular level. Modern-era approaches such as clinical omics, allowing a precise molecular description of disease, are expected to be better linked to postnatal renal survival. However, although knowledge of the underlying genetic causes in CAKUT is increasing, the absence of a clear genotype-phenotype correlation in CAKUT[1,10] suggests that searching

markers of progression should focus on traits beyond the genotype, closer to the phenotype including proteome and metabolome approaches. This has already been shown in adult CKD where a FDA-supported urinary peptide panel outperformed routine clinical measurements in the prediction of disease progression[11]. Therefore, we hypothesized that the use of clinical amniotic fluid peptidome analysis to predict progression of disease could change the *status quo* as well in CAKUT.

Here, using the specifically designed and largest prenatal prospective cohort in this renal developmental disease and a 2-year postnatal follow-up (Clinicaltrials.gov: NCT02675686), we developed and validated an endogenous amniotic fluid peptide signature and evaluated the value of adding this signature to prenatal management.

Results

Characteristics of the study population

During follow-up of the prospectively included 178 bilateral CAKUT patients, 34 pregnancies were excluded for medical or sampling reasons. Four other samples were withdrawn since the amniotic fluid peptidome could not be analyzed (**Figure 1**). For the remaining 140 patients, the major etiologies were hyperechogenic kidneys (40/140) and lower urinary tract obstruction (29/140) representing 49% of the patients (**Table 1**). Forty-nine percent (69/140) of the fetuses had normal or moderately reduced renal function (eGFR>60 ml/min/1.73m2) at 2 years postnatally. Etiologies mostly associated to normal outcome were non-obstructive urinary tract anomalies and upper urinary tract obstruction. In contrast, 71/140 (51%) of the fetuses developed early-onset renal failure (CKD3-5, eGFR<60 ml/min/1.73m2 at 2y) or perinatal death due to ESRD or were subjected to TOP. Bilateral agenesis, MCDK and lower urinary tract obstruction were the main etiologies associated to these compromised outcomes.

Identification of an amniotic fluid peptide signature associated to postnatal renal outcome

For identification of peptides associated to postnatal renal outcome, a training cohort of 53 CAKUT patients (**Figure 1**) including 35 with normal outcome (eGFR>60 ml/min/1.73m2 at age 2 years) and 18 with compromised outcome (early-onset renal failure, perinatal death due to ESRD, or TOP with fetopathology showing severe renal maldevelopment) were used (**Supplementary Table S1**). A total of 7,000 peptides were detected in AF, for 1,008 of which sequence information could be obtained. A first hierarchical clustering analysis using the abundance of these 1,008 peptides separated the CAKUT patients better based on postnatal renal outcome than on CAKUT etiology (**Figure 2 and Supplementary Figure S1**). Next we identified 98 peptides with significantly different abundance (corrected p-values <0.05) and multi-fold changes (up to 100-fold) associated to compromised postnatal renal outcome (**Supplementary**

Table S2). The majority of the peptides were fragments of various collagens (88%, **Figure 3A**). Other peptides included fragments of thymosin-β4, inter α trypsin inhibitor heavy chain H4 or osteopontin (3%, 2% and 1%, respectively, **Figure 3A**). The 98 AF peptides were included in a random forest mathematical model (called the bCAKUTPep signature), which was optimized for the classification of the 53 patients of the training set. A cutoff score of 0.47 was defined, allowing the best prognostic accuracy, with an emphasis on specificity rather than sensitivity. This led to a predictor with an area under the receiver operator curve (AUC) of 0.92 (95%CI: 0.85-1.00), a 78% (95%CI: 52-94) sensitivity and a 94% (95%CI: 81-99) specificity (**Figure 3B and C**).

Independent validation of the amniotic fluid peptide signature

In a first step to clinical application of the bCAKUTPep signature, and in accordance with clinical proteomics guidelines, it is essential to confirm that prognostic biomarkers are generalizable to 'similar but different' individuals outside the set of patients in which they were identified[12,13]. Therefore, in the next step we validated the signature in the holdout set of 51 CAKUT patients (**Figure 1**) composed of 34 fetuses with normal renal outcome and 17 patients with compromised renal outcome (**Supplementary Table S3**). This resulted in prediction of postnatal renal outcome with an AUC of 0.96 (95%CI: 0.90-1.00), a 88% (95%CI: 64-98) sensitivity and a 97% (95%CI: 85-100) specificity (**Figure 4A and 4B**). The prognostic capacity of the peptide signature significantly outperformed routinely used clinical parameters. Indeed, both reduced AF volume (oligohydramnios/anhydramnios) and gestational age at sampling predicted postnatal renal outcome with significant lower AUCs of 0.84 (95%CI: 0.69-0.98; P(one-sided)=0.03) and 0.72 (95%CI: 0.56-0.88; P(one-sided)=0.001), respectively (**Figure 4C**).

We next assessed whether the prognostic performance of the peptide-based signature could be improved by adding these clinical parameters. Combination of the peptides with either AF volume or

gestational age or both did not significantly improve the AUC (0.98 (95%CI: 0.94-1.00), 0.97 (95%CI: 0.94-1.00) and 0.97 (95%CI: 0.94-1.00), respectively) compared to bCAKUTPep alone (0.96 (95%CI: 0.90-1.00), **Figure 4C**).

In our study 60 out of 140 (43%) CAKUT pregnancies were terminated (**Table 1**) but for 28/60 fetopathology was absent (usually due to parental non-consent) or inconclusive (no definite status as to the severity of the renal lesions) (**Supplementary Table S4**). The application of the bCAKUTPep signature to these 28 fetuses led to the prediction of 9 fetuses (32%, **Figure 4D**) with a compromised outcome, thereby suggesting that the present sub-cohort exhibited an important number of fetuses subjected to TOP who would have normal postnatal renal function. Finally, for 8/60 TOP, triple expert reading fetopathology concluded that kidneys were compatible with normal life (**Figure 1, Supplementary Table S5**). The bCAKUTPep signature predicted compromised outcome for only 2 of them (**Figure 4E**).

Robustness of the peptide-based signature

To further evaluate a potential routine application and the robustness of the signature, the performance of the peptides was assessed using other mathematical approaches including support vector machine (SVM), a k-nearest neighbor (KNN) or linear models. These models performed similarly well (AUCs of 0.96 (95%CI: 0.90-1.00), 0.86 (95%CI: 0.71-1.00) and 0.88 (95%CI: 0.75-1.00) respectively, **Figure 5A**), confirming the validity of the selected peptides. Furthermore, the AF peptide signature appears geographically independent since it showed excellent prediction (AUC: 1.00 (95%CI: 1.00-1.00), **Figure 5B**) in a subset of patients from the validation cohort, i.e. 12 patients with CAKUT from Belgium (Belgium was not included in the training phase). We also performed a domain validation of the signature to test the specificity of the signature in individuals having a very different clinical status than CAKUT, even if the bCAKUTPep signature is not intended to be applied in those individuals. For this validation, we used AF

samples of 22 healthy fetuses and of 47 fetuses with primary maternal CMV infection associated with different grades of neurological lesions[14]. The latter cohort was used to preclude for the risk of a prediction only correlating with general fetal damage. The bCAKUTPep signature predicted normal postnatal outcome with a specificity of 82% (95%CI: 60-95) and 94% (95%CI: 82-99) in the two cohorts, respectively (**Figure 5C**). Finally, scoring of samples using the bCAKUTPep signature exhibited excellent short- and long-term precision as well as stability in preparation (coefficient of variation: 0.13; 0.21 and 0.12 respectively, **Supplementary Table S6**). This low analytical variability of the AF signature is similar to the FDA-supported urinary classifier CKD273[15]. Taken altogether, these results confirm the robustness of the selected peptides in the bCAKUTPep signature.

Thymosin β4

The 3 peptides of thymosin β4 included in the AF signature were the non-collagen peptides most significantly associated with postnatal renal outcome of CAKUT patients (**Supplementary Table S2,** column O). We therefore evaluated the prognostic abilities of a new random-forest model combining only these fragments. Interestingly this 3-thymosin β4 peptide model predicted postnatal renal function with a sensitivity, specificity and AUC similar to the bCAKUTPep model (**Figure 6A** and **B**), suggesting a strong association of increased thymosin β4 with compromised postnatal renal outcome. This association was confirmed using a commercial enzyme-linked immunosorbent assay in a subset of patients (**Figure 6C**). Beyond its prognostic potential, the increased level of AF thymosin β4 in severe forms of CAKUT could also inform on the renal physiopathology of CAKUT lesions. Thymosin β4 expression is detected in the human developing kidney (**Figure 6D-I**). In the healthy fetal kidney thymosin β4 is mostly observed in tubules (**Figure 6D-F**); in kidneys from CAKUT patients (unrelated to the current study and without information on the severity of the disease), a tubular localization of thymosin β4 is also observed although

the overall renal parenchyma is disorganized (**Figure 6G-I**). In order to evaluate the role of thymosin β4 during kidney development, we next generated a *thymosin β4* zebrafish mutant (*tmsb4*-/-) using a CRISPR/CAS9-based strategy; the allele generated is a 5 base-pair deletion leading to a frameshift that encodes a truncated Tmsb4 protein of 11 amino acids instead of 44 (**Supplementary Figure S1**). Pronephros development of *tmsb4*-/- was analyzed by *in situ* hybridization at 48h post fertilisation and compared to wildtype siblings (*tmsb4*+/+). Labeling with *slc20a1a* showed increased length of the proximal segment of the pronephros in *tmsb4*-/- (**Figure 7B**). Increased length of the distal segment using *slc12a3* was also observed in *tmsb4*-/- (**Figure 7C**). In contrast, no significant change was observed when using *wt1a*, a podocyte specific marker (**Figure 7A**). No overall morphological differences were detected between *tmsb4*-/- and *tmsb4*+/+ (not shown), suggesting that Tmsb4 plays a specific role in early renal development.

Discussion

We developed and validated a novel method for the prediction of postnatal renal survival in developmental renal disease based on an endogenous AF peptide signature in the largest prospective bilateral CAKUT cohort to date. This signature (bCAKUTPep), assigning a numerical score with a clear-cut cutoff, predicted postnatal renal outcome with high sensitivity and specificity and significantly outperformed routine clinical ultrasound-based measures.

The different antenatal CAKUT etiologies included in our study are similar to the CAKUT populations encountered in other but retrospective studies[5,16], suggesting that the signature will be applicable to CAKUT in general. We believe that the excellent prognostic performance of the signature will be maintained with wider application. Indeed this study was conducted in strict adherence to the clinical proteomics guidelines[12] for a rare disease, using separate and large training and validation multicenter cohorts and stringent statistics. Furthermore, the signature was validated at a small scale in a geographically different setting, in another developmental non-renal (neurologic) disease and in healthy fetuses thereby demonstrating its robust applicability.

Counseling of future parents with a fetus with bilateral CAKUT is emotion loaded as it involves the consideration to terminate the pregnancy in the face of a highly uncertain prognosis ranging from largely normal postnatal kidney function to perinatal death or life-long end-stage kidney disease. The established AF peptide signature provides for the first time an unambiguous prediction of postnatal renal outcome with significantly higher accuracy compared to conventional methods. In addition, the measurement of AF peptides is not subject to personal interpretation, which can be the case for sonographic imaging[5,17,18]. In case a high-risk phenotype is diagnosed, clear-cut knowledge of the outcome of the disease will give time to the future parents to psychologically accept[19] the fact that they will have a child with chronic, potentially severe disease and decide whether they would like their newborn to be offered palliative care or renal replacement therapies (RRT, *i.e.* dialysis or transplantation)[20]. Although RRT has improved over

the last decade long term survival for young infants (<1 y), they still represent a major burden on the quality of life of the children and their families[21]. Therefore, the decision of initiating RRT must be weighed and anticipated carefully.

Sixty out of 140 (43%) CAKUT pregnancies were terminated. This rate is slightly lower than in previous European studies where the rate of pregnancy termination was 55-62[7,22–24], but close to a recent retrospective study from the US (45% (32/71))[16]. Rarely put in the open, and as also observed in our study, a number of CAKUT pregnancies are terminated while subsequent fetopathology analysis reveals fetal kidneys with normal appearance[9]. The added value of the AF peptide-based signature in this context is evident from the fact that the bCAKUTPep predicted a normal outcome for 6 out of the 8 terminated pregnancies in which fetopathology showed kidneys that appeared compatible with normal life. Although shown on a small number, application of such a signature in the management of CAKUT might thus have avoided 75% of TOP displaying kidneys compatible with normal life.

This study focusing on CAKUT employing AF peptidome analysis shows the feasibility of the use of this strategy in other prenatally detected renal diseases such as early onset glomerular diseases.

AF peptide-based clustering analysis suggested that the AF molecular changes in CAKUT are mostly independent of the CAKUT etiology, which could explain the low prognostic value of ultrasound features with respect to postnatal renal outcome[4,5]. In contrast, the excellent prognostic capacity of the signature potentially resides in the fact that early molecular modifications (i.e. changes in AF peptide abundance) precede structural and functional changes. We have recently observed that the presence of specific urinary collagen peptides is related to the degree of in situ kidney fibrosis in adult CKD[25] and that these peptides are prognostic of disease progression[26]. Similarly, we speculate that a focus on the AF peptides may allow assessing the early underlying molecular changes of CAKUT such as connective tissue turnover (collagen fragments) leading to hypo/dysplasia and hyperechogenicity, inflammation (osteopontin, inter α trypsin inhibitor heavy chain H4) and repair (thymosin β4). To further validate the link between these

AF peptides and the severity of CAKUT we investigated in more detail thymosin β4 which showed the strongest association to the severity of CAKUT after the collagen peptides. Thymosin β4 is a small peptide of 43 amino acids with a M_w of ~5 kDa. It is freely filtered by the glomerulus[27] and can be metabolized into shorter peptides like AC-SDKP by meprin-α and prolyl oligopeptidase enzymes mainly localized in proximal tubules[28]. It has already been shown that thymosin β4 invalidation does not lead to a detectable renal phenotype in adult mice[29]. However challenging those mice with nephrotoxic serum led to a more severe renal phenotype than in wild-type mice and, conversely, administration of exogenous thymosin β4 improves animal models of unilateral ureteral obstruction and diabetic nephropathy[30,31]. Thymosin β4 thus appears to have a protective, but non-essential, role in the adult kidney. In contrast, the role of thymosin β4 in the early steps of renal development has never been investigated. Here, we first confirmed the increased thymosin β4 abundance in the AF of severe CAKUT patients by ELISA and the presence of thymosin β4 in the developing human normal and CAKUT kidney. Next we demonstrated that invalidation of thymosin β4 lead to modification of development of proximal and distal tubules in the zebrafish pronephros, thereby demonstrating a possible role of thymosin β4 in fetal kidney development and therefore potentially observed in AF fluid of CAKUT patients with a severe phenotype. Whether the changes in proximal tubule and distal length result from an alteration of tubular shape or from a modification of cell proliferation/differentiation remains to be determined. Although this pronephros phenotype opposes the absence of a phenotype in adult thymosin β4 knockout mice[29], it is possible that alteration of early renal development that we observed in the embryonic zebrafish has no major impact on the establishment of the terminal kidney in adults, but can make the kidney hypersensitive to external aggression during pregnancy (hypertension, diabetes, a nephrotoxic challenge, ...) as observed in adult thymosin β4 knockout animals[29].

A limitation for clinical implementation of the bCAKUTPep signature might be that the analysis is mass spectrometry-based. However, such approach is indispensable since it is currently impossible to

simultaneously analyze 98 peptides using immunological detection. In addition, we have shown in previous studies that samples can be frozen in the clinic, shipped and analyzed in specialized laboratories equipped with CE-MS technology[32,33] with a total turnaround time of less than one week, an acceptable timeframe for clinical decision-making in CAKUT pregnancies. In terms of regulatory support, the CE-MS technology for body fluid peptide profiling was supported by the FDA for a postnatal application[11]. The next challenge will be to develop new tools as specific as the CE-MS technology, but as easy to use clinically as ELISA, to quantify biomarker peptides with high prognostic value such as thymosin β4 fragments (**Figure 6A and B**) in the AF of CAKUT patients. In this context, we believe that an aptamer strategy could provide such tools for a limited set of peptides[34,35]. Another limitation is that compared to ultrasound, AF sampling is invasive. However, recent studies show that the increased risk for miscarriage and stillbirth in women undergoing amniocentesis is almost solely due to the fact that this procedure is exclusively performed in at risk pregnancies[36,37]. We also did not identify adverse outcomes due to AF sampling in the current study. Therefore, the added value of accurate prediction of postnatal renal survival by the AF signature appears to outweigh the invasive procedure associated to AF sampling. Finally, the last limitation of the study is the fact that almost all patients with compromised outcome in the validation cohort (15 out of 17) presented very severe phenotypes, either perinatal death due to ESRD or severely damaged kidneys as assessed by fetopathology after TOP. It would be important to validate the signature on another CAKUT cohort including a larger proportion of patients with less severe postnatal renal dysfunction.

In conclusion, based on an objective numerical score, the introduction of the bCAKUTPep signature in the diagnostic workup of prenatally detected CAKUT provides a long-sought evidence base for rational management of the challenging CAKUT disorder that is currently unavailable.

Methods

Study design and patients

We performed a prospective multicenter observational study. Patient inclusion started on January 2011 and ended on September 2014. Follow up was continued until December 2016. Two-hundred women consented to participate in the study, including 178 originally identified as having a pregnancy with a fetus presenting bilateral CAKUT (**Figure 1, Supplementary Methods**) and 22 from non-CAKUT pregnancies (**Supplementary Methods**). Inclusion criteria for bilateral CAKUT were a singleton pregnancy with bilateral developmental nephropathies with or without urinary tract malformations and a signed consent form. Kidney lesions on ultrasound were defined by hypoplasia (<2 SD) or hyperplasia (>2SD) and/or hyperechogenicity and/or the presence of cysts and/or reduced or absence of corticomedullary differentiation. Non-inclusion criteria were urinary tract anomalies without bilateral involvement of the kidneys, syndromic CAKUT, chronic (VIH, hepatitis B or C) or acute (e.g. chorioamnionitis) infection and refusal of the parents to participate in the study. During follow-up of the 178 CAKUT patients, 34 pregnancies were excluded (**Figure 1, Supplementary Methods**). The trial was performed in accordance with the Declaration of Helsinki[38] and with Good Clinical Practice guidelines. The study endpoint was the renal status after 2 years of postnatal follow-up, obtained in each center for all patients. For live born children, renal function was estimated at 2 years of life using serum creatinine concentrations according the Schwartz method[39]. A fetopathological analysis was performed to confirm the severity of renal damage in case of perinatal death or in case of TOP. The research was approved by national ethics committees (N° RCB 2010-AO1151-38, France and S 55406 and B32220096569, Belgium) and informed consent was obtained from each participant.

Ultrasound observations

Ultrasound observations obtained within 2 weeks of amniotic fluid sampling (see **Supplementary Tables S1** and **S3-5** for the gestational age at sampling) leading to the presence of specific ultrasound annotations were the following: i) Oligohydramnios/anhydramnios: amniotic fluid (AF) volume was estimated using the amniotic fluid index (AFI) which is defined as the sum of the largest vertical fluid pocket in each of the four quadrants of the maternal abdomen. An AFI of <5 indicated the presence of oligohydramnios, an AFI of ~0 was considered as absence of AF (anhydramnios); ii) Hyperechogenicity was defined by a right kidney brighter than the liver and a left kidney brighter than the spleen; iii) Dysplasia was defined by the absence or reduced corticomedullary differentiation, iv) Hypoplasia was defined by a kidney size of <2SD and, v) Hyperplasia was defined by a kidney size of >2SD.

Fetopathology after termination of pregnancy

Post-TOP fetopathology was reviewed and assessed by 3 independent pathologists for attribution of a severity renal score: HS, high severity, defined by extensive dysplasia and/or hypoplasia; S, severe, segmental dysplasia and/or hypoplasia with alternations between healthy and pathological areas; LS, low severity, corresponding to kidneys with nearly normal parenchyma or little segmental dysplasia and/or hypoplasia. Dysplasia was defined by alteration of the renal structure with both glomerular and tubular lesions, persistence of primitive medullar tubules surrounded by fibromuscular cells and cartilaginous islets; hypoplasia was histologically defined by a reduction of structurally normal nephron number (**Supplementary Tables S1** and **S3-5** (column "Renal severity in case of TOP")). At least one HS score without any LS score was interpreted as fetuses with renal lesions incompatible with normal life. At least two LS scores without any HS score was interpreted as compatible with normal life. All other combinations of scores or absence of fetopathology data were considered as inconclusive.

Training and validation sets

The prospective multicenter cohort of 104 bilateral CAKUT fetuses for which we had definite endpoint data was divided in independent training and validation sets (**Supplementary Tables S1 and S3**) for developing and then testing the signature performances, respectively. To be free from potential unintentional inter center differences in both eligibility criteria and outcome, we tried to distribute patients from each center (except for Belgium, see below) in both the training and validation sets. The two sets were balanced in terms of gender, gestational age at sampling, AF volume, postnatal renal outcome and antenatal etiology (**Supplementary Table S7**). All patients from Belgium (n=12) were excluded from the training set in order to evaluate the model performance in a small subset of patients enrolled in another country (geographical validation). The 22 healthy fetuses from pregnancies of healthy women were included together with 47 fetuses with primary maternal CMV infection[14] in another independent set in order to perform a validation in individuals having a very different clinical status than CAKUT (domain validation).

Sample collection and AF peptidome and ELISA analysis

Details can be found in the **Supplementary Methods**.

Adherence to guidelines and data and materials availability

For the entire study we aimed to adhere to "Transparent reporting of a multivariable prediction model" (TRIPOD[40]). Details of adherence to these guidelines can be found in the **Supplementary Materials**.

Immunohistochemistry of human fetal kidneys

Details can be found in the **Supplementary Methods**.

Zebrafish studies

Ethics Statement and Embryos. All embryos were handled according to relevant national and international guidelines. French veterinary service and national ethical committee approved the protocols used in this study, with approval ID: A-31-555-01 and APAPHIS #3653-2016011512005922v6.

Fish were maintained at the CBD-CBI zebrafish facility in accordance with the rules and protocols in place. Embryos were obtained through natural crosses and staged according to[41]. Details on the generation of tmsb4-/- mutants are available in the **Supplementary Methods**.

Disclosure statement

P. Zürbig and P. Magalhães are employees of Mosaiques-Diagnostics GmbH. H. Mischak is the co-founder and co-owner of Mosaiques-Diagnostics GmbH. The other authors declare no conflict of interest.

Supplementary Material

- **Additional BIOMAN consortium collaborators**
- **Supplementary Methods**
- **Tripod Checklist: prediction model development and validation**
- **Supplementary Figure S1. The 1008 sequenced amniotic fluid peptides cluster mostly on postnatal outcome and not on antenatal CAKUT etiology**. A dendrogram representation of the ascending hierarchical classification analysis using peptide abundance. Individuals who are close in the dendrogram are the individuals with a similar peptide signature. This clustering analysis of CAKUT patients based on of amniotic fluid peptide abundance separates patient groups better based on **A)** postnatal renal function than on **B)** antenatal disease etiology.
- **Supplementary Figure S2. Generation of thymosin β4 knockout zebrafish (tmsb4-/-). A)** Structure of the tmsb4 wild type gene, with location of the sgRNA targeted mutation containing a XhoI cleavage site. **B)** The mutation in tmsb4-/- F4 embryo (loss of XhoI cleavage site) was demonstrated DNA by PCR using T1005 (5'-TTGTCTCTCTGTCTGCATGC-3') and T1006 (5'-ATGAACCCGCCTGCTTCTCC-3') primers. **C)** Sequencing of tmsb4+/+ DNA. **D)** Sequencing of tmsb4-/- DNA showing the 5bp deletion (including the XhoI site) and the introduction of a stop codon. Green underline: line quality > 30; yellow underline: line quality 20-29.
- **Supplementary Table S1: Clinical data in bilateral CAKUT fetuses from the training set.**
- **Supplementary Table S2: List of 98 peptides associated to CAKUT progression.**
- **Supplementary Table S3: Clinical data in bilateral CAKUT fetuses from the independent validation set.**

- **Supplementary Table S4: Clinical data in other bilateral CAKUT fetuses displaying inconclusive or not available fetopathology.**
- **Supplementary Table S5: Clinical data in other bilateral CAKUT fetuses displaying a renal phenotype type that was compatible with life.**
- **Supplementary Table S6: Analytical variability of the bCAKUTPep scoring.**
- **Supplementary Table S7: Characteristics of CAKUT patients in the training and validation sets.**

Supplementary information is available at Kidney International's website

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Table 1. Antenatal cohort characteristics of 140 CAKUT patients of which the amniotic fluid peptidome was analyzed.

		N	Gender	Gestational age	Amniotic fluid	Outcome[γ]		
			(f/m)[¶]	(w)[¥]	(n.a, n, o, a)[§]	GFR>60	GFR<60 or death	TOP
Total cases		140	40/82	25.68 +/- 0.50	18, 68, 42, 12	69	11	60
Bilateral hyperechogenic kidneys								
	normal size	17	4/12	26.06 +/- 1.29	2, 12, 3, 0	10	0	7
	enlarged	23	14/8	27.35 +/- 1.30	4, 12, 7, 0	10	2	11
Lower urinary tract obstruction								
	PUV	25	0/20	24.64 +/- 1.40	3, 3, 16, 3	5	4	16
	others	4	1/2	17.50+/- 1.19	1, 3, 0, 0	1	0	3
Abnormal solitary kidney*								
	agenesis	9	2/7	27.44 +/- 1.40	3, 2, 3, 1	4	1	4
	MCDK	12	6/5	28.17 +/- 1.11	1, 8, 3, 0	8	1	3
Upper urinary tract obstruction**		17	1/11	25.12 +/- 1.15	2, 14, 1, 0	15	1	1
Bilateral hypoplasia		10	4/6	25.00 +/- 1.56	1, 4, 4, 1	5	0	5
Nonfunctioning kidneys***		8	3/5	20.75 +/- 1.77	0, 0, 1, 7	0	0	8
Non obstructive urinary tract anomalies****		7	2/1	23.43 +/- 2.16	0, 6, 1, 0	7	0	0
Bilateral dysplasia		5	1/4	28.80 +/- 2.82	1, 3, 1, 0	3	1	1
One hypoplastic and one dysplastic kidney		3	2/1	33.67 +/- 2.73	0, 1, 2, 0	1	1	1

* One nonfunctional (agenesis or multicystic dysplastic kidney (MCDK)) kidney and one kidney with either ureteropelvic junction obstruction (UPJ) with parenchymal lesions or dysplasia or hypoplasia or hyperechogenecity or combinations thereof; ** Bilateral UPJ with bilateral parenchymal lesions; *** Bilateral agenesis or MCDK; **** Vesicoureteral reflux, duplex collecting system, megaureter; [¶] Gender of fetus, female/male (18 missing values); [¥] Gestational age plus or minus standard error in weeks; [§] Amniotic fluid volume: n.a, not available; n, normal; o, oligoamnios; a, anhydramnios; [γ] Postnatal pregnancy outcome at two years of age: GFR>60, normal renal function or moderately reduced renal function (eGFR>60 ml/min/1.73m^2); GFR<60 or death, eGFR<60 ml/min/1.73m^2 or death due to renal dysfunction; TOP, termination of pregnancy; Abbreviation: PUV, posterior urethral valves.

Figure legends

Figure 1. Participant flow in the prospective multicenter fetal bilateral CAKUT study (clinicaltrials.gov NCT02675686) specifically designed for the evaluation of the added value of AF peptides in the prediction of postnatal renal survival.

Figure 2. The 1008 sequenced amniotic fluid peptides cluster mostly on postnatal outcome and not on antenatal CAKUT etiology. The multidimensional scaling distributes patients on a plan using two coordinates (1 and 2). These two coordinates are calculated in order to best represent the distances between all individuals based on the peptide abundance. This graphical representation of CAKUT patients based on amniotic fluid peptide abundance separates patient groups better based on based on **A)** postnatal renal function than on **B)** antenatal disease etiology.

Figure 3. Identification of amniotic fluid peptides prognostic of postnatal renal outcome in bilateral developmental renal disease. A) Identities of the 98 AF peptides that displayed a significantly different abundance between 35 CAKUT fetuses with normal postnatal renal outcome (eGFR>60 ml/min/1.73m2 at age 2 years) and 18 CAKUT fetuses with compromised renal outcome (eGFR<60 ml/min/1.73m2 at age 2 years, perinatal death due to ESRD, or TOP with fetopathology showing severe renal maldevelopment) from the training cohort. **B)** ROC curve of performance of the bCAKUTPep signature based on the random forest mathematical combination of the 98 peptides in the training cohort of 53 CAKUT patients. Confidence intervals given in brackets for AUC are two-sided 95%CI. **C)** Scores of the bCAKUTPep signature in the training cohort of 53 CAKUT patients. The dotted horizontal line indicates the cutoff score of 0.47, which allowed to better discriminate CAKUT patients with normal outcome and CAKUT fetuses with

compromised renal outcome. A bCAKUTPep score above the 0.47 cutoff predicts severely compromised postnatal renal outcome. The abscissa indicates the clinical end-point at 2 years; ****, P < 0.0001, Mann-Whitney test for independent samples; Data are means plus or minus standard errors; normal, normal or moderately reduced postnatal renal function (eGFR>60 ml/min/1.73m^2 at age 2 years); compromised, eGFR<60 ml/min/1.73m^2 at age 2 years, perinatal death due to ESRD, or TOP with fetopathology showing severe renal maldevelopment.

Figure 4. Validation of the performance and comparison to clinical parameters of the AF peptide signature. A) ROC curve of the bCAKUTPep signature in the validation cohort of 51 additional CAKUT patients. Confidence intervals given in brackets for AUC are two-sided 95%CI. **B)** Scores of the bCAKUTPep signature in the validation cohort of 51 CAKUT patients. ****, P < 0.0001, Mann-Whitney test for independent samples; Data are means plus or minus standard errors; normal, normal or moderately reduced postnatal renal function (eGFR>60 ml/min/1.73m^2 at age 2 years); compromised, an eGFR<60 ml/min/1.73m^2 at age 2 years, perinatal death due to ESRD, or TOP with fetopathology showing severe renal maldevelopment. Confidence intervals given in brackets for sensitivity and specificity are two-sided 95%CI. **C)** ROC curve of the bCAKUTPep signature compared to clinical parameters or to its combination with those clinical parameters in the validation hold-out cohort of 51 CAKUT patients. Age, gestational age at AF sampling; AF, amniotic fluid volume; bCAKUTPep-Age, combination of the bCAKUTPep signature with gestational age at sampling; bCAKUTPep-AF, combination of the bCAKUTPep signature with AF volume; bCAKUTPep-AF/Age, combination of the bCAKUTPep signature with both gestational age at sampling and AF volume. * P(one-sided)=0.03 and ***P(one-sided)=0.001 versus bCAKUTPep using Hanley's method for comparing the areas under receiver operating characteristic curves derived from the same cases. Confidence intervals given in brackets for AUC are two-sided 95%CI. **D)** Prediction of postnatal renal outcome by the bCAKUTPep signature of 28 TOPs in CAKUT pregnancies where fetopathology was

inconclusive or not available. **E)** Prediction of postnatal renal outcome by the bCAKUTPep signature of 8 termination of pregnancies (TOPs) in bilateral CAKUT pregnancies where fetopathology, analyzed by three independent pathologists, displayed a renal phenotype type that appeared compatible with normal life. A bCAKUTPep score above the 0.47 cutoff suggests severely compromised postnatal renal outcome.

Figure 5. Robustness and wider application of the signature. **A)** ROC curves in the validation set of 51 CAKUT patients of the 98 peptides combined in different mathematical models. SVM, a support vector machine model; Linear, a linear model; KNN, a k-nearest neighbors model. **B)** ROC curves for the geographical validation of the bCAKUTPep signature. All patients, all patients in the validation set; Belgium patients, 12 patients from the validation set with a distinct geographical origin; All minus Belgium patients, the validation set without the Belgium patients. **C**) Domain validation using 22 healthy fetuses and 47 fetuses with primary maternal CMV infection displaying neurological lesions [14]. The dotted horizontal line indicates the cutoff score of 0.47, previously defined in the training cohort using the random forest mathematical combination of the 98 peptides, above which a patient is predicted to display severely altered postnatal renal outcome. Confidence intervals given in brackets for AUC and specificity are two-sided 95%CI.

Figure 6. Thymosin β4 is associated to the severity of CAKUT. A and B) Validation of the 3 thymosin β4 peptide based-model in the validation cohort of 51 additional CAKUT patients showing similar sensitivity and specificity and an AUC close to the full bCAKUTPep signature composed of 98 peptides. **C)** Enzyme-linked immunosorbent assay analysis (ELISA) of Thymosin β4 in AF of a subset of CAKUT patients. The abscissa indicates the clinical end-point at 2 years; ****P < 0.0001, Mann-Whitney test for independent samples; Data are means plus or minus standard errors; normal, normal or moderately reduced postnatal renal function (eGFR>60 ml/min/1.73m^2 at age 2 years); compromised, eGFR<60 ml/min/1.73m2 at age

2 years, perinatal death due to ESRD, or TOP with fetopathology showing severe renal maldevelopment. **D-I**) Tubular expression of thymosin β4 in human fetal kidney. One healthy (**D-F**: increasing magnification) and 3 different CAKUT (G, H, I: same magnification) fetal kidney sections were stained with a thymosin β4 antibody. The antenatal diagnosis for CAKUT patients was **G)** hypoplastic dysplastic kidney, **H)** hyperechogenic dysplastic kidney and **I)** MCDK. Staining is mostly present in tubules.

Figure 7. Effects of thymosin β4 invalidation in the zebrafish pronephros. Representative pictures of *tmsb4+/+* and *tmsb4-/-* zebrafish embryos (48hpf) subjected to whole mount *in situ* hybridization using antisens DIG-labelled probes [*wt1a* (podocyte), *slc20a1a* (proximal) and *slc12a3* (distal)]. Labeled perimeter of *wt1a* (**A, A'**) and labeled length of *slc20a1a* (**B, B'**) and *slc12a3* (**C, C'**) (arrowhead) in *tmsb4+/+* and *tmsb4-/- (respectively)*, were quantified (**A'', B'', C''**). ** P<0.01, *P<0.05, ns: non-specific (Unpaired t-test with Welch's correction), data are means plus or minus standard errors.

Figure 1

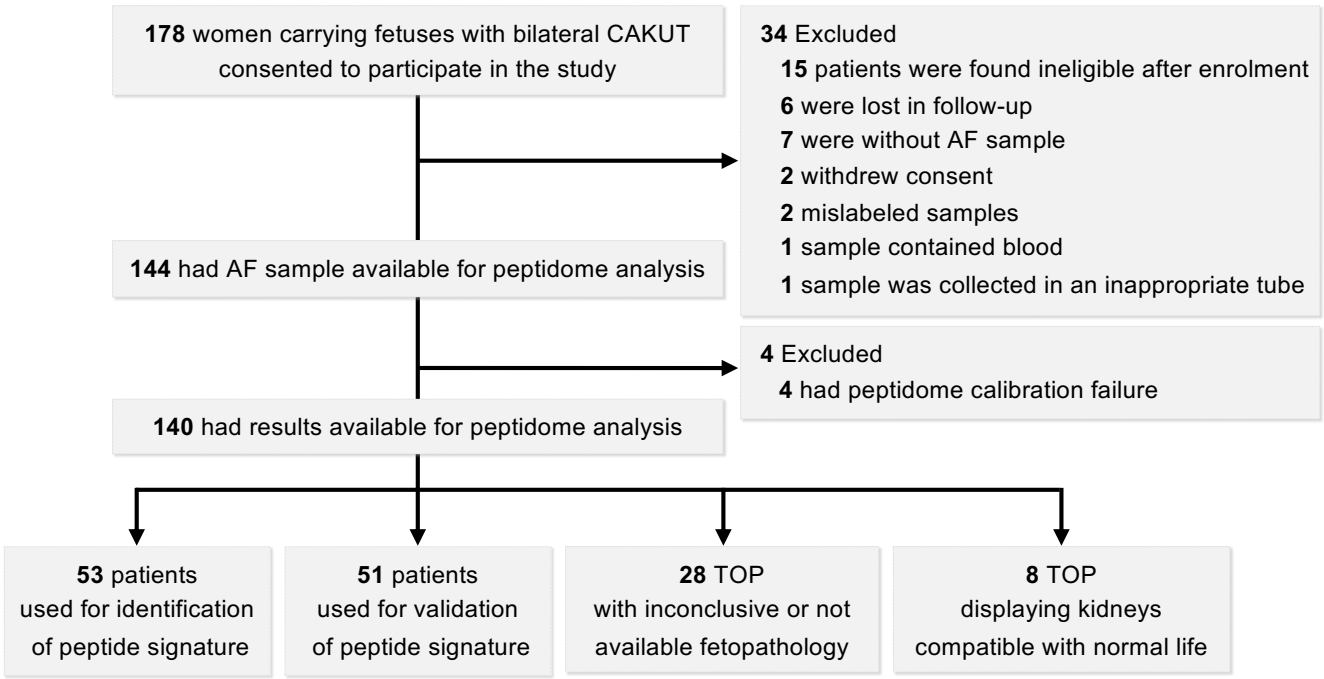
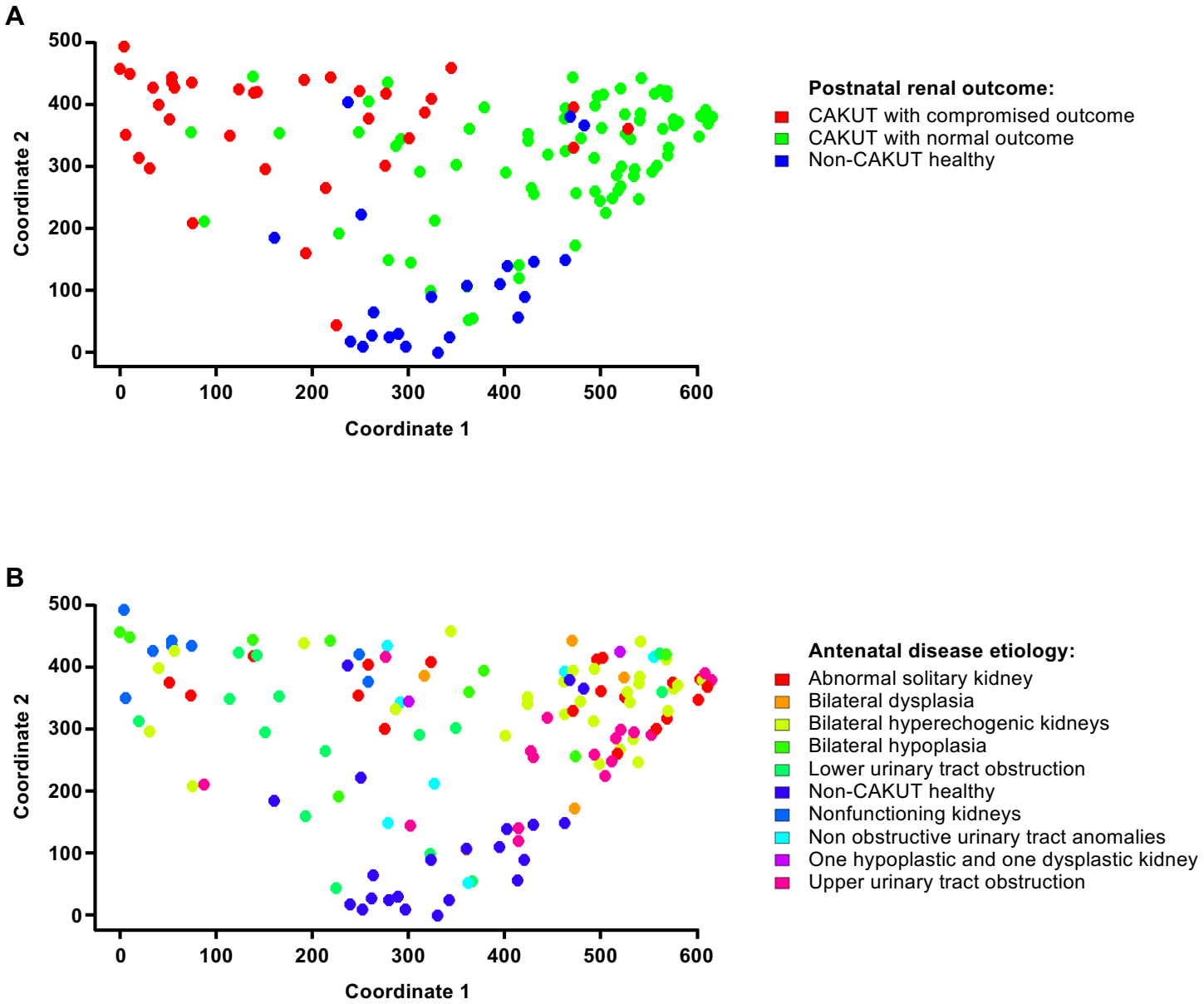

178 women carrying fetuses with bilateral CAKUT consented to participate in the study
34 Excluded
15 patients were found ineligible after enrolment
6 were lost in follow-up
7 were without AF sample
2 withdrew consent
2 mislabeled samples
1 sample contained blood
1 sample was collected in an inappropriate tube
144 had AF sample available for peptidome analysis
4 Excluded
4 had peptidome calibration failure
140 had results available for peptidome analysis
53 patients used for identification of peptide signature
51 patients used for validation of peptide signature
28 TOP with inconclusive or not available fetopathology
8 TOP displaying kidneys compatible with normal life

Figure 2


A
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Coordinate 1
Postnatal renal outcome:
CAKUT with compromised outcome
CAKUT with normal outcome
Non-CAKUT healthy
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Coordinate 2
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Coordinate 1
Antenatal disease etiology:
Abnormal solitary kidney
Bilateral dysplasia
Bilateral hyperechogenic kidneys
Bilateral hypoplasia
Lower urinary tract obstruction
Non-CAKUT healthy
Nonfunctioning kidneys
Non obstructive urinary tract anomalies
One hypoplastic and one dysplastic kidney
Upper urinary tract obstruction

Figure 3

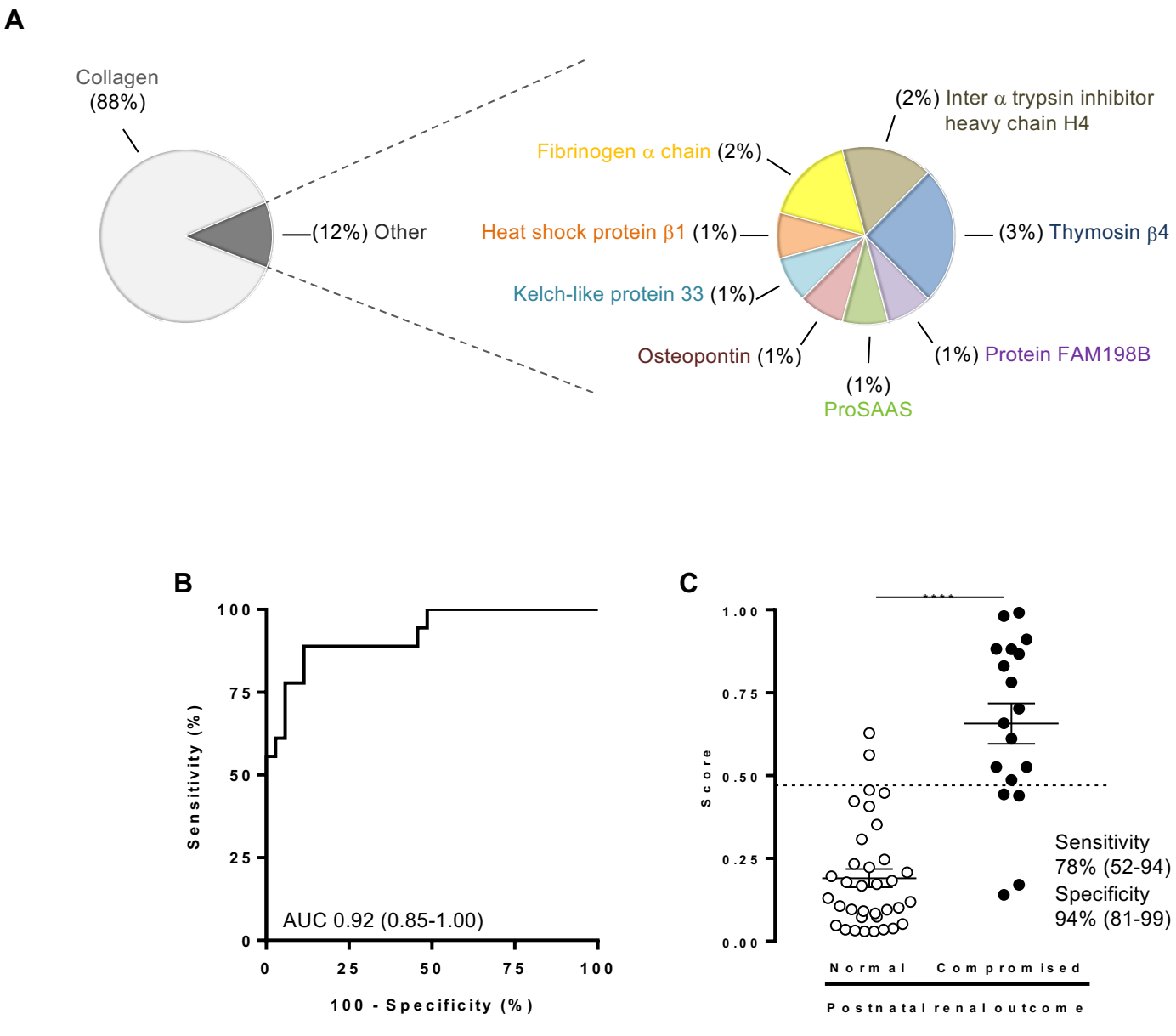

A
Collagen
(88%)
(12%) Other
(2%) Inter α trypsin inhibitor
heavy chain H4
Fibrinogen α chain (2%)
Heat shock protein β1 (1%)
(3%) Thymosin β4
Kelch-like protein 33 (1%)
Osteopontin (1%)
(1%) Protein FAM198B
(1%)
ProSAAS
B
Sensitivity (%)
100
75
50
25
0
0
25
50
75
100
100 - Specificity (%)
AUC 0.92 (0.85-1.00)
C
Score
1.00
0.75
0.50
0.25
0.00
****
Normal
Compromised
Postnatal renal outcome
Sensitivity
78% (52-94)
Specificity
94% (81-99)

Figure 4

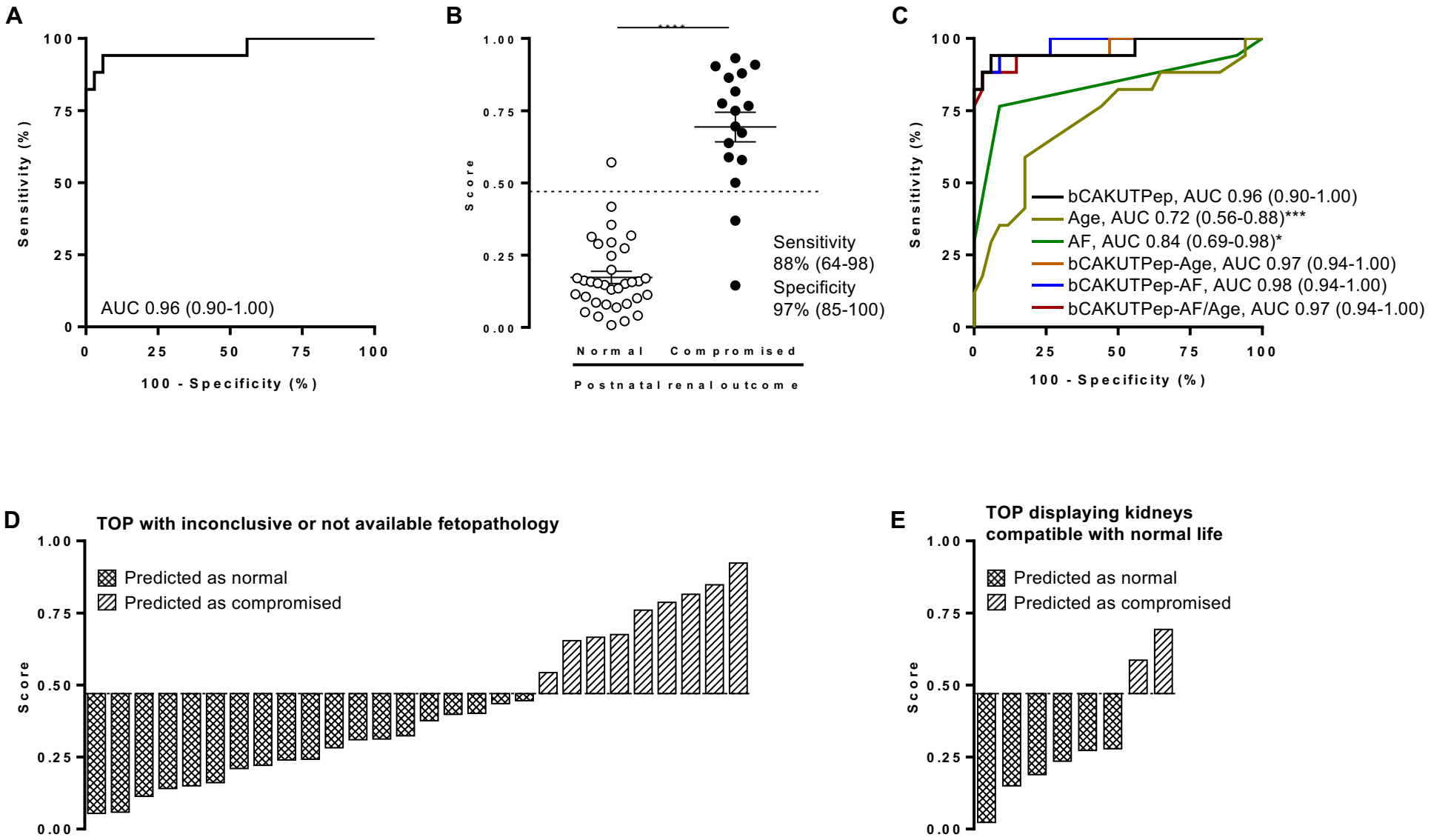
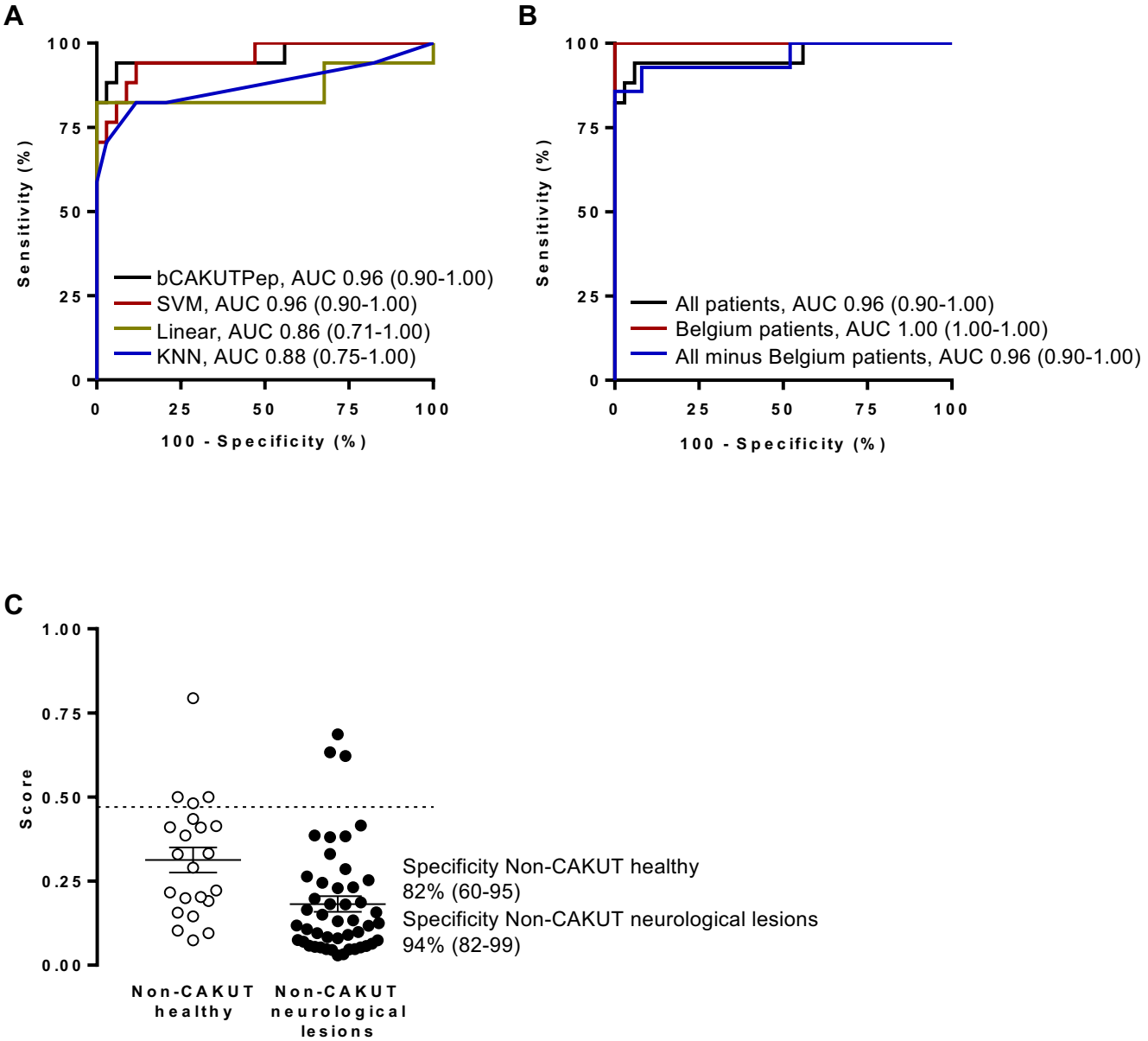

A
Sensitivity (%)
100 - Specificity (%)
AUC 0.96 (0.90-1.00)
B
Score
Normal
Compromised
Postnatal renal outcome
****
Sensitivity
88% (64-98)
Specificity
97% (85-100)
C
Sensitivity (%)
100 - Specificity (%)
bCAKUTPep, AUC 0.96 (0.90-1.00)
Age, AUC 0.72 (0.56-0.88)***
AF, AUC 0.84 (0.69-0.98)*
bCAKUTPep-Age, AUC 0.97 (0.94-1.00)
bCAKUTPep-AF, AUC 0.98 (0.94-1.00)
bCAKUTPep-AF/Age, AUC 0.97 (0.94-1.00)
D
TOP with inconclusive or not available fetopathology
Predicted as normal
Predicted as compromised
Score
E
TOP displaying kidneys compatible with normal life
Predicted as normal
Predicted as compromised
Score

Figure 5


A
100
75
50
25
0
Sensitivity (%)
0
25
50
75
100
100 - Specificity (%)
bCAKUTPep, AUC 0.96 (0.90-1.00)
SVM, AUC 0.96 (0.90-1.00)
Linear, AUC 0.86 (0.71-1.00)
KNN, AUC 0.88 (0.75-1.00)
B
100
75
50
25
0
Sensitivity (%)
0
25
50
75
100
100 - Specificity (%)
All patients, AUC 0.96 (0.90-1.00)
Belgium patients, AUC 1.00 (1.00-1.00)
All minus Belgium patients, AUC 0.96 (0.90-1.00)
C
1.00
0.75
0.50
0.25
0.00
Score
Non-CAKUT healthy
Non-CAKUT neurological lesions
Specificity Non-CAKUT healthy
82% (60-95)
Specificity Non-CAKUT neurological lesions
94% (82-99)

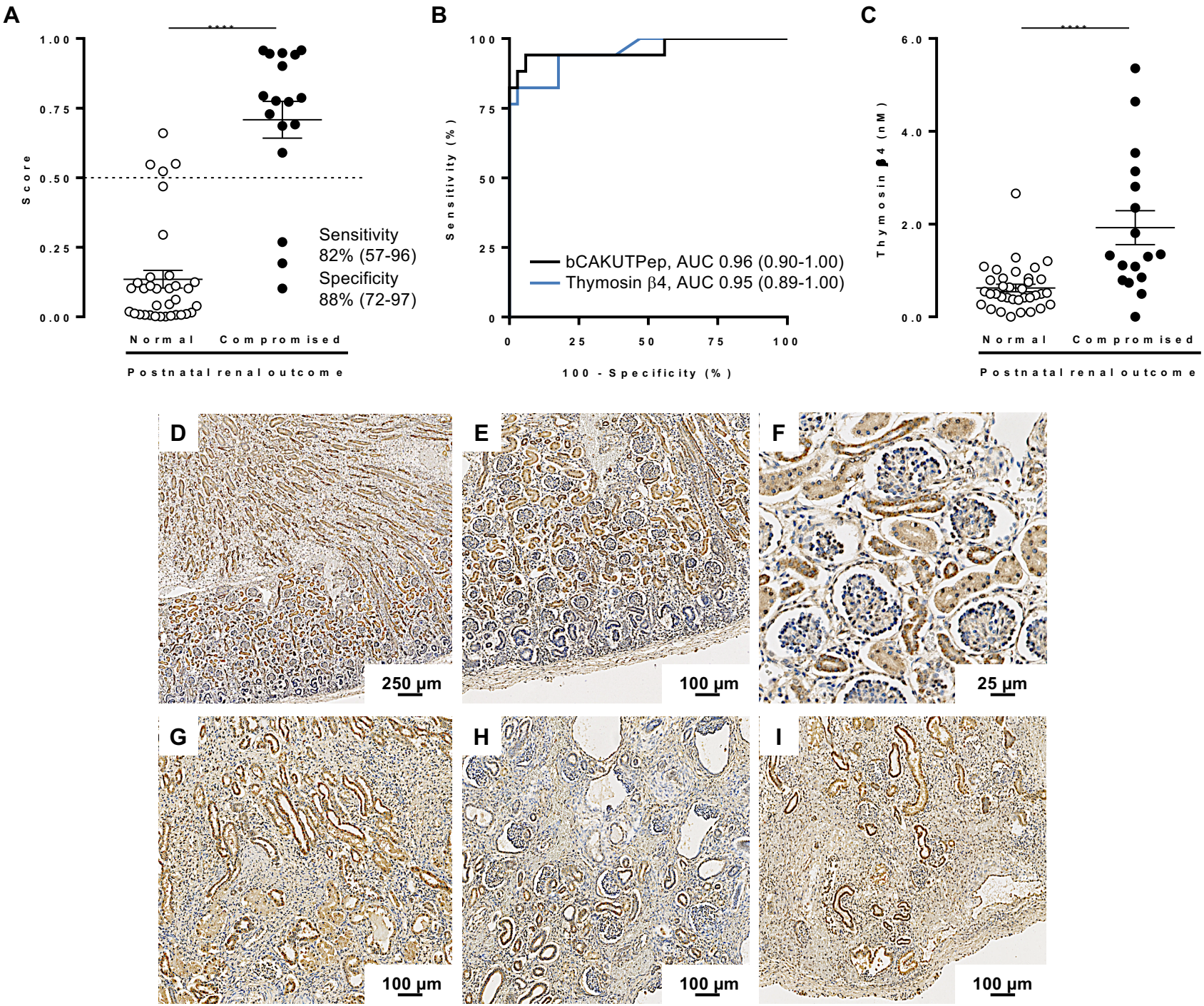

A
B
C
****
Score
1.00
0.75
0.50
0.25
0.00
Sensitivity
82% (57-96)
Specificity
88% (72-97)
Normal
Compromised
Postnatal renal outcome
Sensitivity (%)
100
75
50
25
0
0
25
50
75
100
100 - Specificity (%)
bCAKUTPep, AUC 0.96 (0.90-1.00)
Thymosin β4, AUC 0.95 (0.89-1.00)
Thymosin β4 (nM)
6.0
4.0
2.0
0.0
Normal
Compromised
Postnatal renal outcome
D
E
F
250 µm
100 µm
25 µm
G
H
I
100 µm
100 µm
100 µm

Figure 6

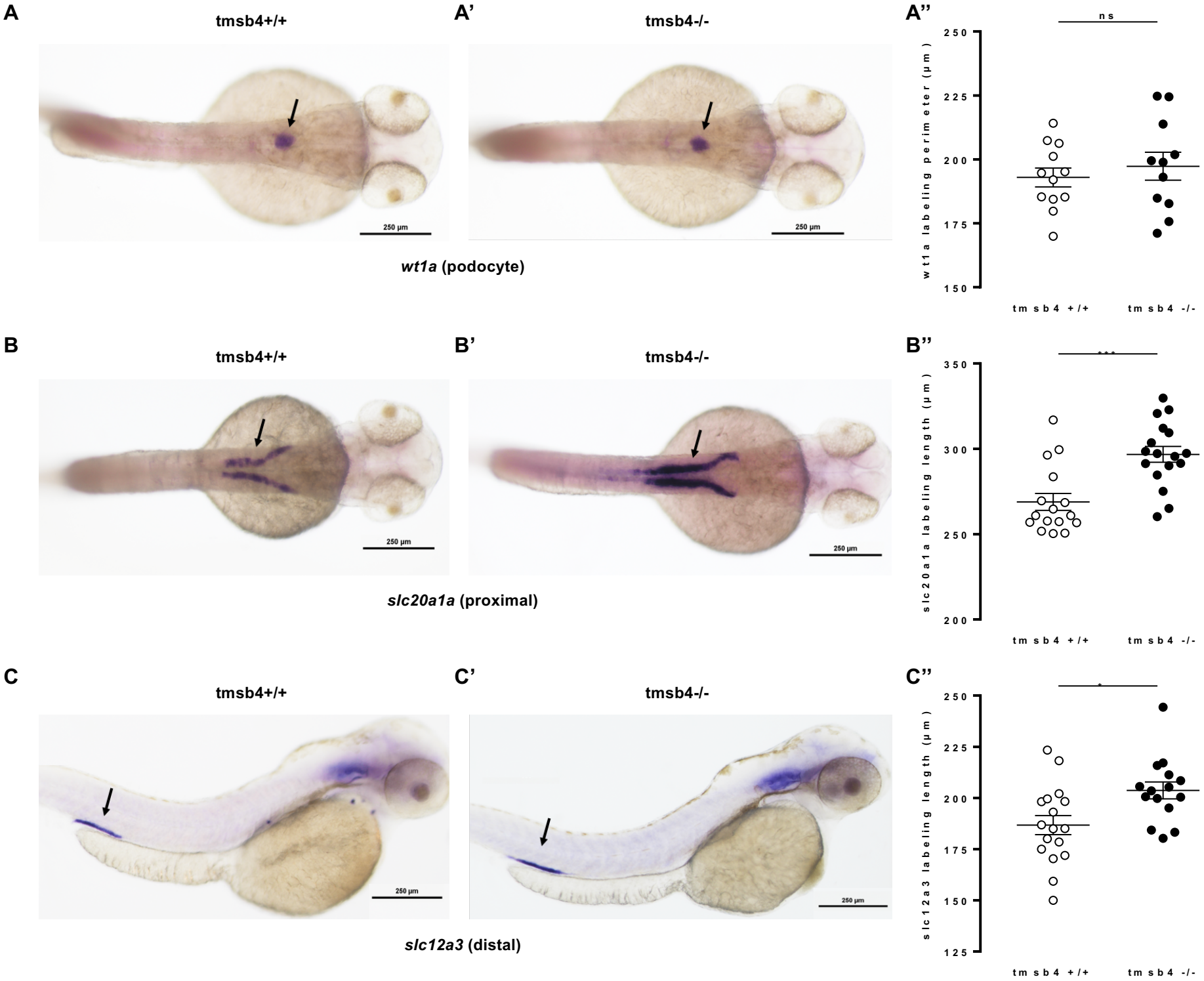

A
tmsb4+/+
A'
tmsb4-/-
A''
ns
wt1a labeling perimeter (µm)
250
225
200
175
150
tmsb4 +/+
tmsb4 -/-
250 µm
wt1a (podocyte)
B
tmsb4+/+
B'
tmsb4-/-
B''
***
slc20a1a labeling length (µm)
350
300
250
200
tmsb4 +/+
tmsb4 -/-
250 µm
slc20a1a (proximal)
C
tmsb4+/+
C'
tmsb4-/-
C''
*
slc12a3 labeling length (µm)
250
225
200
175
150
125
tmsb4 +/+
tmsb4 -/-
250 µm
slc12a3 (distal)

Figure 7