TransBioLine
Translational Safety
Biomarker Pipeline

Innovative Medicines Initiative TransBioLine Drug-Induced Kidney Injury Work Package 1

Letter of Intent

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CONFIDENTIAL

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ABBREVIATIONS

AKI	Acute Kidney Injury
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
ATN	Acute Tubular Necrosis
aVEGFa	Anti-Vascular Endothelial Growth Factor Antibody
BUN	Blood Urea Nitrogen
CLU	Clusterin
CM	Composite Measure
CoU	Context of Use
DIKI	Drug-Induced Kidney Injury
eGFR	Estimated Glomerular Filtration Rate
IA	Interim Analysis
IMI	Innovative Medicines Initiative
IP-LC-MS/MS	Immunoprecipitation coupled to Nano-Liquid Chromatography and Tandem Mass Spectrometry
KIM-1	Kidney Injury Molecule 1
LDH	Lactate Dehydrogenase
LoI	Letter of Intent
LLOQ	Lower Limit of Quantitation
VEGF	Vascular Endothelial Growth Factor
NGB	Novel Glomerular Biomarkers
NHV	Normal Healthy Volunteers
OPN	Osteopontin
PIGF	Placental Growth Factor
QC	Quality Control
RNA	Ribonucleic acid
sFlt-1	Soluble Fms-Like Tyrosine Kinase-1
TMA	Thrombotic Microangiopathy
TransBioLine	Translational Safety Biomarker Pipeline
UPCR	Urine Total Protein/Creatinine Ratio
WP	Work Package

1 Administrative Information

1.1 Submission Title: Letter of Intent for the Qualification of Biomarkers of Drug-Induced Kidney Injury (DIKI)

1.2 Requesting Information:

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2 Introduction

TransBioLine is a fully funded ~30 MM Euro IMI-2 project with a remit to qualify biomarkers for drug-induced injury of critical organ systems, including the kidney. While biomarkers of kidney tubular injury have been extensively studied, qualified for nonclinical safety assessment, and qualified as a composite panel for heathy volunteers in phase 1 clinical trials (Troth et al, 2019a), data on the performance of biomarkers of drug-induced glomerular injury are largely lacking (Troth et al, 2019b).

We aim to identify hitherto unknown glomerular injury biomarkers (that can outperform serum creatinine, BUN and proteinuria) in patients at risk and demonstrate how glomerular specific safety biomarkers or a panel of such biomarkers can optimize insights into drug-induced kidney injury. We also aim to qualify sensitive biomarkers specific to the detection of subclinical (prior to alterations in serum creatinine, BUN, and progressive elevations of proteinuria) glomerular injury in the specified Context of Use (CoU) (see Section 5). To achieve these, we plan to conduct a series of nonclinical and clinical studies as listed in Table 2-1. These studies include 4 learning-phase nonclinical studies, 2 learning-phase clinical studies, and 2 confirmatory-phase clinical studies. The 4 learning-phase nonclinical studies are designed to identify, and characterize sensitivity and specificity of, novel glomerular biomarkers (NGBs) in two nonclinical species (rats and non-human primates). NGBs to be characterized include 12 candidate biomarkers (selected based on literature evidence showing their association with glomerular injury) and potentially additional biomarkers to be identified via an unbiased proteomics approach. These biomarkers will then be measured in two learning-phase clinical studies to establish thresholds defining glomerular injury. The learning-phase nonclinical and clinical studies will also help determine the most promising biomarkers, and/or establish a composite biomarker panel. These prioritized biomarkers, the composite panel, and their thresholds will be further evaluated and validated in the two confirmatory-phase clinical studies. In these confirmatory-phase clinical studies, we will recruit patients with glomerular injury either drug-induced or disease-induced as manifested by progressive proteinuria and impairment of kidney function. For disease-induced glomerular injury we will recruit patients with preeclampsia, and for drug-induced glomerular injury we will recruit oncology (colorectal and gastric) patients treated with inhibitors to vascular endothelial growth factor (VEGF).

Table 2-1 Overview of studies for glomerular injury biomarker identification and qualification

Study Type	Study Description
Learning-Phase Nonclinical Studies	i. A rat study to characterize ***sensitivity*** of NGBs and their correlation to histopathological lesions using glomerular toxicants (Section 8.1).
	ii. A Non-human primate study to characterize ***sensitivity*** of NGBs and their correlation to histopathological lesions using a glomerular toxicant (Section 8.2).
	iii. A rat study to characterize ***selectivity*** and ***specificity*** of the NGBs to glomerular injury by using model tubular toxicants (Section 8.3).
	iv. A non-human primate study to characterize ***selectivity*** and ***specificity*** of the NGBs to glomerular injury by using a model tubular toxicant (Section 8.4).
Learning-Phase Clinical Studies	i. A study in humans to measure NGBs from urine specimens collected in the Boston Kidney Biopsy Cohort Study, and to correlate them with functional end points as well as histopathologic lesions (Section 7.3.1).
	ii. A study in 120 normal healthy volunteer subjects to measure selected NGBs in urine and plasma collected longitudinally across 3 time points, to establish normal variability in urine and plasma levels of those biomarkers and to assess impact of gender and age on that variability (Section 7.3.1).
Confirmatory-Phase Clinical Studies	i. A longitudinal study in humans to test the sensitivity, specificity and thresholds of NGBs in patients with glomerular injury as a result of disease pathogenesis (preeclampsia) (Section 7.3.2.1).
	ii. A longitudinal study in humans to test the sensitivity, specificity and thresholds of NGBs in patients with drug-induced glomerular injury caused by inhibitors of vascular endothelial growth factor (VEGF) in oncology (colorectal and gastric) patients (Section 7.3.2.2).

3 Drug Development Need Statement

Specific and sensitive biomarkers of drug-induced glomerular injury will improve safe dose identification in cohorts or individuals in early clinical trials and enable the safe clinical progression of drug candidates with an *a priori* concern (see Section 5. Context of Use for *a prior* concern scenarios) for glomerular injury. While a panel of safety biomarkers are being qualified to aid in the detection of kidney tubular injury in early clinical trials in healthy volunteers and

subjects with normal renal function, no biomarkers of drug-induced glomerular injury have been qualified. Advantages of having glomerular biomarkers qualified include:

- Sensitive biomarkers which can specifically identify glomerular injury and differentiate glomerular from tubular injury, or those associated with both glomerular and tubular injuries, will fulfill an important drug development need and further improve patient safety.
- If DIKI begins with a glomerular insult that further progresses to tubular injury, then a biomarker strategy limited to measuring tubular injury biomarkers may delay diagnosis. The advantage of the strategy to detect a glomerular toxicant using glomerular-specific biomarkers could be an early and sensitive diagnosis of glomerular damage and subsequent kidney function decline.
- Yet another advantage of biomarkers, whose levels increase selectively as a result of glomerular damage in the absence of tubular injury, is that it provides mechanistic insights into the pathogenesis of kidney damage.

Recent pharmaceutical industry survey results (Troth et al, 2019b) indicate that when kidney injury is observed in drug development animal toxicology studies, the tubular component of the nephron is impacted approximately 75-80% of the time, while glomerular injury involvement is documented in 15-20% of cases (Troth et al, 2019b). While pathology effectively identifies early DIKI in animals, it is not feasible to use biopsy-based pathology assessment for routine monitoring in clinical studies because of the disease/tissue heterogeneity and its invasive nature that involves risks of bleeding and infection. Typically, blood-derived biomarkers such as serum creatinine and BUN, and urinary biomarkers such as albumin and total protein, are serially collected in early phase clinical studies to monitor for drug-induced glomerular injury. However, serum creatinine, the most widely used indicator of damage, is a marker associated with significant (~50%) loss of kidney function, and is delayed, insensitive and non-specific (Waikar et al, 2012). Blood and/or urine-based biomarkers that are sensitive, specific, mechanistic and correlate with severity of glomerular damage have the potential to dramatically improve the way we detect glomerular injury.

4 Biomarker Information and Interpretation

4.1 Biomarker name

Potential NGBs being investigated by the DIKI WP will include:

Glomerular podocyte component biomarkers for which analytical assays are either available or under active development (Kerley, 2018; Bounds, 2017):

Table 4-1 Potential novel drug-induced glomerular injury biomarkers (podocyte component)

	Biomarker Protein	Gene	Uniprot ID	Type
1	Nephrin	NPHS1	O60500	Molecular
2	Podocin	NPHS2	Q9NP85	Molecular
3	Synaptopodin	SYNPO	Q8N3V7	Molecular

Glomerular vascular component biomarkers for which analytical assays are either already available or under active development (Sani, 2019):

Table 4-2 Potential novel drug-induced glomerular injury biomarkers (vascular component)

	Biomarker Protein	Gene	Uniprot ID	Type
1	Angiopoietin 2	ANGP2	O15123	Molecular
2	Matrix metalloproteinase 3	MMP3	P08254	Molecular
3	Neutrophil gelatinase associated lipocalin	NGAL	P80188	Molecular
4	Vascular adhesion protein 1	VCAM1	P19320	Molecular
5	Caldesmon 1	CALD1	Q05682	Molecular
6	Calponin	CNN1	P51911	Molecular
7	Smoothelin isoform b variant	SMTNb	P53814	Molecular
8	P-selectin	SELP	P16109	Molecular
9	Thrombomodulin	THBD	P07204	Molecular

In addition, the DIKI WP will use a discovery proteomics-based strategy in nonclinical studies in both rats and monkeys to identify NGBs for which new assays will be developed. Plasma and urine samples will also be collected for future biomedical research.

4.2 Analytical methods

The initial NGB quantitation method will be immunoprecipitation coupled to nano-liquid chromatography and tandem mass spectrometry read-out (IP-LC-MS/MS).

First, plasma and urinary samples are enzymatically fragmented into peptides. Antibodies are employed to enrich peptides derived from the proteins of interest (see Table 4-3) which can be used to unambiguously identify the protein (proteotypic) (Gautier, 2016). Each protein will be

quantified by adding $^{13}C/^{15}N$ -labelled peptide standards to the fragmented sample. The peptides are measured as protein surrogates by parallel reaction monitoring mass spectrometry. The quantification is achieved by stable isotope dilution (internal $^{13}C/^{15}N$-labelled peptide standard). The readout for the potential protein biomarkers is performed using an orbitrap mass analyzer (QExactive plus, ThermoFisher). Peptide quantification will be based on the calibration curve obtained by peak area ratio (analyte/ internal standard) using a logistic regression model. Protein concentrations are calculated by converting peptide amount (fmol) into ng ml^{-1} and normalized to creatinine (for urine biomarkers) considering the molecular weight of the corresponding protein.

Table 4-3 Assay methods and readout per biomarker

	Biomarker Protein	Gene	Uniprot ID	Matrix	Read Out
1	Nephrin	NPHS1	O60500	Urine	IP-LC-MS/MS
2	Podocin	NPHS2	Q9NP85	Urine	IP-LC-MS/MS
3	Synaptopodin	SYNPO	Q8N3V7	Urine	IP-LC-MS/MS
4	Angiopoietin 2	ANGP2	O15123	Urine	IP-LC-MS/MS
5	Matrix metalloproteinase 3	MMP3	P08254	Urine	IP-LC-MS/MS
6	Neutrophil gelatinase associated lipocalin	NGAL	P80188	Urine	IP-LC-MS/MS
7	Vascular adhesion protein 1	VCAM1	P19320	Urine	IP-LC-MS/MS
8	Caldesmon 1	CALD1	Q05682	Urine	IP-LC-MS/MS
9	Calponin	CNN1	P51911	Urine	IP-LC-MS/MS
10	Smoothelin isoform b variant	SMTNb	P53814	Urine	IP-LC-MS/MS
11	P-selectin	SELP	P16109	Urine	IP-LC-MS/MS
12	Thrombomodulin	THBD	P07204	Urine	IP-LC-MS/MS

4.3 Measurement units and limit(s) of detection

Results will be provided in fmol μl^{-1} and ng ml^{-1}. Since protein standards are not available yet or will not be available due to technical limitation, Lower Limit of Quantitation (LLOQs) are given fmol μl^{-1} referring to the peptide species used as protein surrogate. LLOQs were developed by Signatope in collaboration with the Predictive Safety Testing Consortium.

Note: LLOQ of DIKI markers might deviate, since to date these values have been determined for rat plasma and not human urine.

Table 4-4 Biomarkers and corresponding Lower Limit of Quantitation

	Biomarker Protein	Gene	Uniprot ID	LLOQ (fmol μl^{-1})*
1	Nephrin	NPHS1	O60500	0.011
2	Podocin	NPHS2	Q9NP85	2.000
3	Synaptopodin	SYNPO	Q8N3V7	0.100
4	Angiopoietin 2	ANGP2	O15123	0.091
5	Matrix metalloproteinase 3	MMP3	P08254	0.091
6	Neutrophil gelatinase associated lipocalin	NGAL	P80188	0.357
7	Vascular adhesion protein 1	VCAM1	P19320	0.091
8	Caldesmon 1	CALD1	Q05682	0.823
9	Calponin	CNN1	P51911	0.274
10	Smoothelin isoform b variant	SMTNb	P53814	0.274
11	P-selectin	SELP	P16109	0.823
12	Thrombomodulin	THBD	P07204	0.823

* LLOQ of DIKI markers might deviate, since to date these values have been determined for rat plasma and not human urine.

4.4 Biomarker Interpretation and utility

Once data collection and analysis from the learning-phase nonclinical and clinical studies are completed, we will determine the most promising biomarkers, whether individual biomarkers or composite measures will be used, and whether these individual biomarkers or composite measures will be applied to individuals or a cohort of individuals in a phase 1 clinical trial. Markers indicative of podocyte damage as well as markers released as a result of glomerular vascular injury will be measured. Using the data sets developed from the normal healthy volunteers (NHV) and glomerular injury patient populations proposed in our clinical plan (see Section 7.3.1), we will be able to define thresholds or cut-offs which would indicate an increased risk of glomerular injury. Ideally, such thresholds will be several folds above upper limits of normal in NHV, but still below levels associated with detectable glomerular injury in our patient populations. The clinical utility of these individual biomarkers, composite measures, and their thresholds will be validated in the confirmatory-phase clinical studies.

Statistical considerations for composite measure establishment and threshold determination can be found in Section 7.3.3.

5 Context of Use Statement

NGBs or a composite biomarker panel to be used as safety biomarkers in conjunction with traditional measures to aid in the detection of glomerular injury in early clinical trials in subjects with normal kidney function, when there is an *a priori* concern that the drugs may cause glomerular injury in humans.

A priori concerns include scenarios wherein:

1. Glomerular injury is detected by histopathology in nonclinical studies; or
2. A study drug is from a pharmacological class for which glomerular injury has previously been observed; or
3. Nonclinical toxicology studies with the study drug show reversible histologic glomerular damage with or without changes in standard clinical biomarkers, but with elevation in at least one NGB.

6 Analytical Considerations

Biomarkers will be measured by IP-LC-MS/MS.

We plan assay validation according to the “fit for purpose” approach for the candidate biomarkers as listed in Table 4-1 and Table 4-2 by IP-LC-MS/MS. Assay validation will address (1) selectivity; (2) accuracy, precision, and recovery; (3) the calibration curve; (4) sensitivity; (5) reproducibility; (6) short-term stability (2h and 24h at RT) of analyte in spiked and non-spiked samples (7) and long-term stability (1, 3 and 6 months) in parallel to the study analyses.

Moreover in-study validation will ensure that the assay continues to perform according to predefined acceptance criteria. Biological and technical quality control (QC) samples containing known concentrations of analytes will be used to monitor the quality of assay performance. The biological QC samples will be identified before starting the analyses of the studies.

For transparency, information about the analytical assay and software will be publicly posted if the biomarker test is successfully qualified. Technical parameters and information about the assay will be made public to ensure the biomarker test can be used as a drug development tool by any interested party.

7 Clinical Considerations

7.1 Use in drug development

As indicated in the Context of Use, NGBs or a safety composite biomarker panel will be used in conjunction with traditional measures to aid in the detection of kidney glomerular injury in early clinical trials in subjects with normal renal function, when there is an *a priori* concern that the drugs may cause glomerular injury in humans.

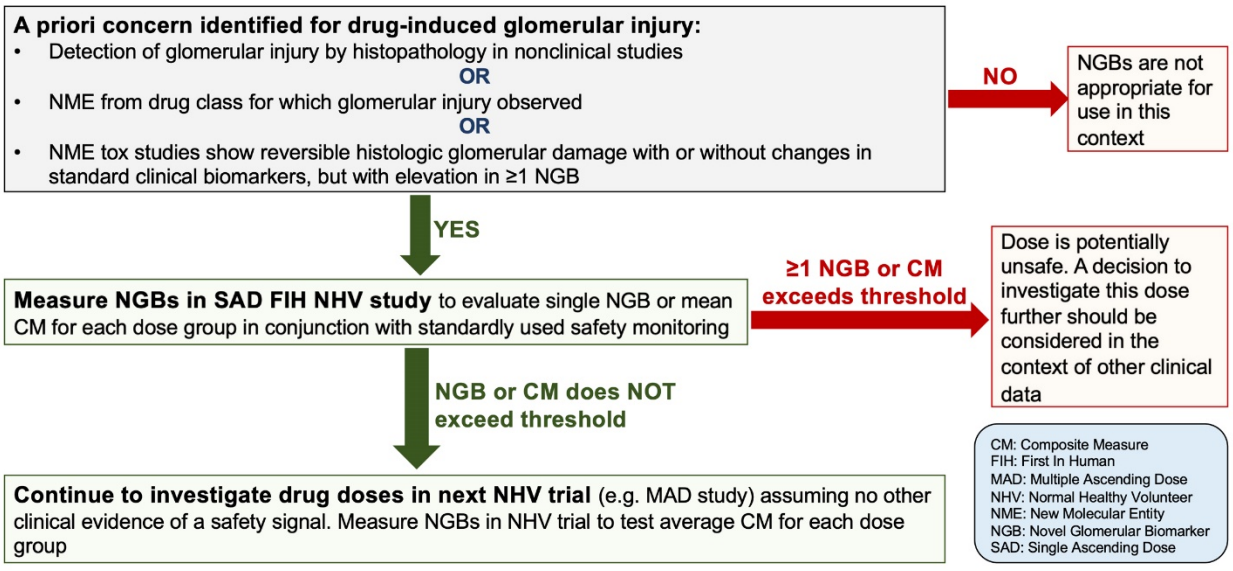
A priori concerns include scenarios wherein:

1. Glomerular injury is detected by histopathology in nonclinical studies; or
2. A study drug is from a pharmacological class for which glomerular injury has previously been observed; or
3. Nonclinical toxicology studies with the study drug show reversible histologic glomerular damage with or without changes in standard clinical biomarkers, but with elevation in at least one NGB.

A decision-tree proposed for the use of novel glomerular biomarkers either alone (single) or as composite measure (CM) in Phase 1 Normal Healthy Volunteer Trials is provided in Figure 7-1 Proposed Decision Tree for the use of glomerular biomarkers in Phase 1 trials

7-1.

Figure 7-1 Proposed Decision Tree for the use of glomerular biomarkers in Phase 1 trials



7.2 Patient population or drug development setting

As described in the COU, the human population in which we propose to use these biomarkers will be subjects in early clinical trials with normal renal function, when there is an *a priori* concern that the drug being evaluated may cause glomerular injury in humans.

7.3 Clinical validation

7.3.1 Learning phase of clinical studies

We plan to conduct the following two clinical studies to help identify the most promising biomarkers, determine whether individual biomarkers or a composite panel will be used, and establish biomarker/composite measure thresholds (for diagram outlining Learning Phase clinical studies planned see Section 11.1.1). These will be confirmed by the clinical studies in the confirmatory phase.

1. A study to measure NGBs from urine specimens collected in the Boston Kidney Biopsy Cohort Study (Srivastava et al, 2018), and to correlate them with functional end points as well as histopathologic lesions of kidney biopsies available from that study. This study will be conducted in collaboration with Dr. Sushrut Waikar, Chief of Nephrology at Boston University Medical Center. In this study, urine, plasma and urine sediment samples from 62 patients with biopsy-proven glomerular damages are available. Candidate NGBs and potential NGBs identified by proteomics from nonclinical studies will be measured. Their levels will be correlated with traditional functional endpoints, quantitative pathological scores, as well as demographic (basic and clinical) information.

2. A study in 120 normal healthy volunteer subjects to measure selected NGBs in urine and plasma collected longitudinally across 3 time points (day 1, 14, and 28). Standard laboratory assessments and Fibroscan will also be performed. The goal is to establish normal variability in urine and plasma levels of those biomarkers and to assess impact of gender and age on that variability.

7.3.2 Confirmatory phase of clinical studies

The prioritized biomarkers (or the composite panel) and their thresholds established in the learning-phase nonclinical and clinical studies will be further characterized and qualified in the two confirmatory-phase clinical studies. To achieve this, we plan to recruit patients with glomerular injury either as a result of disease pathogenesis or drug-induced as manifested by progressive proteinuria and impairment of kidney function. For disease-induced glomerular

injury we will recruit patients with preeclampsia, and for the drug-induced glomerular injury we will recruit oncology (colorectal and gastric) patients treated with VEGF inhibitors. In the confirmatory phase, candidate biomarkers listed in Table 4-1 and Table 4-2, as well as potential NGBs identified by proteomics from nonclinical studies, will be measured, and the biomarker thresholds which were established based on data from the learning phase, will be tested. The biomarkers will be evaluated to determine their capability as an early indicator of potential glomerular injury, and their sensitivity/specificity to detect glomerular injury in comparison to traditional measures.

Biomarkers being studied in these studies will provide no benefit or risk to the trial participants related to their disease progression or drug treatment effects, nor would the urine or blood collection procedure provide additional risks to the trial participants.

7.3.2.1 Preeclampsia

This is a non-interventional, parallel-group study to compare the sensitivity and specificity of NGBs in pregnant study participants with preeclampsia (cases) and participants without preeclampsia (controls) and to compare NGB sensitivity and specificity to performance of standard biomarkers of renal injury (serum creatinine, estimated glomerular filtration rate [eGFR], and BUN) in cases and controls.

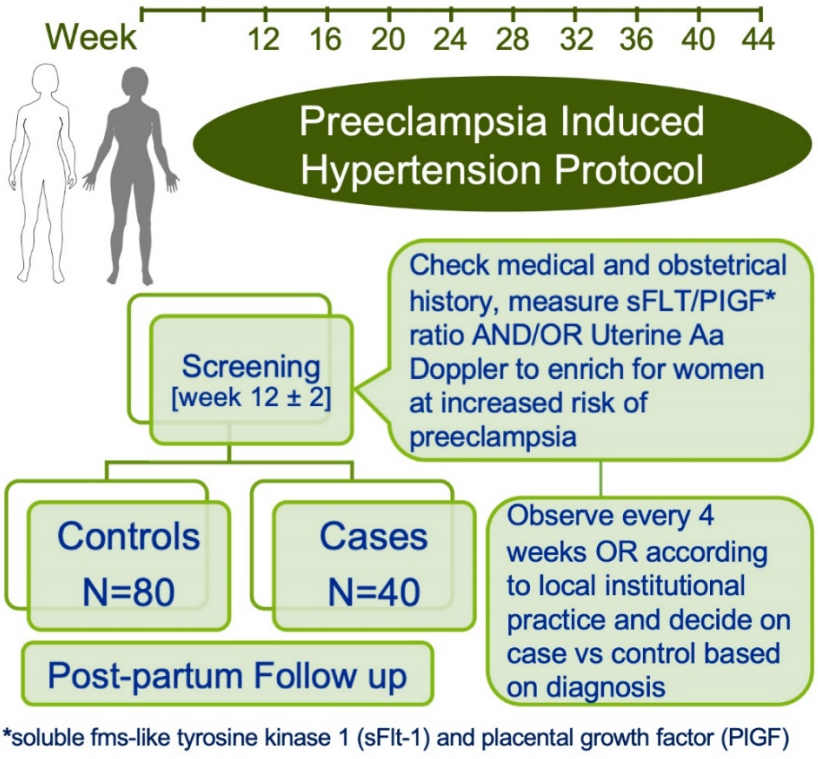
Preeclampsia is diagnosed after the 20th week of pregnancy by new onset hypertension (systolic pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg), proteinuria, and edema. The etiology and pathophysiology of preeclampsia are multifactorial. Changes in local and circulating levels of pro-angiogenic, anti-angiogenic factors, cytokines, hypoxia-inducible factor and others result in increased vascular tone, systemic hypertension, glomerular endotheliosis, increased glomerular permeability culminating in proteinuria, cerebral edema and seizures, and premature labor (Leeman L, 2016). Being a time-limited glomerulopathy, preeclampsia may provide a unique opportunity to observe (i) the onset (2nd trimester or 3rd trimester) and resolution (post-partum) of glomerular protein shedding into the urine and (ii) appearance of serum-based biomarkers coincident with the onset and resolution of hypertension and proteinuria. Since glomerulopathy in the setting of preeclampsia is generally not associated with renal tubular injury, preeclampsia also provides an opportunity to identify specific biomarkers for glomerular injury.

To ensure sufficient recruitment of participants with preeclampsia, an enrichment strategy involving elevated uterine artery Pulsatility Index, soluble fms-like tyrosine kinase-1 (sFlt-1) to placental growth factor (PIGF) ratio >0.85 (Gómez, 2008), and obstetrical medical history and/or general medical history indicating higher likelihood of preeclampsia has been employed. It is anticipated that approximately 120 participants will need to be screened to achieve recruitment of approximately 40 evaluable pregnant participants with pregnancy-induced hypertension and

proteinuria, and 80 evaluable control pregnant participants without pregnancy-induced hypertension and proteinuria. (See Figure 7-2 for an illustration of the study design.)

In addition to standard urine biomarkers (proteinuria and microscopic hematuria) and standard serum biomarkers [e.g. creatinine, BUN, uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH)], the DIKI WP will longitudinally assess urinary and plasma NGBs from pregnant participants. It is anticipated that NGB of injury will appear concomitant with increase in urine total protein/creatinine ratio (UPCR) and prior to the onset of rise in serum creatinine and decline in eGFR. Since glomerulopathy in the setting of preeclampsia is generally not associated with renal tubular injury, it is anticipated that appearance of NGB will be detectable without concomitant rise in urine biomarkers of tubular injury (elevated kidney injury molecule 1 [KIM-1], clusterin [CLU] and/or osteopontin [OPN]).

Figure 7-2 Flow chart of pre-eclampsia clinical study design



7.3.2.2 Cancer patients (colorectal or gastric) treated with anti-angiogenesis drugs

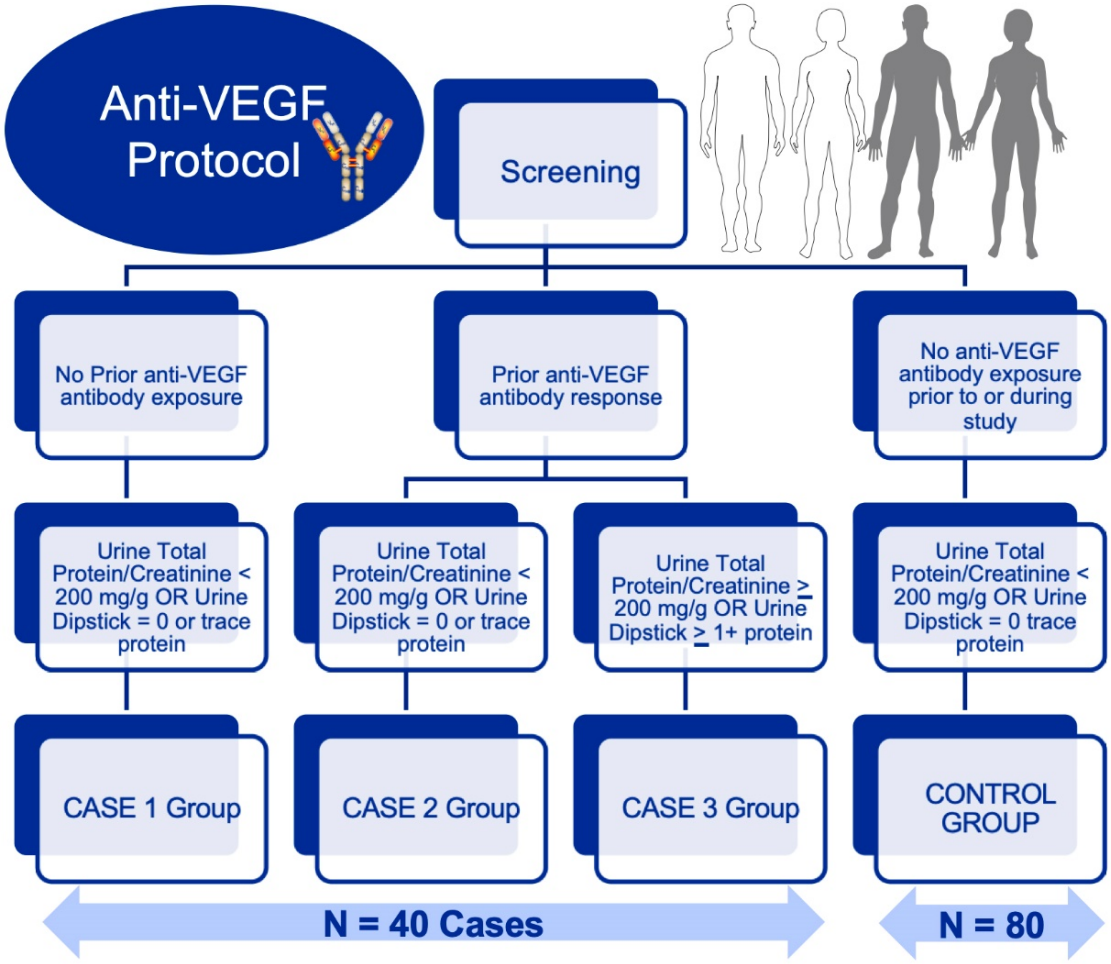
This is a non-interventional, parallel-group study to compare the sensitivity and specificity of NGBs in cancer patients who will begin treatment with an anti-VEGF antibody (aVEGFa) or have previously responded to an aVEGFa (cases), and cancer patients who have never received an aVEGFa and will not receive an aVEGFa during study participation (controls). NGB sensitivity

and specificity will be compared to performance of standard biomarkers of renal injury (serum creatinine, eGFR, and BUN) in cases and controls. (See Figure 7-3 for an illustration of the study design.)

Antibodies against VEGF have been developed to treat malignant neoplasms and neovascular eye disorders. Anti-VEGF antibodies include monoclonal antibodies against VEGF such as bevacizumab (Avastin). As a therapeutic target, aVEGFa effectiveness is based upon the hypothesis that solid tumor proliferation and dissemination rely upon up-regulated tumor angiogenesis through dysregulation of VEGF signaling (Aparicio-Gallego, 2011). Clinical features of aVEGFa-induced kidney injury include Acute Kidney Injury (AKI), hypertension, and proteinuria. Histopathological features include endothelial injury, vascular sclerosis, thrombotic microangiopathy (TMA), and acute tubular necrosis (ATN). Laboratory testing abnormalities include elevated serum creatinine, proteinuria, hemoglobinuria, hematuria, hemolytic anemia, and thrombocytopenia. Nephrotoxicity of an aVEGFa is associated with disturbance of normal housekeeping function and tissue repair of VEGF-associated angiogenesis in the kidney. In the glomerulus, VEGF is secreted by podocytes and it preserves glomerular integrity by binding to endothelial and epithelial receptors. Crosstalk between visceral epithelial cells and endothelial cells may play an important role in maintaining structure and function of the glomerular filtration barrier. Inhibition of VEGF expression in experimental models is associated with glomerular endotheliosis, microaneurysm formation, mesangiolysis, and TMA (Eremina, 2007; Eremina, 2008; Eremina, 2010). Recipients of aVEGFa may provide a unique opportunity to observe (i) the onset (usually within 2 months of initiation of aVEGFa treatment) of glomerular protein shedding into the urine and (ii) appearance and quantitation of serum NGBs as well as changes in standard biomarkers (e.g. serum creatinine, eGFR) with the onset of hypertension and proteinuria. Since aVEGFa-associated glomerulopathy is generally not associated with renal tubular injury, this study also provides an opportunity to identify specific biomarkers for glomerular injury (Eremina, 2007; Eremina, 2008; Eremina, 2010).

In addition to standard urine biomarkers (proteinuria and microscopic hematuria) and standard serum biomarkers (e.g. creatinine, BUN, uric acid, ALT, AST and LDH), the DIKI WP will longitudinally assess urinary and serum NGBs from aVEGFa-treated participants with one or more risk factors for development of aVEGFa-associated proteinuria. In up to 30% of patients treated with aVEGFa, systemic hypertension and glomerular injury are observed with subsequent decline in eGFR and increase in serum creatinine (Eremina, 2007; Eremina, 2008; Eremina, 2010). It is anticipated that injury NGBs in blood will appear concomitant with increase in UPCR and prior to the onset of rise in serum creatinine and decline in eGFR. Since aVEGFa-associated glomerulopathy is generally not associated with renal tubular injury, it is anticipated that appearance of NGB will be detectable without concomitant rise in urine biomarkers of tubular injury (elevated KIM-1, CLU and/or OPN).

Figure 7-3 Flow chart of aVGEFa clinical study design



- Case 1 Group will include participants with no prior treatment with aVEGFa whose current standard of care treatment includes an aVEGFa for the first time; and at screening, urine total protein to creatinine ratio was <200 mg/g or urine dipstick was negative or trace for proteinuria.

- Case 2 Group will include participants previously responsive to a chemotherapy regimen which included an aVEGFa and whose current standard of care treatment includes an aVEGFa; and at screening, urine total protein to creatinine ratio was <200 mg/g or urine dipstick was negative or trace for proteinuria.

- Case 3 Group will include participants previously responsive to a chemotherapy regimen which included an aVEGFa and whose current standard of care treatment includes an aVEGFa; and at screening, urine total protein to creatinine ratio (UPCR) was ≥200 mg/g or urine dipstick was ≥1+ for proteinuria.

- Control Group will include participants with no prior treatment with aVEGFa or during this study; and at screening, urine total protein to creatinine ratio was <200 mg/g or urine dipstick was negative or trace for proteinuria.

7.3.3 Statistical considerations for the planned clinical studies

7.3.3.1. NGB selection for Composite Measure (CM) and threshold(s) establishment

Information on individual candidate biomarkers as listed in Tables 4-1 and 4-2, as well as other potential NGBs identified by unbiased proteomics or transcriptomics from nonclinical studies, will be available from learning phase clinical studies (120 NHV and 62 cases). Based on these data a promising subset of K biomarkers will be identified and a composite measure (CM) will be calculated. The CM is a measure of the geometric mean of the fold change from baseline of K individual biomarkers (selected based on data in learning phase). Note that each urine biomarker will be normalized to urine-Creatinine (uCr). For each participant, CM will be derived using the uCr-normalized fold-change from baseline in biomarker data and will be calculated at time t (= 1, 2, 3) following equation below:$CM_{it} = \exp(\sum_{j=1}^{K} w_j \log(FC_{ijt}))$,

where

$w_j = \frac{1}{K}$: the weight for biomarker j

FC_{ijt}: the uCr-normalized fold-change from baseline for subject i for biomarker j at time t

Note that log refers to the natural log transformation of the fold changes from baseline. In the event values are below the limit of quantitation, the value should be assigned as < LLOQ =0.90* LLOQ (i.e. 90% of the LLOQ value).

The geometric mean (GM) of CM for a group (g=Case or NHV) with n subjects at timepoint t is calculated as:

$$GM\ CM_{tg} = exp\left(\sum_{i=1}^{n} \log\left(CM_{itg}\right)/n\right)$$

Fold change from baseline (FCB) of CM at a given time point versus CM at baseline will be calculated and an acceptable threshold will be defined based on data (120 NHV and 62 cases) in Learning Phase (e.g., “at any post-baseline timepoint there is less than a 5% chance of observing such a result when the true underlying population is NHV”). Similar threshold will also be calculated for each of the individual biomarkers.

7.3.3.2. Adaptive trial design and statistical considerations

For the planned confirmatory clinical studies, we will incorporate the adaptive trial design to potentially make the trial more efficient. The adaptive trial design would allow a unblinded

assessment at predetermined accrual points by a separate panel of professionals not directly involved in the study conduct. The panel would review the number of cases and controls, and the measurements of prioritized and pre-determined promising novel glomerular injury biomarker candidates. This panel would consist of nephrologists at the University Hospital of Zürich, namely Prof. Rudolf Wüthrich and Prof. Thomas Müller. The purpose of interim analysis (IA) would be to assess futility or success before 90 subjects are accrued, or to determine that a greater number of subjects may be needed.

The thresholds established for CM and individual biomarkers will be used at the IA for confirmatory phase (both Anti-VEGF protocol and Preeclampsia Induced Hypertension Protocol) to stop the trial for possible futility.

P1: Proportion of normal participants (n1) with FCB in CM (or individual biomarker) ≥ Threshold (TBD)

P2: Proportion of patients with glomerular damage (e.g. preeclampsia) (n2) with FCB in CM (or individual biomarker) ≥ Threshold (TBD)

Hypothesis: P1 = 0.05 vs. P2 = 0.30

Type I Error level = 2.5% using one-sided test

In the confirmatory phase, each of the two protocols (Anti-VEGF protocol and Preeclampsia Induced Hypertension Protocol) will enroll n=120 subjects with n1=80 NHV and n2=40 cases (2:1 ratio). For each protocol, we propose two IA at (n1 + n2) = 60 and 90 and a final analysis at (n1 + n2) =120. At interim analysis Z-score will be calculated comparing two binomial proportions (P_1 vs. P_2) using pooled estimate of the variance

Boundary values will be calculated using Len-DeMets (OF) method in East software. An example of stopping boundaries at a given interim analysis is provided in Figure 5.

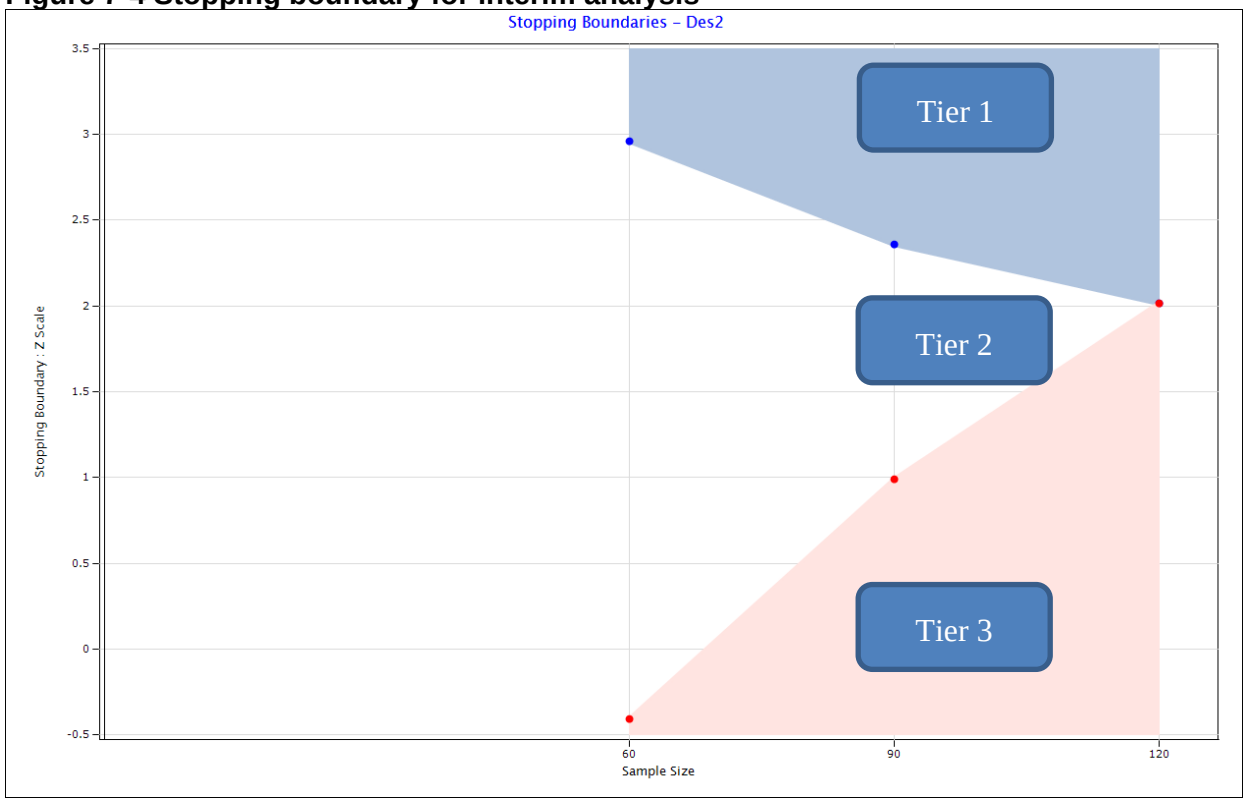
At the interim analysis a given CM will be considered

- Promising if Z-score is > Tier 1 boundary (similar to efficacy)
- Not-Promising if Z-score is < Tier 3 boundary (similar to futility)
- Inconclusive (need more data) if Tier 3 ≤ Z-score ≤ Tier 1

Results of interim analysis at ($n_1 + n_2$) = 60 or 90

- Not-promising (either individual biomarker or CM) would support stopping for futility
- Inconclusive would support possible increase in sample size
- Study will continue even either individual biomarker or CM are found to be promising

Figure 7-4 Stopping boundary for interim analysis



The sample of 120 subjects in a given study yields 93% (α=0.025) power to detect a proportion difference from 5% in NHV to 30% in patients with glomerular damage. Assuming two proportions are equal (both 5% under null), the probability of crossing boundary for futility at IA is 49% by 60 subjects and 77% by 90 subjects.

If the data from both studies (Anti-VEGF protocol and Preeclampsia Induced Hypertension Protocol) are available at the time of analysis, if appropriate (homogenous within group), data may be pooled before either the IA and/or final analysis.

7.4 Benefits and Risks

A clear benefit for specific and sensitive biomarkers of drug-induced glomerular injury will be to improve safe dose identification in cohorts or individuals in early clinical trials and enable the safe clinical progression of drug candidates with an *a priori* concern.

The risks associated with the use of the proposed biomarkers in a clinical setting include the potential for false negative results and false positive results. To mitigate the risk of false negatives, we proposed to use the NGBs in conjunction with the traditional kidney injury/functional biomarkers. The level of risk caused by false negative biomarker results therefore would not be higher than that of the current practice. False positive biomarker results, however, may lead to protracted monitoring of patients and delay in drug development. A weight of evidence approach will be taken in the case of false positives where decision will be taken based on other factors along with biomarker results such as a) preclinical evidence of kidney injury, b) drug metabolism and clearance by kidneys, c) drug route of administration and d) drug mechanism of action. We will also optimize the predictivity of our assay and statistical criteria in order to minimize both risks.

7.5 Current knowledge gaps, limitations, and assumptions

We chose the preeclampsia study as one of our confirmatory studies because preeclampsia is a time-limited glomerulopathy and may provide a unique opportunity to observe the onset and resolution of glomerular protein shedding into the urine and appearance of biomarkers coincident with the onset and resolution of hypertension and proteinuria. Since glomerulopathy in the setting of preeclampsia is generally not associated with renal tubular injury, preeclampsia also provides an opportunity to identify specific biomarkers for glomerular injury (Phipps, 2016; Possomato-Vieira, 2016). No drug will be involved in this study. We are mindful that some biomarkers identified in this study might be specifically associated with preeclampsia pathogenesis rather than glomerular injury in general, and some biomarkers might not be applicable to glomerular injury in drug-induced settings. However, we believe this study will provide great insight, and data interpretation can be corroborated or refuted by the other clinical study in the confirmatory phase (Section 7.3.2.2).

8 Supporting Information

The DIKI WP is also conducting four nonclinical studies. The nonclinical study data are expected to contribute significantly to the weight of evidence supporting the clinical qualification.

Data from the following nonclinical studies in conjunction with the data from clinical studies in learning phase will be used in three ways: 1) assess whether the candidate biomarkers listed in Table 4-1 and Table 4-2 are differentially expressed following glomerular injury due to a variety of conditions irrespective of the species; 2) additional promising biomarkers may be added to the list of biomarkers mentioned in Table 4-1 and Table 4-2 if the discovery studies using nonclinical samples identify promising mechanistic biomarkers that are likely to outperform the traditional measures; 3) data obtained from measuring candidate biomarkers in nonclinical and clinical

studies in the learning phase will inform about thresholds to be used in confirmatory studies. (nonclinical study outline diagrams are provided in Section 11.1.2).

Candidate biomarkers (listed in Table 4-1 and Table 4-2) will be measured and correlated with histopathological gradings. In addition, a discovery proteomics- and transcriptomics-based strategy will be used to identify additional NGBs.

8.1 A rat study to characterize ***sensitivity*** of NGBs and their correlation to histopathological lesions using glomerular toxicants .

1. Merck compound X

Forty (40) female SD rats were be divided into 2 groups of 8 control and 32 treated rats. The treated group were dosed with 30 mg/kg/day of Compound X via oral gavage for up to 13 weeks. Urine samples were collected longitudinally on weeks 3, 5, 7, 9, 11 and 13 with albumin levels monitored throughout the study. Animals with albuminuria >300 µg/mg Cr were binned to recovery or necropsy groups. At time of sacrifice, urine and kidney tissue collection, standard serum chemistry and urinalysis were conducted. Kidney tissues were examined for histopathological changes and a portion of all collected urine samples were used for identification of candidate glomerular safety biomarkers.

2. Puromycin

Thirty-six (36) male Wistar rats will be divided into 2 groups of 12 control and 24 treated rats. The treated group will be dosed with 150 mg/kg single dose of puromycin via IV injection. 4 control and 8 treated rats will be sacrificed on study days 4, 8 and 22 with urine and kidney tissue collection, standard serum chemistry and urinalysis conducted on those days. Kidney tissues will be examined for histopathological changes and urine will be used for measurements of candidate kidney safety biomarker concentrations.

3. Doxorubicin

Thirty-six (36) male Wistar rats will be divided into 2 groups of 12 control and 24 treated rats. The treated group will be dosed with 8 mg/kg single dose of doxorubicin via IV injection. 4 control and 8 treated rats will be sacrificed on study day 4, 8 and 15 with urine and kidney tissue collection, standard serum chemistry and urinalysis conducted on those days. Kidney tissues will be examined for histopathological changes and urine will be used for measurements of candidate kidney safety biomarker concentrations.

8.2 A non-human primate study to characterize ***sensitivity*** of NGBs and their correlation to histopathological lesions using a glomerular toxicant.

Anti-glomerular basement membrane (GBM) antiserum

Fifteen (15) male Cynomolgus monkeys will be divided into 2 groups of 5 control and 10 treated monkeys. The treated group will be dosed with one intravenous bolus injection of an anti-GBM antiserum at 2 mL/kg produced by the company Probetex from sheep immunized with GBM from Cynomolgus kidney cortex. The animals will be monitored for urinary microalbuminuria throughout the study. 5 out of these 10 animals will be necropsied at the onset of minimal increase of microalbuminuria (expected at Day 8) and the 5 other animals will be necropsied when moderate microalbuminuria corresponding to an Albumin/Creatinine ratio of approximately 100 mg/mmol is reached (expected at Day 10). The pre-immune serum will be administrated intravenously at 2 mL/kg to 5 vehicle control animals which will be necropsied on Day 10. Urine and plasma samples will be collected on Day -6 and Day -3 for pretest, Day 3, 5, 8, 10, while kidney tissues will be collected at the end of the study. Kidney tissues will be examined for histopathological changes and a portion of all collected urine samples will be used for identification of candidate glomerular safety biomarkers.

8.3 A rat study to characterize ***selectivity*** and ***specificity*** of the NGBs to glomerular injury by using model tubular toxicants.

1. Cisplatin

Twenty-four (24) male Wistar rats will be divided into 2 groups of 8 control and 16 treated rats. The treated group will be dosed with 5 mg/kg single dose of cisplatin via Intraperitoneal injection. 4 control and 8 treated rats will be sacrificed on study day 4 and 8 with urine and kidney tissue collection, standard serum chemistry and urinalysis conducted on those days. Kidney tissues will be examined for histopathological changes and urine will be used for measurements of candidate kidney safety biomarker concentrations.

2. Gentamicin

Twenty-four (24) male Wistar rats will be divided into 2 groups of 8 control and 16 treated rats. The treated group will be dosed with 120 mg/kg/day of gentamicin via Intraperitoneal injection. 4 control and 8 treated rats will be sacrificed on study day 4 and 8 with urine and kidney tissue collection, standard serum chemistry and urinalysis conducted on those days. Kidney tissues will be examined for histopathological changes and urine will be used for measurements of candidate kidney safety biomarker concentrations.

8.4 A non-human primate study to characterize ***selectivity*** and ***specificity*** of the NGBs to glomerular injury by using a model tubular toxicant

Gentamicin

Ten (10) male Cynomolgus monkeys will be divided into 2 groups of 5 control and 5 treated monkeys. The treated group will be dosed with intramuscular injections of gentamicin at 25 mg/kg/day for 10 days to produce moderate tubular degeneration/necrosis. Urine and plasma samples will be collected on Day -6 and Day -3 for pretest, Day 3, 5, 8, 10, while kidney tissues will be collected at the end of the study. Kidney tissues will be examined for histopathological changes and urine will be used for measurements of candidate kidney safety biomarker concentrations.

9 Previous Qualification Interactions and Other Approvals (if Applicable)

A Pre-LOI Meeting was held on 30 January 2020 with CDER Biomarker Qualification Program team.

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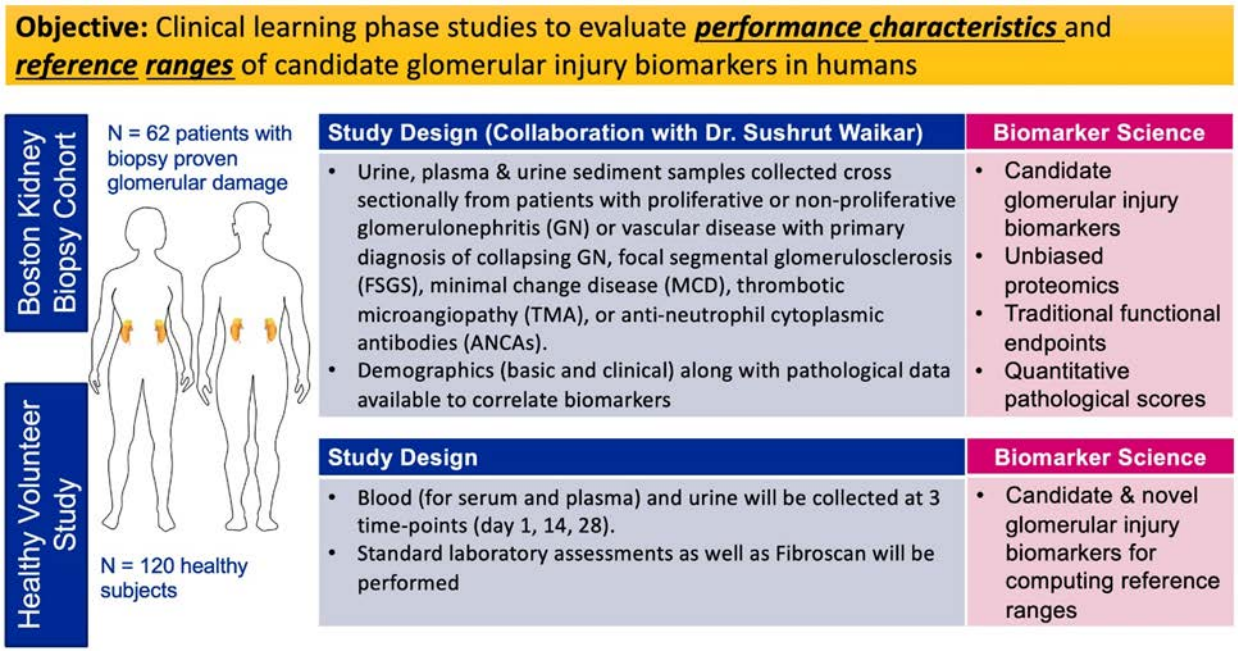
11 Appendix

11.1 Learning Phase studies – diagrammatic outline

11.1.1 Clinical studies

Figure 11-1 Clinical studies in learning phase



11.1.2 Supporting Nonclinical Information

Figure 11-2 Nonclinical learning phase studies to characterize sensitivity of NGBs

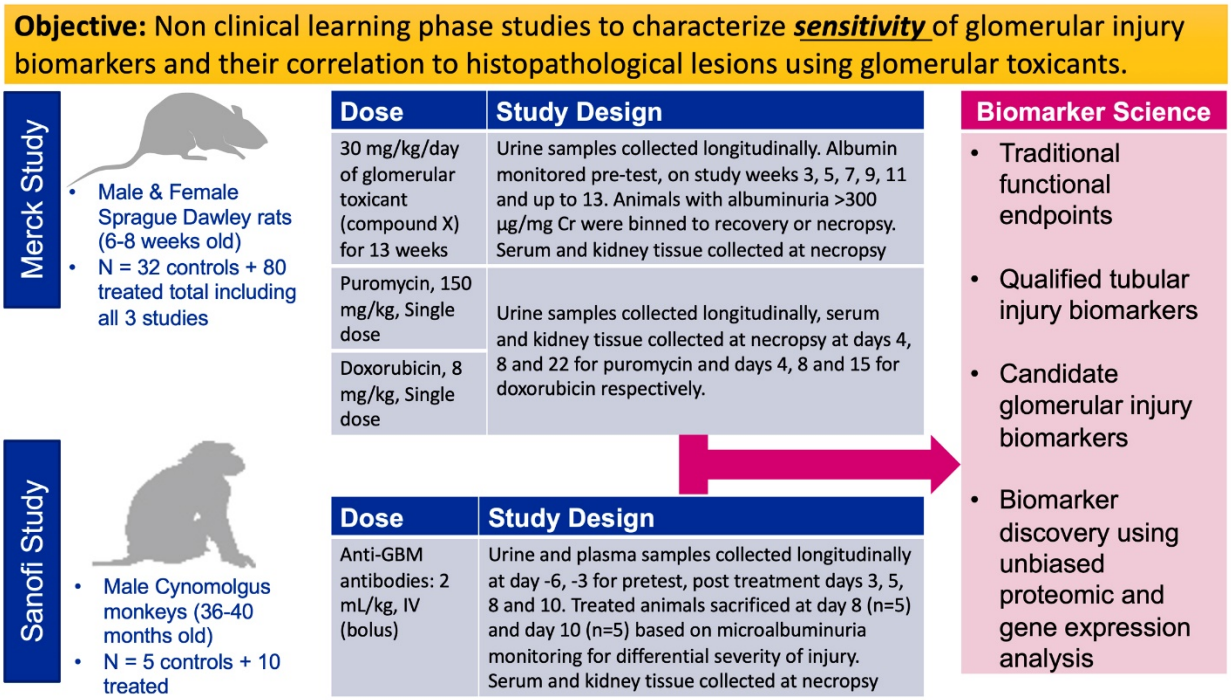


Figure 11-3 Nonclinical studies to characterize selectivity and specificity of NGBs in the context of DIKI