Methodology for the Development and Validation of New Stent Designs: *In Vitro* and *In Vivo* Models

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1 *In-Vitro* Encrustation Models: A Critical Review

Implantation of biomaterials into the urinary tract is hampered by crystal formation, bacterial adherence and, ultimately, encrustation through biofilm formation resulting from a multifactorial disturbance of the delicate balance between numerous physico-chemical and biochemical processes. Non-infectious stone formation and encrustation usually result from metabolic imbalances, often on the tubular level. In contrast, infectious stone formation and biofilm-induced encrustation are linked to the enzymatic activity of bacteria. Best known are urease-producing species such as *Proteus mirabilis*, which increase the pH of the urine. This alkalization, in turn, decreases the solubility of urinary calcium and magnesium salts and thus facilitates encrustation.

Consequently, the use of urinary implants is complicated by several factors stent surface encrustation through deposition of crystal-forming urinary ions, bacterial colonization and biofilm formation despite antibiotic treatment and prophylaxis, mechanical irritation of the urothelium by encrustation, and alterations of urine flow in and around the stent due to encrustation [1].

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The development of *in vitro* models to simulate bacterial infections and biofilm formation started after the initial observation of sessile bacteria and their role in chronic infections in humans. Biofilms form an irregular network matrix. They protect the bacteria from physical, chemical and biological stresses. Shear stress caused by the flow of the fluid medium is hereby one of the main factors impacting on the formation of a stable biofilm.

Early approaches focused on the use of continuous flow systems, such as the chemostat model, which had the advantage of a regular supply of fresh fluid medium whilst maintaining a constant volume [2]. Many *in vitro* models designed to mimic encrustation on urological devices have been derived from classical microbiological approaches, and often do not reflect important physiological factors such as the complex and variable physico-chemical urinary environment *in vivo,* or infection with mixed species.

In 1973, Finlayson and Dubois described a dynamic flow *in vitro* encrustation model which used both, a constant flow of artificial urine and a magnetic stirrer [3]. A number of adaptations to this model have been devised over time to enable the study of urinary encrustations utilizing both, human and artificial urine [4]. Depending on particular research questions, two groups of *open* systems were designed: The Continuous Flow Stirred Tank Reactor (CFSTR) and the Plug Flow Reactor (PFR). The Modified Robbins Device (MRD) was designed to monitor biofilm formation with different flow speeds in an axial direction, and in a completely mixed reactor using diffusion. This PFR-system consists of a pipe with multiple threaded holes containing coupons. The biofilm reactor of the Center for Disease Control (CDC) is a current, commercially available flow-based CFSTR-system. A vessel with a polyethylene lid bears independent rods housing removable coupons. Inside the reactor, there is a rotating magnetic stirrer exerting a constant high shear force on the coupons. The number of revolutions can be varied and is independent of the feed speed. The system allows for a perfect mixing and operates at a steady state. With this system, structure and physiology of biofilm formation can be monitored by confocal laser scanning microscopy (CLSM) in a non-invasive fashion [5]. The CDC biofilm reactor is indispensable for prototype testing, but less suitable for screening testing. Another disadvantage of the semi-open design of the CDC reactor is its susceptibility to contamination.

This led to the development of high-throughput static biofilm models. Microtiter plate (MTP)-based static systems are the perhaps most commonly used biofilm model systems. They are an important tool to study especially the early stages of biofilm formation. In these systems, biofilms are typically grown on either the bottom or the sidewalls of a MTP. MTP-based systems are *closed* systems without in- or outflow from the reactor. Consequently, during an experiment the composition of the environment inside the well of an MTP changes. Nutrients are depleted whilst signaling molecules accumulate. It has been suggested that a part of the accumulated biomass may not result from biofilm formation, but rather from cell sedimentation and subsequent entrapment of cell sediments within the matrix of extracellular polymeric substances (EPS).

The Calgary Biofilm Device (CBD) represents a modification of the MTP-based systems, where biofilms are formed on lids with rods that fit into the bacteria-containing wells of the MTP. A newer system to study biofilm formation and encrustation on implants uses this CBD as a commercially available high-throughput screening assay. However, the lid is configured in such a way that materials are held in a matrix. The bottom is a welled plate into which the implant materials to be tested can be inserted. The matrix in combination with the high-throughput capability of the assay allow the study of several encrustation parameters. The use of MTP-based assays offers many of advantages. MTP are cheap and they provide the opportunity for multiplexing, as multiple organisms and treatments can be incorporated in a single experimental run [6].

Both, MTP/CBD-based and flow-based systems share some limitations. One common pitfall in designing *in vitro* biofilm models is the use of bacterial strains with a low virulence which, in turn, results in a low translation rate from *in vitro* to *in vivo* studies.

Most *in vitro* encrustation models use synthetic urine, based on urease reactions or urease-producing bacteria. However, in real life most urinary tract infections are caused by *E. coli*. These are acid-producing, and, consequently the urinary pH does not increase. Whilst models using urease-related alkalization are relatively easy to design, the multifactorial physiological conditions in stone- and encrustation formation are not properly represented. In fact, 80% of all urinary stones and probably most urinary implant encrustations consist to a large part of calcium and oxalate. Only 10% of urinary stones contain uric acid crystals, and struvite as a typical infectious stone is clinically found in less than 10% of urinary stones, typically in alkaline urine with a pH > 7. Yet, alkalization models do focus on this group of stones.

In clinical practice, guidelines mandate that urinary catheters and stents with such infectious stone encrustations must always be removed due to the presence of inactive bacteria protected by the biofilm [7]. Using these models seems therefore non-relevant for the development of new stents for a large target population of patients.

The above-mentioned encrustation models could be complimented by *in vitro* calcium oxalate crystallization methods from urolithiasis research. There are different options to choose from. These vary from simple experiments in defined inorganic solutions to whole human urine experiments replicating urine flow dynamics [8]. Currently, models are being developed that combine the advantages of continuous flow and static models. One such system is the *stent-on-chip* microfluidic model (SOC). SOC tries to simulate the hydrodynamic areas of a stented ureter under physiological conditions, including drainage holes and the cavity formed by a ureteral obstruction. Encrustation formation over time is monitored and measured by optical microscopy [9].

For the future, examination of the urinary microbiome may provide promising insight into the underlying mechanisms of biofilm formation and encrustation on urinary implants. It has been suggested that the urinary tract is not, contrary to earlier assumptions, a perfectly sterile environment and that commensal bacteria may

play a role in patient susceptibility to infection and in the composition of the urinary microbiome associated with stent complications [10].

OMICs (genomics, transcriptomics, proteomics and metabolomics) have improved our understanding of microbial interactions in the urinary tract. It is now possible to identify all microbial species that colonize the urinary tract. Combining results from OMICs studies with *in vitro* biofilm research has the potential of making a real impact in clinical practice in the future.

2 Preclinical *In Vivo* Evaluation of Urinary Stents

Experimental *in vivo* trials represent the final step in the preclinical validation of a medical device. These *in vivo* evaluations should be preceded by the corresponding *in silico* simulations, *in vitro* and *ex vivo* studies of the newly developed device. The urinary tract constitutes a complex dynamic environment with a high variability, where *in vitro* and *ex vivo* models often fail to reflect certain factors that are decisive for the safety and effectiveness of a urinary stent. These factors include urodynamic behavior of the urinary tract, the changing physico-chemical conditions and the multifactorial nature of urinary tract infections, biofilm and encrustations. Besides, ureteral peristalsis and the potential presence of vesicoureteral reflux may play a crucial role in the success of new designs of ureteral stents [1, 11, 12].

Prior to its translation into a clinical setting, the safety and performance of a urinary stent requires to be tested in a whole organism, provided currently by animal models. Animal models overcome the aforementioned limitations of reproducibility in laboratory setting and also allow the evaluation of the systemic effect of a new device on the host, including its potential systemic toxicity [13]. The rational sequence of the preclinical assessment of a new stent design or innovation should follow the order from in silico, *in vitro* and *ex vivo* studies, to finally *in vivo* trials. This thus allows the reduction of the number of animal models used to a minimum that provides adequate statistical power, increasing the likelihood of success of these experimental trials and preserving animal welfare [14, 15].

Concerning animal welfare in experimental studies, ethical evaluation of projects involving animal testing is mandatory in the EU since January 2013, through the Directive 2010/63/EU of the European Parliament and of the Council [16]. Establishing the basic rules applicable to the protection of animals used in experimentation and other scientific purposes [15, 16]. In order to ensure moral standards, scientific validity, and public trust, all projects must be evaluated and approved by an ethical committee prior to development. The use of animals for research should be justified by carefully evaluating each procedure, as to the scientific validity, usefulness and relevance of the expected result of that use. The potential harm to the animal will be balanced against the expected benefits of the project [15, 17].

With regard to the translational perspective of animal research, the choice of the species should be based on the similarity of the conditions studied with those of the human being. Ideally, we should seek for the model that provides anatomic,

urodynamic, pathophysiological, histological and biochemical levels as identical as possible to that of humans. Non-human primates represent the closest model in this regard, except for two anatomic variations, they possess unipapillary kidneys and the left kidney lies lower in the abdomen, as opposed to human kidneys [18]. Nevertheless, the scientific literature has not reported the assessment of urinary stents in primates, which may be due to ethical, legal, economic and logistical considerations [16, 19].

2.1 Porcine Model

The porcine species are the animal models most frequently used for the assessment of urinary stent designs. The anatomy of the human and porcine urinary tracts are highly similar, rendering this model ideal for analyzing the behavior of the urinary tract in the presence of new devices [20] (Fig. 1). Pigs have multipapillary kidneys, with 8–12 papillae compared to humans, which usually have 4–18 [21]. Porcine ureters tend to be longer and more tortuous than those of humans [20, 22, 23]. Moreover, porcine renal physiology parallels that of humans with respect to

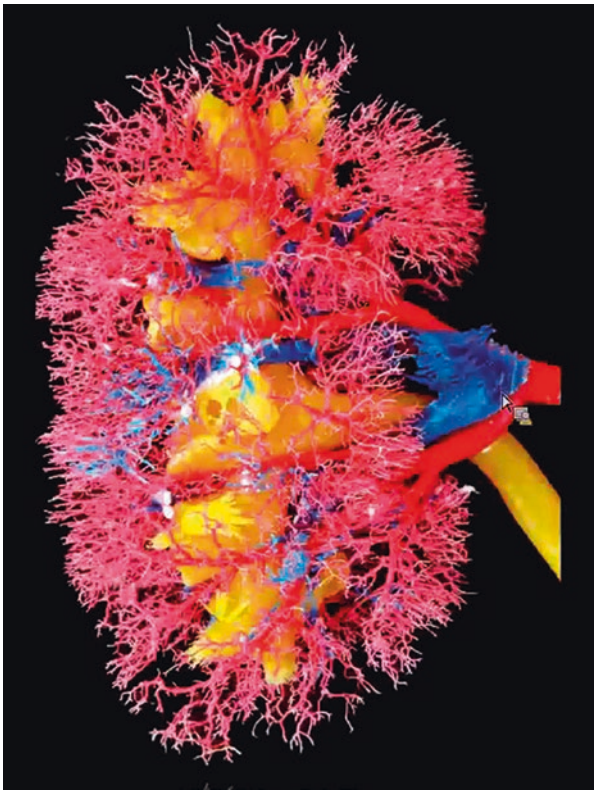

Fig. 1 Corrosion endocast shows pelvicalyceal system and renal vessels. Dorsal view

maximal urine concentration, glomerular filtration rate and total renal blood flow [24]. Since the male porcine urethra prevents retrograde approach due to its sigmoid morphology, research involving endourologic procedures is performed on females. Ideally, interventions should be carried out on 35–40 kg models, as the dimensions of their urinary tract at that weight are comparable to a human adult [25, 26].

The devices assessed in the porcine model are mainly ureteral stents, including polymeric stents, antireflux, biodegradable, drug-eluting and metallic stents [24–29]. This animal enables the transurethral retrograde insertion of the devices, although antegrade and cystostomy approaches have also been described [24, 29–32]. The evaluation of the performance *in vivo* of the urinary devices involves blood and urinalysis, urine culture and imaging tests that include the ultrasonographic assessment of the hydronephrosis degrees [33] (Fig. 2). Radiologic tests comprising excretory urography and retrograde ureteropyelography, provide valuable information on urinary patency, stent migration, radiopacity and fashion of degradation of biodegradable devices [12, 34, 35] (Fig. 3). As a limitation, this animal model prevents the assessment of vesicoureteral reflux by means of a voiding cystourethrography; which can be examined via a simulated voiding cystourethrography [27, 36] (Fig. 4). Histological analysis may be performed for the analysis of biocompatibility, tissue damage and more specifically, of the ureteral healing provided with the stents [34, 36, 37]. In addition, intravesical and renal pressures in stented ureters have also been measured, as well as ureteral peristalsis and contractility [29, 38, 39]. Research on urinary stents in the porcine species is generally performed on healthy intact models. However, pigs may undergo the surgical and pharmacological induction of pathologic features such as ureteral strictures and urolithiasis [31, 35, 40].

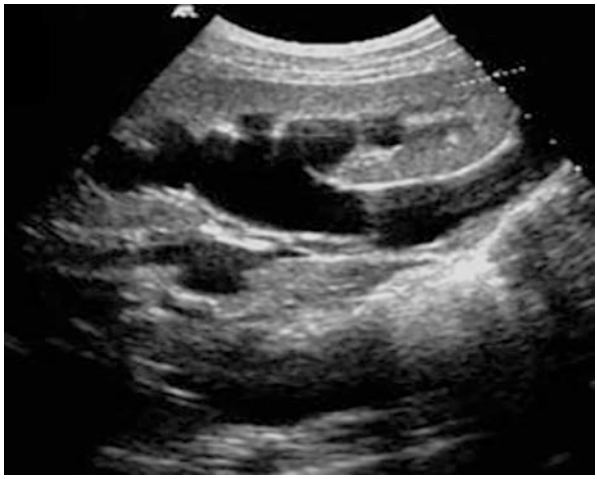

Fig. 2 Ultrasonographic assessment of the hydronephrosis degrees in a porcine model of obstructive uropathy

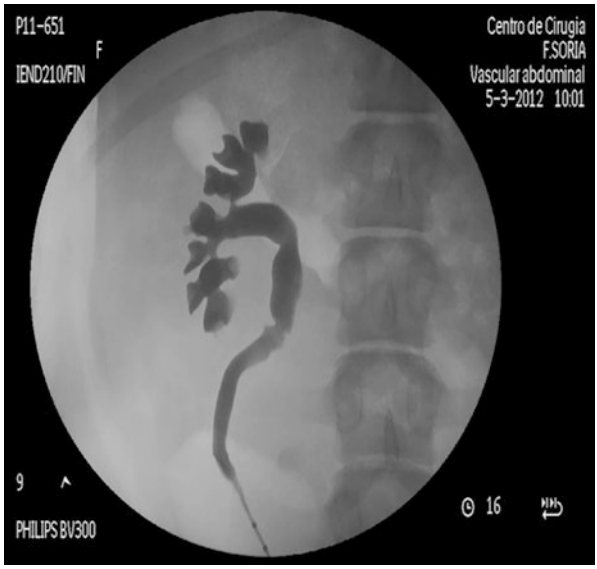

Fig. 3 Retrograde ureteropyelography of the proximal ureter, renal pelvis and calyxes of a healthy porcine model. The use of radiologic catheters with radiopaque marks enables the measurement of upper urinary tract dimensions and perform a follow-up of their development

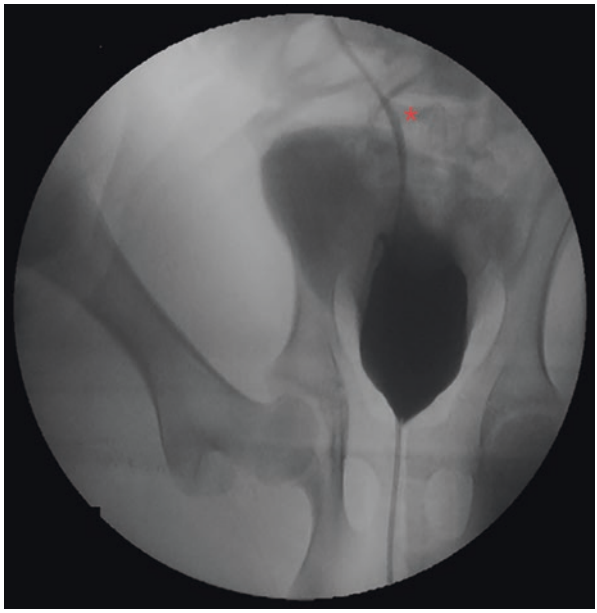

Fig. 4 Simulated voiding cystourethrography in a porcine model stented with a double-j ureteral stent. *Vesicoureteral reflux reaches the lumbar ureter

2.2 Canine Model

The validation of urethral and prostatic stents is generally not performed on pigs, given the particularities of male porcine urethra and the anatomical differences of the accessory sex glands [22]. The dog has proven to be an adequate model for the study of prostate diseases, as it develops benign prostatic hyperplasia (BPH) and prostate cancer both spontaneously and experimentally induced [41, 42]. Metallic, covered, drug-eluting and biodegradable urethral stents have been assessed in healthy and in BPH induced canine models, via transurethral insertion [43–45]. Urethral diameter is measured by means of a retrograde urethrography, which enables the monitoring of position, expansion, patency and migration of the stents [44–46]. Besides, histological evaluation is also included for the follow-up of stent-related urethral damage and urothelial hyperplasia [44, 47]. Nevertheless, the use of urodynamic studies for testing the therapeutic response in BPH canine models does not seem reliable as, unlike humans, canine hyperplastic prostate produces rectal obstruction rather than lower urinary tract symptoms [42].

The canine model has occasionally been chosen for the evaluation of biodegradable ureteral stents [48–50]. Noteworthy, the group of Lumiaho et al., tested their first prototypes of their biodegradable ureteral stent in dogs, placing them with an open surgical approach [49, 50]. The analysis of renal function, ureteral patency and the presence of vesicoureteral reflux are carried out similarly to the methodology in pigs, in addition to renograms [48–50].

2.3 Rat Model

Smaller laboratory animals, such as rabbits and rats, provide the advantages of easier handling, are more cost effective and require less infrastructure and logistics [40]. Unlike porcine and canine models, whose dimensions and anatomy allow the evaluation of the urinary stents that will be tested in future clinical trials, the devices inserted on rabbits and rats may differ from the definitive prototype under development. Small laboratory animals are therefore of great use for the assessment of stent upgrades including biomaterials, coatings and the release of substances [51, 52].

As for the rat model, it enables the analysis of the antimicrobial and anti-encrustation potential of new stents, since urolithiasis and urinary tract infection (UTI) can be experimentally induced in a controlled manner [40, 52]. UTI models are performed by the intravesical instillation of bacterial suspensions, being the most common *S. aureus*, *E. faecalis* and *P. aeruginosa* [52–54]. The induction of urolithiasis in rats to promote stent encrustation is carried out with dietary manipulations, gastrointestinal resections and the administration of lithogenic agents [40]. These animals are often chosen for the validation of both urethral and ureteral stents. Ureteral stents are inserted through a cystotomy in either the bladder or the ureter [51, 55, 56]. Besides the evaluation of the device's performance, when placed in the ureter, ureteroureteral anastomosis may also be performed for the histological analysis of ureteral healing and scarring processes [13, 55, 56]. Urethral stents are tested in the bladder

and the urethra, and depending on stent size and characteristics, transurethral placement may be feasible [57–59]. The rat's urethra allows the detection, as well as the histological analysis, of injuries during stent placement and the development of urethral strictures secondary to fibrotic and hyperplastic tissue formation [59].

2.4 Rabbit Model

The rabbit has been used for biocompatibility studies of stent materials. To this end, stent samples can be inserted in the muscle by blunt dissection, preferably the dorsal muscle to prevent the animal from self-mutilation [60]. The scientific literature regarding urinary stent validation in this animal model is scarce, probably due to the significant differences between rabbit's and human's urine composition [61]. The potential of biomaterials and drug-release against stent-related urinary tract infections has been assessed by transurethral intravesical placement of ureteral stent samples, for the performance of microbiological cultures and histological analysis [62, 63]. The rabbit's urethra enables the evaluation of urethral and prostatic stents, including placement, degradation of materials, therapeutic success and histology in both healthy and urethral stricture models [64, 65].

3 Guidelines for Animal Research

Finally, for reporting animal research, it is recommended to follow the ARRIVE guidelines [66]. These guidelines have been developed to ensure that studies involving live animals follow methodological rigour, are reported in enough detail and enable reproducibility. This tool is primarily aimed for the writing and revision of scientific publications. However, they are also valuable for study planning and conducting, as they help researchers to design rigorous and reliable *in vivo* experiments, minimize bias and to record important information about study methods. Besides, ethical review boards, funders, institutions and learned societies may rely on them to help promote best practice and ensure rigorous design and transparent reporting of *in vivo* preclinical research [66].

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