Biomarkers and *in vitro* strategies for nephrotoxicity and renal disease assessment

Abstract

Acute kidney injury (AKI) is a global public health concern, impacting nearly 13.3 million patients and resulting in three million deaths per year. Chronic kidney disease has increased by 135% since 1990, representing the pathology with the fastest growth rate worldwide. The annual costs of dialysis and kidney transplants range between US$35,000 and US$100,000 per patient. Despite its great impact, kidney disease has remained mostly asymptomatic for many years. AKI continues to be a major, unmet medical condition for which there are no pharmacological treatments available, while animal models are limited to provide direction for therapeutic translation into humans. Currently, serum creatinine is the standard biomarker to identify nephrotoxicity; however, it is a late stage biomarker. Hence, there is a pressing need to study *in vitro* biomarkers for the assessment of nephrotoxicity in order to develop new and safer drugs. Understanding of the mechanisms by which molecules produce nephrotoxicity is vital in order to both prevent adversity and treat kidney injury. In this review, we address new technologies and models that may be used to identify earlier biomarkers and pathways involved in nephrotoxicity, such as cell culture, omics, bioinformatics platform, CRISPR/Cas9 genome-editing, *in silico*, organoids and 3D bioprinting, considering AOP.

Key words:

- ✓ Nephrotoxicity
- ✓ *in vivo*
- ✓ *in vitro*
- ✓ New technologies
- ✓ Biomarkers

1. Introduction

Kidney disease is a relatively silent disease and, in most cases, only becomes apparent to those suffering from it in the later stages of the illness. This can be partially attributed to the fact that most people are not aware of the early signs associated with kidney disease, coupled with the fact that these signs are not always recognized by healthcare providers (MacLeod, 2009). Nonetheless, it is estimated that, annually, around three million deaths worldwide are caused by acute kidney injury (AKI). However, these numbers may be underestimated, given that half of the patients admitted to intensive care units develop AKI during hospitalization, and 27% of these die before being released from the hospital. It is also well-known that these figures constitute only a small proportion of the total number of reported cases (Kellum et al., 2016).

AKI is an expected event that occurs relatively quickly, such as a few hours or days, after organ failure. The full process is still not clear. AKI is characterized by a reduction in the glomerular filtration rate (GFR), which leads to an increase in serum creatinine or blood urea nitrogen (BUN). However, measuring BUN levels is not a reliable factor to predict AKI (Kramann et al., 2015).

The frequency of AKI has significantly increased in recent decades and is now recognized as a significant cause of Chronic Kidney Disease (CKD) and kidney failure in general (Levin et al., 2017). AKI is characterized as kidney disease that persists up to 90 days; this disease is considered chronic CKD when it surpasses 90 days (KDIGO, 2013; Kellum et al., 2016).

Between 19% and 26% of the cases of AKI among hospitalized patients are caused by medication-induced adverse effects that injure the kidney (Murugan et al., 2010). AKI is still a major, unmet medical problem for which no efficient pharmacological treatments exist (Mehta et al., 2015). Therefore, an understanding of the mechanisms by which molecules may cause harm (such as those leading to AKI) is vital in order to both prevent toxicity and treat kidney injury.

Acknowledging the need for action, an increasing number of global advocacy initiatives, such as World Kidney Day, the International Society of Nephrology 0by25, and the Lancet Kidney Campaign aim to raise public awareness regarding the consequences, costs, and impact of both CKD and AKI (Kellum et al., 2016).

2. **Acute Kidney Disease**

Acute Kidney Injury (AKI) is a worldwide public health concern, impacting nearly 13.3 million patients per year (Mehta et al., 2015). AKI is associated with high morbidity and mortality - over 1.7 million deaths per year among adults and children worldwide (Ho et al., 2014). AKI is disseminated throughout 72 countries and is higher in developing countries. The mortality rates of critically ill patients with AKI in hospitals ranges between 20% and 70% (Fortrie et al., 2019; Schmitt et al., 2008). In the United States alone, half a million hospital admissions each year are complicated by AKI, resulting in total costs of approximately $10 billion (Chertow et al., 2005). Worldwide, only half of the individuals who need renal replacement therapy can be treated, due to difficulties related to access to care or lack of diagnosis. In fact, estimates suggest that 2.5 to 5 million patients are not properly treated (Liyanage et al., 2015). In addition, the costs of dialysis and kidney transplants range between $35,000 and $100,000 per patient annually.

Prescription drugs and other traditional low-level medication is the major cause of AKI, resulting in nearly 25% of all patients who use at least one nephrotoxic drug. The other 75% of the cases stem from other etiologies. However, the main cause of mortality and morbidity by AKI can be explained by drug-induced nephrotoxicity in over 20% of hospital-acquired AKIs (Bellomo, 2006; Kaufman et al., 1991; Nash et al., 2002; Wonnacott et al., 2014). Despite this high rate, data show that only 7% of new drug candidates fail in preclinical trials because of nephrotoxicity (Frost and Sullivan Rang, 2007), whereas the incidence of patients in intensive care units developing acute kidney injury (AKI) is about 30–50% (Devarajan, 2005). This discrepancy may help to explain the underestimation of nephrotoxicity in

preclinical trials (Fuchs and Hewitt, 2011). Therefore, AKI is still a major medical condition for which no pharmacological treatment has been developed (Kumar et al., 2014; Lameire et al., 2013; Naughton, 2008).

The incidence of AKI has greatly increased over the past two decades, and it is now recognized as an important cause of CKD and kidney failure (Jha et al., 2013; Levin et al., 2017). However, the causal pathway leading to CKD is complex and will require a broad multidisciplinary response (Horton and Berman, 2015). Fortunately, the kidney carries an extremely regenerative potential and, in some cases, can be completely recovered '*restitutio at integrum*' after an AKI. Nevertheless, there is strong evidence suggesting that repair can be incomplete and an episode of AKI may lead to CKD. The appearance of tubular response after AKI will shed light on the connection between incomplete repair and future risk of CKD (Kramann et al., 2015). Thus, the 'AKI to CKD transition' might involve incomplete renal tubule repair after AKI, eventually triggering interstitial renal fibrosis, making it critical to investigate the cells responsible for normal repair. Hence, by developing new targeted therapies to reduce injury and promote healing in AKI, it will be possible to determine which cells should be targeted.

In clinical practice, the early detection of AKI has had no impact on clinical decisions. In addition, therapeutic possibilities for AKI are very limited, and it is unclear if there is any benefit in either early or late onset of renal replacement therapy. As a result, the early or late detection of AKI, in most cases, does not impact the clinical course of the patient. To some extent, this may explain why most biomarkers have not been included in the panel of classic laboratory parameters used in the clinical setting. Hence, advances are needed in this area in order to understand the gap related to renal disease.

3. **Chronic kidney disease (CKD)**

According to the "Kidney Disease: Improving Global Outcomes (KDIGO)" guidelines, CKD is characterized by abnormalities of kidney structure or function that are present for more than 3 months (KDIGO, 2013).

There are few markers, such as neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule1 (HAVcr-1, also named KIM-1), and nephronectin (NPNT), as well as the validated markers of proteinuria and microalbuminuria, that can detect the progression of AKI into CKD, which makes it an important area for investigation (Kashani and Kellum, 2015; Zuk and Bonventre, 2016). Among those who survive the long-term outcomes of AKI, patients with CKD can progress into the end-stages of renal disease (ESRD) or into the aggravation of pre-existing CKD, which can quickly lead to ESRD (Jha et al., 2013; Liyanage et al., 2015; Waheed et al., 2013).

Thus, AKI, which was previously thought to have no major consequences for recovered patients, may lead to poor life quality as well as long-term financial costs (Stephen C. Noble et al., 2006), which increases the need for public awareness regarding the early signs of the disease and investment in further research (Zuk and Bonventre, 2016). CKDs represent an important risk factor for cardiovascular and cerebrovascular diseases and progress toward ESRD (Balogun et al., 2017; Eckardt et al., 2013), which can only be treated by renal replacement therapies such as hemodialysis, peritoneal dialysis, or transplants

(Cazorla-Vázquez and Engel, 2018; Eckardt et al., 2013). Kidney failure is the most severe form of CKD and is fatal if not treated by dialysis or kidney transplant. Because of the uncertainty of what causes CKD, many patients do not receive the correct treatment (Levin et al., 2017; Pickkers et al., 2017).

The resources required to treat CKD-associated complications (including kidney failure) impose a huge burden in such countries as Malaysia, Tunisia, Turkey, Chile, Mexico, and Uruguay (Muralidharan and White, 2015). In fact, it has been shown that the burden of CKD is on the rise, as indicated by the increase in both deaths and ESRD (Jha et al., 2013; Levin et al., 2017; Luyckx et al., 2018; Norton et al., 2016; Ojo, 2014).

Nevertheless, CKD is not on the priority list of non-communicable diseases, and only a few countries have explicit policies or public programs focused on preventing and controlling CKD. This unfortunate scenario is of concern because there is a gap between recent advances in treatment and the increase in mortality worldwide, thus creating barriers that limit the progress of basic research that would ultimately lead to new treatments (translational research type 1) (Sanchez-Niño et al., 2017).

CKD represents the pathology with the fastest growing incidence worldwide (Kam Tao Li et al., 2013). Its incidence has virtually duplicated between 1990 to 2010 (Lozano et al., 2012). The global prevalence of CKD is estimated at around 10% to 15%, with similar estimates in men and women, and is more frequent in high-income countries. The impaired renal function is caused by diabetes mellitus in 30–40% of the cases, hypertension in 20%, inflammation of the glomeruli (glomerulonephritis) in 13%, interstitial nephritis in 10%, and polycystic kidney disease in 6%. Regardless of the cause, the common final outcome of almost all progressive CKDs is renal fibrosis (Humphreys, 2018; Liu, 2011).

The actual incidence of renal disease seems to be underestimated because many deaths attributed to diabetes have actually been caused by renal disease (Rao et al., 2012). Moreover, hypertension and obesity are among the growing non-communicable diseases and represent important risk factors for CKD (Jha et al., 2013). Thus, identifying CKD has become imperative not only to prevent the progression of the disease, but also to reduce the risk of cardiovascular morbidity and mortality. Hence, new biomarkers are paramount for early identification of the disease.

4. **Biomarkers for nephrotoxicity**

Currently, AKI diagnosis is based on serum creatinine's (sCr) rise and/or fall in urine production. These are not renal-specific markers and have major limitations. Alternatively, there are two sets of biomarkers available for clinical use: neutrophil gelatinase-associated lipocalin (NGAL) and the combination of urinary insulin-like growth factor-binding protein 7 (IGFBP-7) and tissue inhibitor of metalloproteinases 2 (TIMP-2), two markers of cell-cycle arrest. These biomarkers seem to be specific and sensitive enough to be used together with additional markers for AKI to better stratify renal injury (Pickering and Endre, 2016).

In 2008, the FDA designated seven biomarkers of nephrotoxicity to test in animals and, on a case-by-case basis, to be used in humans. These included urinary KIM-1, β2-microglobulin, cystatin C, clusterin, trefoil factor 3, albumin, and total protein. These markers, among others (e.g., urinary NGAL, urinary IL-

18, and L-FABP - liver fatty acid binding protein), have been evaluated in many conditions but have rendered inconclusive results as a predictive model. In most cases, the data obtained with biomarkers tested in animals are compared to changes in serum creatinine (sCr) concentrations in humans, but results are questionable. In 2014, the NephroCheck test, which helps to determine if critically ill patients are at risk of developing moderate-to-severe AKI following 12-hour testing, was approved by the FDA. However, it is unknown at which time-point the measurement should be performed to ideally predict AKI (Alge and Arthur, 2015; Koyner et al., 2012; Zuk and Bonventre, 2016). Thus, there are few markers for progression of AKI to CKD. Moreover, this strategy of using biomarkers also serves to determine a specific point in the course of AKI when the predictive value of the biomarker should be tested (Chertow et al., 2005; Marx et al., 2018).

AKI is an extremely heterogeneous and complex condition. Even though there is an effort to identify AKI biomarkers that could be used for risk assessment, diagnosis, severity, and/or outcome of AKI with high predictive power, an ideal, universal AKI marker is unlikely to be discovered. Thus, considering the heterogeneous nature of AKI, a complex multimodal approach, including a detailed risk assessment and the implementation of new biomarkers, is advisable to prevent and manage AKI (Meersch and Volmering, 2017).

For this reason, several biomarkers that comprise the different aspects of AKI are required. Sensitive detection of an acute reduction in kidney function and injury in various renal structures, as well as the evaluation of the degree of renal injury, are the types of information provided by marker panels. Ideally, these markers should also be mediators of other disease pathways in AKI. Even better, these markers are the key to understanding the pathological mechanisms, to indicating the etiology of AKI, to providing references for future therapies, as well as to following therapy progress. Early biomarkers to estimate renal tissue damage is an advance that replaces the old low-sensitive methods for diagnosis by using better AKI criteria (Mehta et al., 2015; Schrezenmeier et al., 2017).

AKI biomarkers may be used to reveal higher serum creatinine levels after ischemia reperfusion injury (IRI) and can also be used an early indication of the progression from AKI to CKD. This may also indicate that the process used was not as effective as intended. For instance, fibrosis is still found in patients, despite the reacquisition of normal biochemical parameters, such as plasma creatinine removal; progression to CKD is still the most frequent result (Block and Schoolwerth, 2006).

KIM-1 is a type I cell membrane glycoprotein with extracell immunoglobulin and mucin domains (Devarajan, 2008; Hillege et al., 2006; Ichimura et al., 2004). The mRNA and protein for KIM-1 are expressed at very low levels in the normal rodent kidney; however, expressions increase dramatically after an injury in the proximal tubule epithelial cells in postischemic rodent kidney and in humans during ischemic acute renal ups and downs of using KIM-1, and according to Medić et al. (2015) (Medić et al., 2015), larger trials are still necessary before KIM-1 can be tested in broader clinical trials. Although KIM-1 is a sensitive and specific marker for kidney tubular injury, it is difficult to measure it in acute settings (Miao et al., 2017).

By contrast, Ichimura et al. (1998) (Ichimura et al., 1998) demonstrated that human *KIM-1* exhibits homology to *HAVCR1*, and studies using gentamicin (Campos et al., 2018; Silva et al., 2017),

amphotericin B (Grossi et al., 2017), and cisplatin (no publishing data) have shown that the genes *HAVCR1 (KIM1)* might be used as *in vitro* biomarkers of nephrotoxicity.

To find new drugs for AKI and prevent its transition into CKD, it is crucial to better understand how tubular injury and repair work. Once this knowledge is acquired, it would be possible to use translational biomarkers to assess drug targets and their pathways. Furthermore, the expected biological effect of the drug can be revealed by specific biomarkers, i.e., whether the drug has reached the pathophysiological pathway for which it was developed and what is the expected clinical outcome (Block and Schoolwerth, 2006; Kramann et al., 2015; Schrezenmeier et al., 2017).

5. Preclinical studies for nephrotoxicity

5.1. Animal models

Animal models have been widely used to understand the pathogenesis and underlying mechanisms of renal disease. As shown in the FIG.1, mice and rats are the most commonly used to study nephrotoxicity and therapeutic targets, as well as to identify new biomarkers, mainly because these are easily bred and are relatively inexpensive to house and maintain (Camacho et al., 2016). Nonetheless, few advances have been achieved in understanding nephrotoxicity mechanisms or in identifying either a new biomarker or a panel of biomarkers. According to our search, monkeys are also used; however, regardless of the size and breed of the animals, results did not show a good predictability to identify adverse effect in humans.

FIGURE 1

In fact, the failure of existing animal models to accurately predict nephrotoxicity has been a major barrier to the development of safer drugs. Extensively studied for several decades, animal models of human AKI have, to date, yielded no specific therapy that benefits the human disease, either in preventing its occurrence, ameliorating its severity, hastening its recovery, or retarding the risk of ensuing CKD. Such inability to translate these models' uncovered salutary strategies into therapies for human AKI has led to a questioning of the value of these models: specifically, their resemblance and fidelity to human AKI. Overlapping this kidney-centered discussion are broader debates in relevant biomedical communities, national as well as international, regarding a worrisome, relative lack of congruence of conclusions in preclinical studies and the slow pace of translating findings obtained in animal models into new therapies for human disease (Nath, 2015).

Ischemia–reperfusion is one of the main causes of AKI; by definition, human AKI implies decreased GFR. Hence, conventional AKI models are mainly the result of kidney ischemia–reperfusion, exposure to endogenous or exogenous toxins and urinary tract obstruction (Sanz et al., 2017). However, this may not be the case for animal models if only one kidney is injured. Hence, despite the abundant literature on the pathogenesis of ischemia–reperfusion, no novel therapy has been able to be applied in clinical scenarios (Ortiz et al., 2015).

Exogenous drugs or poisons, as well as endogenous toxins, are widely used to stimulate AKI for their side or poisoning effects. Among these models, 6–20 mg/kg cisplatin can result in acute tubular injury within 72 h, whereas the administration of 40–200 mg/kg gentamicin in rats for 4–10 days can induce acute renal failure (Bao et al., 2018).

Chemotherapy comprised three cycles of high-dose (80-100 mg/m^2) cisplatin administered on days 1, 22, and 43. Cisplatin is a drug widely used for chemotherapy (80-100 mg/m^2 – IV administered on days 1, 22, and 43) to effectively treat various cancers and gentamicin is an aminoglycoside antibiotic (6-12 g/day, IV administered, for 2-6 weeks) commonly used to prevent gram-negative bacterial infection. However, both drugs have limitations in clinical practice due to nephrotoxicity. For many years, nephropathy caused by cisplatin and gentamicin have been widely studied, but it is still unclear, although different models using different animals have been used and many hypotheses have been suggested (Hayward et al., 2018; Lavergne et al., 2018; Quintanilha et al., 2019; Uccelli et al., 2008). Nevertheless, these hypotheses do not fully reproduce human clinical diseases. Hence, we do believe new *in vitro* strategies are necessary to study the molecular mechanism involved in nephropathy so as to reach proper clinical diagnoses and implement effective therapeutic interventions.

Actually, some AKI models in mice have been used with both advantages and disadvantages (Table 1). Even so, models tend to have a high clinical relevance, for example, classical models contain a high knowledge background and are technically simple and reproducible. The types of models are incomplete and many details, such as model techniques and modeling time, are not mentioned. In addition, the majority of these have a variable response between models, expected acute renal necrosis is not always achieved, AKI is not produced clinically and pathologically, surgery is required, reproducible outcome is dependent on accurate Ischemia/Reperfusion time, and the renal function can be compensated by a non-ligated kidney (Bao et al., 2018).

Table 1: Advantages and limitations of conventional AKI animals models.

	Model	Renal target	Design	Advantages	Limitations
Drug induced	Isquemia-reperfusion	Proximal tubule and endothelium	Clamping time: 30-45 min Reperfusion time: 24-48 hours	Pathology similar to human disease	Requires surgery
	Cisplatin	Proximal tubule	Sinlge IP injection (6-20 mg/kg) 72 hours	Pathology, timing and drug doses similar to human disease	No clinical correlate
	Gentamicin	Proximal tubule and glomerulus	Serial IP injections (40-200 mg/kg/day) 4-10 days	Induces rapidly progressive CKD	No fully reproduce human clinical diseases
	Aristolochic acid	Proximal tubule	Serial IP injections (5 mg/kg/day) 5 days	Induces rapidly progressive CKD	No clinical correlate
	Folic acid	Proximal tubule	Single IP injection (250 mg/kg) 24-48 hours	Pathology findings similar to human AKI from other causes	No clinical correlate
	Glycerol	Proximal tubule	Single IM injection (8 mL/kg of glycerol 50%) 24-48 hours	Clinically relevant, resembling human rhabdomyolysis	Severe and focuses in the symptoms, not the mechanism
	Warfarin	Proximal tubule and glomerulus	5/6 nephrectomy for 3 weeks and 8 days on warfarin	Useful to study AKI caused by anticoagulants	Not guarantee the same mechanism

IP, intraperitoneal; IM, intramuscular.

Aristolochic acid (AA) and high dose folic acid (FA) are also frequently used to study AKI-CKD transition, with AKI models developed by warfarin and glycerol (Bao et al., 2018).

Nephrotoxicty caused by AA was first reported in Belgium in a group of patients who ingested weight loss pills containing extracts of Chinese herbal powdered roots(Vanherweghem et al., 1993). The findings in humans are supported by laboratory animal studies showing that oral exposure to aristolochic acids caused tumors in the urinary tracts of various animal species. Although this model showed good results it did not work for other drugs. In 2001, the U.S. Food and Drug Administration (FDA) advised consumers to stop using any products that may contain aristolochic acids, after seeing an increase in nephropathy, or kidney disease, among users (Food and Drug Administration, 2001).

Fink et al. (1987) (Fink et al., 1987) demonstrated that single intravenous doses of FA in rats causes direct nephrotoxic effects; hence, it has been used to study AKI-CKD transition. Some studies have used intraperitoneal injection (Linkermann et al., 2014, 2012; Martin-Sanchez et al., 2017). However, FA is the synthesized form of folate present in fortified foods and supplements, such as grains, and has a higher bioavailability than naturally occurring folate (Khan and Jialal, 2019).

Thus, AA and FA are used at low doses for oral administrations in human and at high dosages and intravenous and peritoneal doses when studying nephrotoxicity in animals. Hence, AA and FA do not represent good models, since the profiles for causing nephrotoxicity are completely different.

Warfarin (WRN) therapy can result in AKI by causing glomerular hemorrhage and renal tubular obstruction by red blood cell (RBC) casts (Brodsky et al., 2009). Analysis of more than 15,000 warfarin-treated patients showed that WRN affects approximately 33% of CKD patients and 16% of non-CKD patients who experienced an international normalized ratio > 3.0. This study also identified that mortality rates in patients with WRN was significantly higher than in patients without WRN (Brodsky et al., 2011). However, excessive anticoagulation in control animals was not associated with changes in creatinine levels, and renal morphology was normal. Therefore, Ware et al. (2011) (Ware et al., 2011) developed a new method to study nephrotoxicity using an excessive amount of warfarin combined with a 5/6-nephrectomy model. Although this method could cause functional and morphological damage of the remnant kidney, it does serve to demonstrate the effect. Nevertheless, it does not guarantee the same molecular mechanism responsible for nephrotoxicity, which is the main element to be discovered.

AKI is also a common complication of rhabdomyolysis and accounts for the high mortality (Bao et al., 2018; Elterman et al., 2015; Zhang et al., 2012). To reproduce the typical symptoms observed in humans, rats or mice are deprived of water for 24 h, after which a 8–10 mL/kg dose of 50% glycerol is administrated in the hindlimb muscle (Geng et al., 2015; Kim et al., 2014). We do not believe glycerol could be a good model, given that the model is focused on the symptoms, which is not the main point of our study. In addition, glycerol is used in human food at low doses. Thus, it does not represent a good model to study nephrotoxicity.

Mechanisms of disease generation and progression in AKI and CKD have yet to be fully understood (Singh et al., 2013; Tampe et al., 2017). Although several clinical studies have investigated early stage predictive biomarkers of kidney disease, few have been applied in clinical practice (Endre and Pickering, 2013). CKD models can include mainly renal mass reduction, diabetic nephropathy, hypertension glomerular injury, IgA nephropathy, polycystic kidney disease, and chronic tubulointerstitial nephritis (Table 2).

Table 2: Advantages and limitations of experimental CKD animals models.

Pathology	Model	Transition AKI-CKD	Advantages	Limitations	Comments
Renal mass reduction	5/6 nephrectomy (rats)	Depends on the mice strains	Equivalent to humans	Two surgery requires. The second one having a high mortality rate, especially in mice, and requiring technical expertise; mice strains are differentially responsive to renal mass reduction with respect to CKD progression; it is irreversible and the amount of kidney left to study is small and may be distorted by surgical procedure	No clinical translation of successful
Diabetic Nephropathy	Streptozotocin mice/rats; NOD mice BB-DP rat; ob/ob mice db/db mice; STZ-eNOS-/-; db/db-eNOS/mice	No	Gene modified; commercially available; available on multiple strains; useful to study mechanisms of diabetic nephropathy. Reproduce human physiopathology	None of the many animal models perfectly replicate the human disease; expensive; some strains are infertile; cause modest albuminuria without decreased GFR	No clinical translation of successful
Hipertensive Nephropathy	SHR rats+UNX; angiotensin II infusion models	Depends on the dose/time	Highly relevant to hypertension nephropathy; useful to study AngII effect over kidney	SHR rats are more resistant to streptozotocin-induced diabetes; uninephrectomy is required to promote significant kidney injury; no progressive GFR loss; higher dose or long exposure time periods increase serum creatinine levels; high cost; slow progression	No clinical translation of successful; long exposure time periods stress animals
Acquired Glomerular Injury	Focal segmental Glomerulosclerosis: adriamycin (rat, mice) or puromycin (rat)	Depends on the strains	Mimic acute glocmerular injury	Usually no adequate reproduction of slowly progressive human disease	No clinical translation of successful NOD
IgA Nephropathy	ddY mouse, HIGA mice; Uteroblobin-deficient mice		Reproduces human pathology	Mild disease development usually without progression towards end-stage renal disease; no progressive GFR decrease	No clinical translation of successful
Polycistic Disease	Genetically engineered mouse model	Yes, but no specific to renal disease	Useful to study PKD mechanisms and to find therapeutic targets; new insights into the molecular mechanisms of cystogenesis and associated progression of CKD; resulted in regulatory approval for Tolvaptan in Japan for human ADPKD	Mouse models usually lack the variety of human phenotypes	ARPKD mouse models usually lack the variety of human phenotypes
Chronic Tubulointersticial Nephritis	Simple, dose-dependent decreased GFR; Possibly reversible (rats)	Model no specific for human	Simple, dose-dependent decreased GFR; possibly reversible	Adenine-ged male rats had a more severe decline in kidney; models CKD extrarenal complications; function and molecular changes	Adenine intoxication is not an issue in human
Hereditary Glomerular Injury	*Pkd1* or *Pkd2* gene engineered; *Col4a43* gene knockout mouse; Alport syndrome	Depends on the strains	Reproduce features of human disease, including progressive GFR loss; develop proteinuria and renal failure	Difficulty to translation of genetic findings into improved patient care; high costs	Resulted in clinical recommendations
Stone Disease	Natural development (dogs and cats)	No information	Without external factors to cause disease	Few studies	Long-term

GFR glomerular filtration rate; SHR, Spontaneously Hypertensive rat; BB-DP, BioBreeding-diabetes prone; nod, nonobese diabetic; ob/ob, obese; db/db mouse, type 2 diabetes; DBA/2J, model for congenital experimental glaucoma; STZ, streptozotocin; Enos, endothelial nitric oxide synthase; UNX, uninephrectomy; ddY mouse, outbread; HIGA, Nephropathy with Hyperserum IgA; pkd, polycystic kidney disease; COL, collagen genes.

Renal mass reduction (remnant kidney model) is one of the most widely used models to study CKI. Subtotal (5/6) nephrectomy has been a mainstay of studies of progressive CKD. The right kidney is removed and the upper and lower poles (2/3 of the left kidney) are resected after ligation of the left renal artery. After surgery, activation of the renin-angiotensin system (RAS) can cause glomerular hypertension/hyperfiltration (Bao et al., 2018; Ergür et al., 2015; Ortiz et al., 2015; Tapia et al., 2012).

Glomerular injury may be acquired or hereditary. Acquired diabetic nephropathy (DN) is the leading cause of ESRD. There are many rodent models for diabetic nephropathy; however, none of them perfectly mimics the human disease (Deb et al., 2010). The Animal Models of Diabetic Complications Consortium (AMDCC) defines the ideal rodent model of human diabetic nephropathy and complications (Kitada et al., 2016; Kong et al., 2013). Several models of gene targeting have reproduced mutations to generate murine models of hereditary podocyte-based nephropathies, including focal segmental glomerulosclerosis and Alport syndrome (Ortiz et al., 2015). Hypertension-induced renal injury is usually investigated by using spontaneous hypertension. Additionally, uninephrectomy is required to promote significant kidney injury in the form of albuminuria without reduced GFR (Nishimura et al., 2018). Chronic exposure to pressure-increasing doses of angiotensin II in rats and mice caused renal dysfunction (Ruiz-Ortega et al., 2006).

Focal segmental glomerulosclerosis (FSGS) is a common primary glomerular injury, characterized mainly by podocyte loss, glomerular fibrosis, and marked proteinuria (Fogo, 2015). Although there is currently no primary FSGS model available, several secondary FSGS models have been established. Non-genetic models mimic acute glomerular injury but not the spontaneous slowly progressive focal segmental glomerulosclerosis observed in the clinic. Experimental podocyte depletion, using adriamycin or puromycin aminonucleoside, in turn leading to CKD, emphasizes the key role of podocytes in the preservation of the renal function (Daehn et al., 2014; Ortiz et al., 2015).

IgA nephropathy (IgAN) is the most common human glomerulonephritis, but no specific therapy has been discovered. However, none of these models enabled the identification of the factors that control the transition between disease onset and progression to ESRD. Thus, the underlying mechanism of IgAN is still not fully understood (Bao et al., 2018; Ortiz et al., 2015).

Polycystic kidney disease (PKD) includes a group of human monogenic disorders inherited in an autosomal dominant (ADPKD) or recessive (ARPKD) fashion. Genetically engineered mouse models, in which disease-causing genes were targeted, now mimic human ADPKD, ARPKD, and atypical PKD. Those models have already provided new insights into the molecular mechanisms of cystogenesis and the associated progression of CKD. However, ARPKD mouse models usually lack the variety of human phenotypes (Ortiz et al., 2015).

Primary chronic tubulointerstitial nephritis is associated with an immune-mediated infiltration of the kidney interstitium by inflammatory cells, which may progress to fibrosis. This is characterized by tubular atrophy, interstitial fibrosis, and interstitial inflammation in the absence of significant glomerular disease. Most animal models of chronic tubulointerstitial nephritis are secondary to ischemia, proteinuria, AKI, or other primary kidney diseases (Joyce et al., 2017; Ortiz et al., 2015). Thus, the processes

involved are different and do not demonstrate similar mechanisms; hence, it does not represent a good model.

In addition to the animals already mentioned dogs and cats also have been suggested to study the calcium oxalate stone formation, considering that a naturally occurring animal model could be more useful. This strategy could represent a good model; however, the pathologic and etiologic mechanism surrounding calcium oxalate nephrolithiasis is incomplete (O'Kell et al., 2017).

Thus, despite the valuable new insights into kidney disease gained from existing models, many do not fully reproduce human clinical diseases. In the AKI, models are not stable and reproducible, while in the CKD, the disease is quite complex (Bao et al., 2018). However, in addition to each model's specific limitations, all animal-model research into human diseases is ultimately restricted by the need to translate findings across species. This calls for the wider use of human-based models to complement and reduce the use of experimental *in vivo* research (Fonseca et al., 2017).

In this scenario, we do believe that new strategies and models without animals are required to better mimic processes involved in AKI and CKD. These models must mainly address molecular mechanisms, since the mere understanding of them may help to develop therapeutic interventions and clinical diagnoses, as well as to identify new biomarkers for nephrotoxicity. Hence, we also believe that new strategies are paramount for advances in this area. Moreover, alternative methods, such as those addressed in this review, represent important tools that can shift the paradigm of nephropathy and the way new, more effective, and safer molecules can be developed.

5.2. *In vitro* models

In vitro toxicity studies, whether cell or tissue, have been used to study the mechanisms of action and toxic effects of drugs in order to assess efficacy and safety in humans regarding exposure to xenobiotics, and may also help to classify them according to their toxic capacity. The knowledge of the mechanism of toxic action involving a xenobiotic is of paramount importance, since it provides significant aid in the evaluation of predictive values, in addition to improving the selection of new drugs to treat specific pathologies (Eisenbrand et al., 2002; Descotes, 2003; Bernauer et al., 2005).

The number of *in vivo* studies has grown proportionally to *in vitro* studies (FIG.2) according to our search. To find these numbers we used the keywords listed below.

FIGURE 2

Despite this, such models have demonstrated limitations in generating knowledge of nephrotoxic processes in humans. By contrast, there is an increasing demand for new methods that refine, reduce, and replace animal use. Cell culture techniques are extremely relevant tools for *in vitro* studies of nephrotoxicity. The number of studies with animal models and *in vitro* renal toxicity has grown in the last 20 years. Despite this increase of both strategies, no reported results have provided a better understanding of nephrotoxicity mechanisms (FIG.2).

It is difficult to study specific epithelial cell types because the renal cortex carries a heterogeneous population of renal tubule segments. Fortunately, improvements in the methods used to grow homogeneous cultures of kidney cells allow one to access proper cells over different portions of the nephron. Other possibilities are the 3D printing of scaffolds to test the efficacy of new drugs, which will be different in scale and throughput from the assays used in the initial toxicological screening of chemicals (Innovate UK, 2015).

Regardless of these advances, the kidneys produce very complex structures with blood filtration and urine re-absorption units needed for their function and homeostasis. It will also be highly important to consider the quality control of kidney organoids for disease modeling, added to the potential sources for developing kidney regenerative therapies. Conversely, there is currently no consensus regarding the methods that should be used for the evaluation of the quality of kidney organoids, mainly in terms of their functional characterization (Morizane and Bonventre, 2017).

Recently, the mouse ENCODE Consortium reported that, while there are many similarities between human and mouse genomes, there are important differences as well. Consequently, animals are not good models for nephrotoxic effects in humans. Many discrepancies in DNA and differences in gene expression patterns have been found, potentially restricting the usefulness of some disease models based on mice (Yue et al., 2014). More than 160 inherited genetic kidney diseases are now known.

Alternatively, by establishing human induced pluripotent stem cells (hiPSCs) from patients with a given genetic disease, such as degenerative disorders and cancer, it might be possible to study customized disease mechanisms and to perform drug screening *in vitro* instead of using animal models. Most importantly, updated advances in genome editing have provided new approaches to modeling genetic kidney diseases by using human pluripotent stem cells (hPSCs) *in vitro* (Cheng et al., 2014; Dakhore et al., 2018; Devuyst et al., 2014; Stergachis et al., 2014).

In addition, the market potential for NATs (Non-Animal Tests) is enormous. Approximately $100 billion was spent on R&D in 2014 by the top 25 pharmaceutical companies (based on recorded global sales for 2014) and a recent survey of the members of Pharmaceutical Research and Manufacturers of America estimated that more than 20% of total R&D expenditure was on preclinical research (Innovate UK, 2015; Pharmaceutical Research and Manufacturers of America, 2013).

In the long term, there is the potential to use NAMs (Non-Animal Models) to support the development of personalized medicines through the use of human-based approaches that will enable the identification of the possibility for side effects caused by drugs, or variations of efficacy in the population. The selection of patients on the basis of their predicted response allows a drug to continue through its development into clinical use in circumstances where its progress would otherwise have been suspended. This could bring enormous benefits to both patients and companies (Innovate UK, 2015).

In addition, there is an increasing demand for new methods which refine, reduce, and replace animal use. Cell culture techniques are extremely relevant tools for *in vitro* studies of nephrotoxicity. The number of studies with animal models and *in vitro* renal toxicity has grown in the last 20 years. And despite this increase in both strategies, it has not resulted in a better understanding of nephrotoxicity mechanisms (FIG.2).

6. New technologies

While *in vitro* models have helped us to understand nephrotoxicity mechanisms, it is paramount to use new technologies, such as omics (transcriptomics, proteomic, and metabolomics), bioinformatics platforms, CRISPR/Cas9 genome-editing and *in silico* modeling to advance this process. As shown in FIG.3, these new technologies have been increasingly applied to study renal toxicity.

FIGURE 3

6.1. Omics technologies

Omics technologies include transcriptomics, proteomics, and metabolomics and are methodologies applied for biomarker identification useful in understanding the mechanisms of nephrotoxicity. Such technologies offer unbiased approaches to identify new biomarkers of AKI. Circulating or urinary microRNAs are being evaluated, and kidney-specific DNA methylation patterns are also being analyzed. Researchers have also been paying attention to urinary extra cell vesicles, which contain mRNA, microRNA, and proteins that mimic the cell physiology and pathophysiology along the nephron. The components of urinary extracellular vesicles are a source of information about disease pathogenesis, and they may serve as diagnostic and prognostic biomarkers, since they may enable target identification for drug discovery or provide evidence of biological activity in response to therapeutic agents. Among other objectives, omics studies seek to identify those that are mainly descriptive in nature, through the integration of experimental approaches and new computational modeling, such as in systems genetics, which will be generated in order to help identify marker sets to guide diagnoses, monitor disease progression, and identify new therapeutic targets (Ho et al., 2014; Kumar et al., 2014; Marx et al., 2018).

The European Consortium for High Throughput Research in Rare Kidney Diseases (EURenOmics) has been working on a cohesive bioinformatics platform to study rare nephropathies. Additionally, a renal phenomenon database is being created, using the Human Phenotype Ontology website. The phenotype information will be linked to genomic, transcriptomic, proteomic, and metabolomics studies, omics datasets, and the public domain knowledge-base in a systems biology approach to identify molecular pathways associated with phenotypic features (Levy and Myers, 2016).

This situation thus becomes a great opportunity to advance *in vitro* studies for renal disease by analyzing gene expression. A bioinformatics platform will be an important tool to identify biomarkers that could be paramount in developing new medicines as well as in identifying nephrotoxicity and stopping the processes of AKI and CKD. Bioinformatics platforms are able to respond to such challenges as renal disease.

6.1.1. **Transcriptomics**

Studies on transcriptional profiling have revealed molecular markers and potential regulatory pathways of renal repair. A few key markers become active on the developmental pathways that have been reported during repair. By using high-resolution technologies, such as RNA sequencing and translational profiling specific to cell compartments within the kidney, further insights into injury and repair processes will be gained through the study of repairing transcriptome and cell-specific translatome. An enhanced understanding holds promise for both the identification of modern therapeutic targets and biomarker-based evaluation of the damage-repair process (Kumar et al., 2014).

Both proximal and distal tubular epithelial cells mount an acute transcriptional response to ischemia reperfusion injury (IRI). The earliest genes to be induced after *in vivo* injury (within 4 h after injury) include *Fos*, *Jun*, and *Egr1* (Ouellette et al., 1990). *Fos* is induced predominantly in the thick ascending limb (TAL) (Witzgall et al., 1994). The latter observation suggests that the distal tubule, in addition to the proximal tubule, also senses the acute insertion. Subsequent microarray-based gene expression profiling studies encountered a similar immediate-early response, including *Fos* and *Egr1*, after IRI (Supavekin et al., 2003; Yuen et al., 2006). It is possible to identify toxicity on proximal and distal tubules, which will create an enormous advantage. Consequently, gene expression studies using tubular and renal cells may help to identify earlier nephrotoxicity (Kumar et al., 2014).

Adler et al. (2016) showed that primary human proximal tubular epithelial cells (HPTEC) are a good in vitro model for screening for renal toxicity because they have differentiated epithelial cell characteristics. In addition, HO-1 expression proved to be a better option than currently used to predict toxicity in HPTEC, especially when combined with the total number of cells in the same assay. In addition, the approach used by the authors can be used to screen a large number of compounds besides can be used in combination with existing cytotoxicity assays (Adler et al., 2016).

The understanding of how the several cell types in the kidney communicate, in order to regulate the intrinsic repair mechanisms and their contribution to post injury fibrosis, remains a substantial challenge. Nonetheless, a continued focus is still placed on cell-type–specific signatures and a broadening of analysis beyond early injury, which in turn makes it feasible to provide important new insights. Clearly, the major clinical objective is to develop new analytical tools to diagnose both short- and long-term outcomes, as well as to develop new therapeutic strategies to improve existing repair processes and reduce the long-term risk of CKD after AKI (Devuyst et al., 2014). In sum, all of these strategies could help to develop new agents to treat renal disease.

6.1.2. **Proteomics**

Proteomics is considered one of the most promising ‘omics techniques for biomarker discovery in AKI, as it may identify specific molecular targets for early injury in different renal compartments. Almost all human diseases are characterized by a complex panorama at the molecular level, and it is imperative to acquire a global proteome picture to depict pathways and proteins with pivotal roles in pathogenesis.

Proteomics studies have indicated that a single biomarker cannot fully account for the complexity of human diseases, and thus it is preferential to use biomarker panels.

Hence, it is thought that proteomics is a good tool for discovering and identifying new premature biomarkers. The main target of proteomics is to characterize the information flow within the cell and the organism, through protein pathways, interactions and networks, thereby allowing a hypothesis-free identification of disease biomarkers (Rosner and Okusa, 2006). The search for biomarkers using urinary proteomics approaches has proven to be effective in CKD, diabetic nephropathy, acute kidney injury, ureteropelvic junction obstruction, vesicoureteral reflux, renal Fanconi syndrome, acute renal allograft rejection, cancer, diabetic nephropathy, chronic rhinosinusitis, atherosclerosis and calcific aortic valve disease, and posterior urethral valves (Di Carlo et al., 2018; Marx et al., 2018; Mulligan et al., 2018; Neufeld et al., 2014; Patti et al., 2012; Salvadori and Tsalouchos, 2017; Van et al., 2017).

6.1.3.**Metabolomics**

Metabolomics is defined as the analysis of molecules smaller than 1,000 Da, which are transformed as a result of, and in support of, an organism's metabolism. The metabolome accounts for a complete set of metabolites that can be produced and consumed by organisms (Alonso et al., 2015; Patti et al., 2012). The results of metabolomics experiments constitute big data and require sophisticated data handling strategies and advanced statistical tools. In addition, before any statistical analysis, data preprocessing must take place to ensure the best possible results from the applied statistics (Marx et al., 2018). Spectral alignment, normalization, transformation, and scaling are indispensable stages in metabolomic data preprocessing. Data normalization is required for accurate metabolite quantification. To normalize data, a common method is based on endogenous metabolites, such as urinary creatinine. However, creatinine itself may be somewhat variable, and subject to variation owing to diseases such as AKI (Alonso et al., 2015; Frank Dieterle et al., 2006; Marx et al., 2018).

The potential to identify drug targets from metabolomics will be amplified by the coordination of efforts to analyze samples of human kidney tissue and other biomaterial, such as urine and blood, by using state-of-the-art genomic, proteomic, and metabolomic approaches, together with detailed patient phenotyping and the use of existing biomarkers to discover and qualify new therapeutic purposes. Genetic data should be linked to existing phenotypic information or generated from customized human tissue models, by using induced pluripotent stem cells and targeted mutation, followed by differentiation to human kidney tissue. To give support to these efforts, enhanced disease prototypes are needed to reflect the complexity of CKD (Nicholson and Lindon, 2008; Patti et al., 2012).

Equally challenging is the multifactorial origin of AKI and the fact that the changes of molecular expression induced by AKI are difficult to distinguish from those of the diseases associated with or causing AKI, such as shock or sepsis(Marx et al., 2018). In the past, these experiments were focused on a single metabolite that was attributed to a specific disease or enzymatic reaction (German et al., 2005). Nowadays, such technologies as GC/MS (gas chromatography-mass spectrometry) and computational tools allow for a more extensive and wide-ranging investigation of many metabolites within a single

measurement, providing a broader insight into mechanisms of diseases (Marx et al., 2018). For this reason, it is very important to employ a panel of biomarkers.

New and cutting-edge techniques have been, and continue to be, developed; the challenge with the present status of metabolomics is its transition to clinical use. Integrating metabolomic data with other omics data for the purpose of drug discovery, and development is one direction in which this field may proceed (Kell and Goodacre, 2014; Robertson and Frevert, 2013).

The low sensitivity of current clinical markers (serum creatinine and blood urea nitrogen- BUN) in early stages of the development of acute kidney injury limits their utility. Rapid LC/MS (liquid cromatography-mass spectrometry)-based metabolic profiling of serum was demonstrated in a key study showing that metabolomics could provide new indicators of AKI. Currently, omics technologies provide a great opportunity for research in rare renal diseases, as it is possible to probe the diseased organ directly (Sun et al., 2012). Hence, we believe it is important to compare results obtained from patients and to evaluate them using cells, mainly because there are several different types of renal disease. This would thus be a safer strategy for using clinical results as a starting point.

6.2. CRISPR/Cas9 genome-editing

The fast advancement of genome-editing technologies has provided new pathways in the study of the genetic basis of human diseases and in the development of targeted therapeutic strategies that would not have been possible with traditional pharmacological drugs. Chemical toxicants act by many different mechanisms, however, the genes involved in adverse outcome pathways (AOPs) and AOP networks are not yet characterized. Functional genomic approaches can reveal both toxicity pathways and susceptibility genes, through knockdown or knockout of all non-essential genes in a cell of interest, and the identification of genes associated with a toxicity phenotype following toxicant exposure (Lin and Musunuru, 2016; Shalem et al., 2014; Shen et al., 2015).

In this environment, new strategies could improve the engineering of genome-editing tools, and appropriate regulatory practices, together with genome editing, will accelerate discoveries in basic science and clinical translation, which will in turn aid in the process of identifying new biomarkers to become more robust. Hence, advances in genome editing with CRISPR/Cas9 will promote the use of kidney organoids to study inherited genetic kidney diseases (Lin and Musunuru, 2016).

The potential use of kidney organoids derived from hPSC combined with genome engineering technologies, particularly the CRISPR/Cas9, is a novel approach for the targeted modification of the renal epigenome to study renal organ dysfunction, such as CKD (Hurtado Del Pozo et al., 2018). In addition, this technology could be further used to produce kidney phenotypes.

6.3. *In silico*

In silico, or computational, approaches to predicting and identifying compounds associated with kidney disease are based on the use of existing data and the ability to draw chemistry-based inferences

from those data (Cronin and Madden, 2010). The concepts are relatively trivial in that, should data of acceptable quality be available for a compound, then there is no need for further testing. If no suitable data are available for a compound, then inferences can be made from it, e.g., making an analogy with similar compounds or identifying chemical / molecular features that may be indicative of the ability to cause disease. All of these resources together form a battery of computational approaches, or even of a compound, that can shed light on the disease-causing potential (Cronin and Yoon, 2018). The general availability of data related to the ability of a compound to cause damage to the kidney has been reviewed by Pletz et al. (2018) (Pletz et al., 2018). The resources can be classified according to the type of data they represent. The most relevant for human risk assessment are those for the effects of compounds in humans; these are available as part of clinical trial data or from reports of adverse drug reactions. Websites, such as www.clinicaltrials.gov, are useful to retrieve such data. While potentially relevant, they can only be used for the drugs to which they relate and lack consistency between clinical trials. To develop and evaluate *in silico* models, the results of animal tests done in the past are now useful, particularly low-dose chronic toxicity testing that show the superior predicting power of *in silico* models. Several resources have been compiled of repeated dose toxicity values and are freely available. These become useful in understanding and evaluating effects to the kidney when they record organ-level effects. The COSMOS database was developed specifically to achieve this and can be searched for organ-level toxicity, including effects to the kidney and bladder (Mostrag et al., 2018). Other similar databases of repeated-dose toxicity data include REPDOSE (Bitsch et al., 2006) and HESS (Sakuratani et al., 2013). Other significant data resources of repeated-dose toxicity values that may require licensing and/or payment include Chemtunes (MN-AM, https://www.mn-am.com/products/chemtunes), Vitic Nexus (Lhasa Ltd, https://www.lhasalimited.org/products/vitic-nexus.htm), and LeadScope Toxicity Database (Leadscope Inc, http://www.leadscope.com/product_info.php?products_id=78). It is important to note that while these data resources may shed light on which compounds may promote disease, or damage, to the kidney, they were not developed specifically for this purpose.

There are fewer databases for *in vitro* data. However, one important resource that will aid in understanding, from the mechanistic point of view, is the compilation of the United States Tox21 initiative. Tox21 set about to screen large numbers of compounds (currently over 10,000) with high content assays, some of which are relevant to the effects and mechanisms of kidney disease and damage(Richard et al., 2016). This immense and freely accessible resource is available through the US EPA's Chemistry Dashboard(Williams et al., 2017). The Chemistry Dashboard is a cutting-edge and rapidly evolving resource for *in silico* assessment. It enables a user to input a chemical structure, retrieve existing (*in vivo* and Tox21) data, make predictions, and build knowledge about a substance. It is currently focused on a number of environmental and human health effects and could be applied to provide information on kidney disease if used appropriately, i.e., the data can assist in the identification and rationalization of chemicals associated with disease. Another important *in silico* tool that brings together data through the development of chemistry-based groups is the OECD QSAR Toolbox (https://www.qsartoolbox.org/). Designed for grouping and read-across to allow for data gap filling, the Toolbox includes access to mechanistically based profilers to find similar molecules.

One of the most powerful methods to use existing data to make predictions for compounds with no data (the aforementioned process of data gap filling) is to form groups of similar compounds and "read-across" data from one molecule to another (Patlewicz et al., 2018). The use of the read-across paradigm has seen growth in recent years, most notably due to its application in the European Union's Registration, Evaluation, Authorization and restriction of Chemicals (REACH) legislation (Spielmann et al., 2011). The identification of similar compounds is a broadly applied concept and requires a certain basis. One approach has been to use markers of organ-level toxicity to define similarity (Cronin and Richarz, 2017). What seems particularly relevant here are the chemistry-based structural alerts or biomarkers for kidney disease. Thus, should compounds be associated with the same chemistry alerts or biomarkers, then similarity in activity may be assumed, i.e., a prediction of harm can be made by analogy to the similar substance. While conceptually simple, there are numerous caveats to the approach and care must be taken (Schultz and Cronin, 2017). One means of providing relevance from the *in silico* side is to ensure that the alerts are anchored on a mechanistic basis and specifically to relevant AOPs (see section below).

The logical identification of structure-activity relationships related to kidney disease, i.e., the sub-molecular features intimately associated with damage, has enabled the concepts of structural alerts to be defined. Such alerts are easily coded into software. Some structural alerts for kidney damage are available in the literature; for example, Myshkin et al. (2012) (Myshkin et al., 2012) published alerts for a number of effects. Efforts are currently underway to rationalize the small number of publicly available structural alerts for harm caused to the kidney and to add new alerts where they are missing. This work is being supported by a compilation of over 200 compounds known to cause harm to the kidney (Pletz et al., 2018), which have assisted in the evaluation of existing alerts and will enable efficient screening of new molecules for these effects.

Historically there have been many efforts to form quantitative associations between chemical structure and biological activity – the development of the so-called Quantitative Structure-Activity Relationships (QSARs) (Cronin and Madden, 2010). It is fundamental that QSARs require suitable data for development and, ideally, should be mechanistically based. As a result, particularly of the need for robust data sets, there have been relatively few QSAR models developed for nephrotoxicity; available models have been reviewed by Pletz et al. (2018) (Pletz et al., 2018). For instance, Lei et al. (2017) (Lei et al., 2017) used machine learning approaches to develop models for damage to the urinary tract. While some progress has been made, the development of QSARs for the full suite of harmful effects to the kidney is limited at the current time, but it can be expected to improve due to the better access to reliable data for modeling.

Using human renal proximal tubular cells (PTC) and a set of 44 compounds, subdivided into PTC-toxic, non-PTC-toxic and non-nephrotoxic compounds, Su et al. (2016) described an approach based on high-throughput imaging, phenotypic profiling and machine learning methods to predict human nephrotoxicity. This approach does not require characterization of injury mechanisms and is capable of identifying structurally distinct xenobiotic-induced nephrotoxicity that lead similar phenotypic endpoints as from six sets of nuclear and actin cytoeskeletal features with high efficiency and accuracy (Su et al., 2016).

In addition to using information from chemistry for modeling, according to Soo et al. (2018) (Soo et al., 2018), integration of information obtained from *in vitro* models into computational algorithms that incorporate patient-specific physiological parameters will not only reduce late drug loss from the development pipeline, but also facilitate the development of safer drugs and improve compound management of clinically important adverse nephrotoxic effects.

6.4. Stem cells

Stem cell therapy is an innovative approach to ameliorate IRI due to its antioxidative, immunomodulatory, and anti-apoptotic properties. However, the majority of the studies are confined to experimental animal models, although, several studies have investigated Mesenchymal stem cells -based therapies for both acute and chronic kidney disease in clinical trials. Hence, it is paramount to understand the biological effects and mechanisms of action of stem cell therapy to improve its therapeutic benefits. In addition, more translational studies are needed to provide a more comprehensive understanding of stem cell-based therapies and to ensure their safety for future clinical applications (Lee et al., 2019; Li and Li, 2019).

7. New models

New models such as organoids and 3D bioprinting also are important tools which may help us to understand nephrotoxicity mechanisms.

7.1. Organoids

Nephrons are complex architectural 3D structures. Hence, to recapitulate these structures *ex vivo*, the development of 3D culture systems is required. Organoids are 3D organ-like tissues that mimic *in vivo* organs structurally and functionally in culture plates. Kidney organoids derived from hPSCs are an attractive approach for studying mechanisms of human inherited kidney diseases, which might be applied to more common diseases as well as to the development of new drugs using human tissues, which may facilitate the translation of the findings obtained with this methodology to humans. However, to optimize this approach, differentiation protocols, genetic background, and epigenetic variation need to be considered in depth when disease phenotypes are analyzed in kidney organoids (Morizane and Bonventre, 2017).

One goal of hPSC studies is to regenerate the kidney function. Kidneys form complex structures with blood filtration, and the urine re-absorption unit is paramount for their functioning and homeostasis. There are many challenges in the use of organoids to generate functional bioengineered kidney tissues. Vascularization of kidney organoids needs to be induced in an organized way to direct blood flow from arteries in order to drain into venous structures. This represents one of the great challenges related to vascularization (Morizane and Bonventre, 2017).

7.2. 3D bioprinting

In addition, the use of 3D bioprinting has proven to be an option to study nephrotoxicity, namely a functional bioengineered kidney with vasculature, multiple cell types, which include proximal tubular cells, and an extracell matrix. A new system is being used to generate heterogeneous structures in 3D that enable the perfusion of liquid into lumens lined with vascular or tubular cells. It may be employed to mimic blood flow and intratubular flow in vascular and tubular channels (Kolesky et al., 2016, 2014; Morizane and Bonventre, 2017).

8. Adverse Outcome Pathways (AOP)

The AOP framework is a systematic, transparent approach used to organize existing toxicological knowledge and translate mechanistic information to adverse effects at higher levels of organization based on causal relationships between endpoints (FIG. 4). As such, the AOP framework has been proposed as a means to relate alternative types of data (*in silico, in vitro* and *in vivo*, biomarker-type data) to endpoints of concern to chemical risk assessors (e.g., survival, growth, and reproduction). This application in the regulatory field is timely given the current momentum to move toxicology from broadly empirical *in vivo* assessments of unique chemical effects to predictive approaches that make use of newer tools and alternative data (Ankley et al., 2010; Fay et al., 2017; National Research Council, 2007).

With reference to AOPs for renal disease, a small number of studies have been performed using molecular initiating events (MIEs) to identify the following AO: adenomas and carcinomas, and renal failure and mortality, respectively. Most authors do not inform about the progress of the studies (https://aopwiki.org/aops).

FIGURE 4

Hence, it is paramount to use new tools in order to develop renal AOPs. Some studies have been performed utilizing biochemistry (Ferreira et al., 2016; França et al., 2014b, 2014a) and genomics (Campos et al., 2018; Grossi et al., 2017; Silva et al., 2017). However, it is important to make these data public through database, like the Comparative Toxicogenomics Database, in an attempt to achieve faster and more reliable results.

Thus, in terms of using the biomarker data, the AOP paradigm is a useful starting point. There are many advantages to the use of AOPs, including the rationalization of response and the ability to form the basis of quantitative AOPs, dissemination through the AOP-wiki, as well as the elucidation of the MIE (Cronin and Richarz, 2017). In addition, the use of the MIE of an AOP is a robust means of developing structural alerts and has been applied for other organ-level toxicities (Cronin and Richarz, 2017; Nelms et al., 2015). At the time of writing this chapter, structural alerts for nephrotoxicity that allow for grouping and read-across are still under development (Pletz et al., 2018).

It is clear that AOP will greatly help reduce animals in research involving nephrotoxicity, but it also uncovers the molecular mechanisms that may speed up the discovery of biomarkers for disease staging and therapy individualization, as well as the design and testing of novel therapeutic strategies that are safer to be developed, together with diagnoses and more accurate treatments.

Conclusions

The failure of existing animal models to accurately predict nephrotoxicity has been a major barrier to the development of safer drugs. The failure has mainly been due to the complexity of the physiology of the kidney, coupled with the variety and mechanisms of potential adverse effects, which animals are not able to identify. The application of advances in the understanding of molecular signaling and function would be a great benefit to find a means through which to prevent toxicity and/or treat kidney injury. Thus, new tools have been implemented, using alternative methods in order to elucidate mechanisms of nephrotoxicity. The use of *in vitro* models is at the forefront of the understanding of functional maturity and injury responses caused by drugs. However, it is essential to integrate existing *in vitro* methods with new technologies. For instance, organoids, 3D bioprinting, omics (transcriptomics, proteomics, and metabolomics), bioinformatics platforms, CRISPR/Cas9 genome-editing, and *in silico* modeling have been proposed as potential new tools to understand and identify nephrotoxicants. The main goal of the use of new and existing technologies will be to generate an integrated model in which new biomarker(s) and/or pathway(s) involved in nephrotoxicity can be established as early as possible. The use of AOPs is helping to organize the information from the new technologies and the Molecular Initiating Event is expected to represent a robust means to detect toxicity. Therefore, combining a panel of biomarkers may improve the performance of the interpretation and predictability of nephrotoxicity. Gene expression integrated with computational analysis is also a key area that will facilitate the understanding of nephrotoxicity mechanisms and will consequently aid in the development of safer new drugs and be useful in the detection and monitoring of adverse effects from nephrotoxicants.

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Supplementary files

S1: Animals utilized in renal toxicity

((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND ((animal model) AND (monkey)); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND ((animal model) AND (rat)); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND ((animal model) AND (mouse)); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND ((animal model) AND (rabbit)); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND ((animal model) AND (guinea pig)); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND ((animal model) AND (zebrafish)); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND ((animal model) AND (pig OR swine)).

S2: *In vivo* and *in vitro* models utilized in nephrotoxicity

((renal OR kidney or nephron) AND (animal model or in vivo) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2000-2002; ((renal OR kidney or nephron) AND (animal model or in vivo) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2003-2005; ((renal OR kidney or nephron) AND (animal model or in vivo) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2006-2008; ((renal OR kidney or nephron) AND (animal model or in vivo) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2009-2011; ((renal OR kidney or nephron) AND (animal model or in vivo) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2012-2014; ((renal OR kidney or nephron) AND (animal model or in vivo) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2015-2017.

((renal OR kidney or nephron)) AND (In Vitro) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2000-2002; ((renal OR kidney or nephron)) AND (in vitro) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2003-2005; ((renal OR kidney or nephron)) AND (in vitro)AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2006 -2008; ((renal OR kidney or nephron)) AND (in vitro) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2009-2011; ((renal OR kidney or nephron)) AND (in vitro) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2012-2014; ((renal OR kidney or nephron)) AND (in vitro) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) 2015-2017

S3: New technologies used to study renal toxicity

((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (organoid); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (computer simulation); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (genomics); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (transcriptomics); ((Renal OR Kidney OR nephron) AND

(nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (proteomics); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (metabolomics); ((renal OR kidney OR nephron) AND (nephrotoxicity OR toxicity OR nephrotoxic OR toxic)) AND (computational biology); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (bioprinting); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (CRISPR); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (genome editing); ((Renal OR Kidney OR nephron) AND (nephrotoxicity OR Toxicity OR nephrotoxic OR toxic)) AND (cell spheroids)