Radboud Repository

Radboud University Nijmegen

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/160311

Please be advised that this information was generated on 2017-12-06 and may be subject to change.

MULTI-HIT HYPOTHESIS IN KIDNEY DEVELOPMENT:

DRUG EFFECTS AND INTERACTIONS WITH EXTRAUTERINE GROWTH RESTRICTION

Ruud Bueters

ISBN
978-94-6233-340-6

Design/lay-out
Promotie In Zicht, Arnhem

Print
Gildeprint BV, Enschede

Copyright © R. Bueters, 2016

All rights are reserved. No part of this book may be reproduced, distributed, stored in a retrieval system, or transmitted in any form or by any means, without prior written permission of the author.

MULTI-HIT HYPOTHESIS IN KIDNEY DEVELOPMENT:

DRUG EFFECTS AND INTERACTIONS WITH EXTRAUTERINE GROWTH RESTRICTION

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op woensdag 5 oktober 2016
om 10.30 uur precies

door

Ruud Robèrt Gérard Bueters
geboren op 9 mei 1983
te Eindhoven

Promotor
Prof. dr. L.P.W.J. van den Heuvel (KU Leuven)

Copromotor
Dr. M.F. Schreuder

Manuscriptcommissie
Prof. dr. F.G.M. Russel
Dr. ing. B. Smeets
Prof. dr. A.J. van der Heijden (Erasmus Universiteit Rotterdam)

Table of contents

Chapter 1	Introduction and aims of the thesis.	7
Chapter 2	Background review: Effect of drugs on renal development. (**Schreuder *et al.*** Published in Clinical Journal of the American Society of Nephrology. 2011 Jan;6(1):212-7.)	17
Chapter 3	Adult renal size is not a suitable marker for nephron numbers: an individual patient data meta-analysis. (**Bueters *et al.*** Published in Kidney Blood pressure Research. 2013;37(6):540-6.)	31
Chapter 4	Antibiotics and renal branching morphogenesis: A comparison of toxicities. (**Bueters *et al.*** Published in Pediatric Research. 2014 Dec;76(6):508-14.)	45
Chapter 5	Early postnatal gentamicin and ceftazidime treatment in Wistar rats: implications for kidney development. (**Bueters *et al.*** Submitted.)	61
Chapter 6	Effect of NSAIDs and diuretics on nephrogenesis in an ex vivo embryogenic kidney model. (**Bueters *et al.*** Published in Birth Defects Research B Developmental Reproduction Toxicology, 2013 Dec;98(6):486-92.)	77
Chapter 7	Impact of early postnatal NSAIDS treatment on nephrogenesis in Wistar rats. (**Bueters *et al.*** Published in Birth Defects Research B Developmental Reproduction Toxicology, 2015 Oct;104(5):218-26.)	93
Chapter 8	The effects of early postnatal diuretics treatment on kidney development and long-term kidney function in Wistar rats. (**Bueters *et al.*** Published in Nephron: Experimental Nephrology and Genetics:2016 Jan;132(2):110-8.)	115
Chapter 9	Discussion and future perspectives	139
	A. Methodological considerations	141
	B. General discussion	149
	C. Perspectives for future studies	155
Chapter 10	Summary	167
	Samenvatting	171
Chapter 11	Dankwoord	179
	List of Abbreviations	183
	Curriculum Vitae	187
	Publications	189

1

Introduction and aims of the thesis

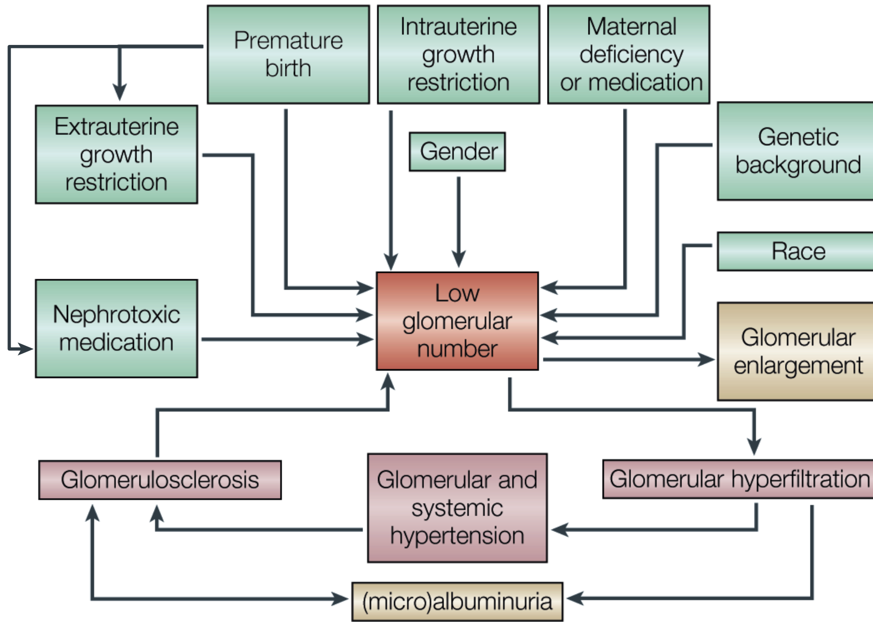
Outline and aims of the thesis

Currently premature neonates have a relatively good survival rate from 24-25 weeks of gestation onwards (1,2) and this will likely improve even more in the future due to the continuing expansion of knowledge of neonatal physiology, drugs and the better integration of this knowledge with engineering and computational power. With improved survival rates, new questions surfaced: How does this impact (late) gestational organ development and are there long term consequences of preterm birth and all interventions associated with it? This field of research was established by David Barker, who hypothesized that early life events have a definitive programming effect on our physiology, which may only show an effect in later life (3,4). Barker postulated that this is a result of a misbalance in nutrient state during organ development causing the body to redirects the available energy to vital organs for survival, relatively neglecting the other ones. This hypothesis is named 'the developmental origins of health and disease (DOHaD) and has its own international society formed around it (www.dohadsoc.org). With more efforts invested, evidence for this hypothesis is starting to accumulate for metabolic, cardiovascular and brain organ systems (5-8), however with regards to the kidney there is limited data available, but it was suggested that a lower energy state could result in less nephrons being formed (9).

The Barker thesis is closely related to another well-known renal paradigm, namely the hyperfiltration hypothesis established by Brenner in 1982 (10,11). The hypothesis states that a low glomerular number in the kidney results in additional stress for the remaining glomeruli, which first results in hypertrophy and subsequently hyperfiltration. In the next stage local- and later on systemic blood pressure is increased resulting eventually in scarring of the glomeruli and loss of functional nephrons. Progressively, this cycle results in renal disease in the end. Although originally established as a chronic kidney disease paradigm, the starting point for both paradigms is a low glomerular number. Both hypothesis can therefore be linked as shown in **figure 1**. Additionally Luyckx and Brenner have published quite compelling and convincing overviews of all supporting evidence in both animal and human for the hyperfiltration hypothesis, identifying many risk factors for a lower glomerular number (13,14). Some of these, such as intrauterine growth restriction, are well studied, but for others stressors many uncertainties remain on how they affect kidney development.

In the last few decades evidence is accumulating for a 'two-hit hypothesis' in kidney disease, where a first hit such as a congenital defect (e.g. low birth weight, intrauterine growth retardation) leads to a predisposition and a second hit (e.g. Aging, renal disease) results in progressive renal disease (15).

Figure 1 Integration of renal programming ('Barker' hypothesis) with hyperfiltration hypothesis. (reprinted with permission from Schreuder et al. (12))



Most of these studies so far have focused on evaluating the effect of a single congenital factor, however neonates are often exposed to a combination of these factors. In light of the DOHaD the concept that multiple stressors have an increased chance to affect the normal nutritional balance is very plausible. Therefore it is important that the interactions between individual factors for low glomerular numbers are studied as well.

Therefore we chose to further study the impact of two factors and their interaction, namely drug treatment (nephrotoxic medication) and the 'environmental' stressor extrauterine growth restriction.

We chose to investigate drugs for three different neonatal indications: (1) sepsis, (2) closure of a patent ductus arteriosis and (3) fluid retention, which are normally treated with antibiotics, NSAIDs and diuretics respectively. These complications are quite prevalent in preterm born children as shown in statistics from the Netherlands Perinatal Registry. Antibiotics are administered to 62%, NSAIDs to 16% and diuretics to 19% of neonates born before 32 weeks in the Netherlands (16). Additionally to neonatal treatment, the drugs may also be taken by pregnant women (although contraindicat-

ed) for similar or other indications (infections, water retention/high blood pressure, pain killer or tocolycant).

It is known that some drugs for these indications have a risk of causing acute kidney toxicity (aminoglycosides, Non-steroidal anti-inflammatory drugs (NSAIDs). However with regard to the DOHaD hypothesis we have to consider that these drugs may have an impact on (late) development of the kidney and may cause a predisposition to disease in later life. Therefore we have to reconsider the safety of these drugs even though the acute risk/benefit ratio may be very good in sight of survival. The developmental toxicity profiles of these drugs are well defined in preclinical safety testing but these experiments cannot comprise all the complex interactions that may be present in a clinical environment. Due to these drug-drug or drug-environment interactions, dose levels that were proven safe may still have a delayed (adverse) effect. We focused our work on a first-line treatment drug used in the Netherlands (gentamicin, indomethacin and furosemide) and a second drug that is also suitable to treat the aforementioned indications (ceftazidime, ibuprofen and hydrochloro-thiazide).

We selected our second stressor in the form of extrauterine growth restriction to evaluate interactions between drug treatment and this 'environmental' stressor. Due to all the necessary treatments (and accompanied side effects) in preterm born children, extrauterine growth restriction is one of the most difficult stressors to prevent in the neonatal care unit. Furthermore, models for extrauterine growth restriction have shown to negatively impact glomerulogenesisis. (17-20). This makes this stressor suitable as a second-hit for studying additive or synergic interactions with drug treatments.

My hypothesis is that drug treatment within the neonatal clinical dose range can cause subtle programming effects on kidney development and can thereby cause a predisposition for renal disease in later life. Secondary I hypothesize that adding a second stressor in the form of extrauterine growth restriction can increase the toxicity of drug treatment.

In order to test this hypothesis, we aimed to provide answers to the following questions in this thesis:

1. Are currently used kidney morphometric parameters such as size or surface area good predictors for the state of kidney development?
2. What is the impact of drug treatment on early- and late kidney development? How does such an impact affect the function of the kidney in later life? Are there alternatives for drugs used in the current first-line treatment?
3. Does an additional stressor in the form of extrauterine growth restriction indeed modulate drug toxicity according to the two-hit hypothesis?

To answer these questions we have performed experiments described in the following chapters of this thesis. A more detailed outline of the chapters is presented below.

To supplement this short introduction to the thesis, we provided more background information on the known drug effects of our selected compounds on renal development In **chapter 2**.

Currently there is no direct way of counting glomeruli in humans *in vivo*, therefore estimations are often made based on renal weight and/or renal size. We questioned the value of these morphometric predictions and investigated the validity of renal size in **chapter 3** by comparing the available literature on individual patient data. Papers were included in which both renal sizes were reported as well as unbiased results for glomerular numbers using stereology.

We further examined renal morphology markers in **chapter 4** where we assessed the validity of metanephron surface area for ureteric bud differentiation, a well-studied kidney development endpoint. In chapter 3 we also investigated the effect of gentamicin, ceftazidime and meropenem on early kidney development by evaluating ureteric bud differentiation and mRNA expression of specific targets in important differentiation pathways.

In **chapter 5** we investigated the effect on glomerular numbers of both gentamicin and ceftazidime when administered in a clinical dose range at a later phase of kidney development, which is comparable to the developmental stage of a human premature neonate. Additionally, we introduced a 'second hit' in this chapter in the form of extra uterine growth restriction to study the interaction between this environmental stressor and drug treatment. The effects on glomerular numbers, proliferation/apoptosis and expression of important genes in renal development or renal function were studied by means of stereology, immunohistochemistry and quantitative polymerase chain reaction.

Other drug classes such as non-steroidal anti-inflammatory drugs (NSAID's) and diuretics are also frequently administered to premature neonates, but also have their uses in pregnant women. Therefore the effects of the NSAID's ibuprofen and

indomethacin, and the diuretics furosemide and hydrochlorothiazide were studied in **chapter 6**. Similar endpoints compared with chapter 3 were used to evaluate toxicity.

The impact of NSAIDs with or without extrauterine growth restriction on late kidney development and long term sequelae were further studied in an *in vivo* rat study in **chapter 7**, where in addition to the parameters measured in chapter 5, kidney function and blood pressure were followed up to 9 months by determining electrolyte and creatinine clearance in serum and urine, and using radio telemetry blood pressure measurement devices.

Finally we studied the effects of furosemide and hydrochlorothiazide treatment with or without extrauterine growth restriction during late nephrogenesis and their long -term consequences for kidney function in **chapter 8**. The same study set-up was chosen as in chapter 7, with exception of the blood pressure measurements. Due to the difficulties of obtaining physiological blood pressure readings by telemetry in chapter 7, tail cuff measurements were used to determine blood pressure.

All considerations for our choice of techniques used in this thesis and the overall thesis findings and future recommendations for research are discussed in **chapter 9**.

Reference list

1. Effer SB, Moutquin JM, Farine D, Saigal S, Nimrod C, Kelly E, Niyonsenga T. Neonatal survival rates in 860 singleton live births at 24 and 25 weeks gestational age. A Canadian multicentre study. *BJOG* 2002;109(7): 740-745.
2. Kutz P, Horsch S, Kuhn L, Roll C. Single-centre vs. population-based outcome data of extremely preterm infants at the limits of viability. *Acta Paediatr* 2009;98(9): 1451-1455.
3. Barker DJ. The fetal and infant origins of adult disease. *BMJ* 1990;301(6761): 1111.
4. Barker DJ. The origins of the developmental origins theory. *J Intern Med* 2007;261(5): 412-417.
5. Richards M, Hardy R, Kuh D, Wadsworth ME. Birthweight, postnatal growth and cognitive function in a national UK birth cohort. *Int J Epidemiol* 2002;31(2): 342-348.
6. Rogers LK, Velten M. Maternal inflammation, growth retardation, and preterm birth: insights into adult cardiovascular disease. *Life Sci* 2011;89(13-14): 417-421.
7. Martinez SR, Gay MS, Zhang L. Epigenetic mechanisms in heart development and disease. *Drug Discov Today* 2015;20(7): 799-811.
8. Sosa-Larios TC, Cerbon MA, Morimoto S. Epigenetic alterations caused by nutritional stress during fetal programming of the endocrine pancreas. *Arch Med Res* 2015;46(2): 93-100.
9. Reyes L, Manalich R. Long-term consequences of low birth weight. *Kidney Int* 2005;Suppl(97): S107-111.
10. Brenner BM, Meyer TW, Hostetter TH. Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease. *N Engl J Med* 1982;307(11): 652-659.
11. Brenner BM, Lawler EV, Mackenzie HS. The hyperfiltration theory: a paradigm shift in nephrology. *Kidney Int* 1996;49(6): 1774-1777.
12. Schreuder MF, Nauta J. Prenatal programming of nephron number and blood pressure. *Kidney Int* 2007;72(3): 265-268.
13. Luyckx VA, Brenner BM. Low birth weight, nephron number, and kidney disease. *Kidney Int* 2005;Suppl(97): S68-77.
14. Luyckx VA, Brenner BM. Birth weight, malnutrition and kidney-associated outcomes--a global concern. *Nat Rev Nephrol* 2015;11(3): 135-149.
15. Nenov VD, Taal MW, Sakharova OV, Brenner BM. Multi-hit nature of chronic renal disease. *Curr Opin Nephrol Hypertens* 2000;9(2): 85-97.
16. The Netherlands Perinatal Registry. The Netherlands Perinatal Registry: Perinatal care in the Netherlands 2005. 2008 Utrecht.
17. Langley-Evans SC, Welham SJ, Jackson AA. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sci* 1999;64(11): 965-974.
18. Huizinga CT, Engelbregt MJ, Rekers-Mombarg LT, Vaessen SF, Delemarre-van de Waal HA, Fodor M. Ligation of the uterine artery and early postnatal food restriction - animal models for growth retardation. *Horm Res* 2004;62(5): 233-240.
19. Schreuder MF, Nyengaard JR, Remmers F, van Wijk JA, Delemarre-van de Waal HA. Postnatal food restriction in the rat as a model for a low nephron endowment. *Am J Physiol Renal Physiol* 2006;291(5): F1104-1107.
20. de Oliveira JC, Grassiolli S, Gravena C, de Mathias PC. Early postnatal low-protein nutrition, metabolic programming and the autonomic nervous system in adult life. *Nutr Metab (Lond)* 2012;9(1): 80.

2

Impact of drugs on renal development

MF Schreuder[1]
RR Bueters[1]
MC Huigen[2]
FGM Russel[3]
R Masereeuw[3]
LP van den Heuvel[1,2]

Radboud University Nijmegen Medical Centre, [1]Department of Pediatric Nephrology,
[2]Department of Laboratory Medicine, Laboratory for Genetic, Endocrine and Metabolic Diseases,
and [3]Department of Pharmacology and Toxicology, Nijmegen, The Netherlands.

Schreuder MF, **Bueters RR**, Huigen MC, Russel FG, Masereeuw R, van den Heuvel LP.
Effect of drugs on renal development. Clin J Am Soc Nephrol. 2011 Jan;6(1):212-7.

Abstract

Many nephrotoxic effects of drugs have been described, whereas the impact on renal development has received less attention. Nephrogenesis ceases around the 36th week of gestation, indicating that drugs administered both to pregnant women as well as to preterm born neonates may influence kidney development.

Such impact on renal development may lead to a wide spectrum of renal malformations (congenital anomalies of the kidney and urinary tract, CAKUT), ranging from renal agenesis to a reduced nephron number. Any of these anomalies may have long-term sequelae and CAKUT is the primary cause for renal replacement therapy in childhood. This review focuses on research into the impact of drug treatment during active nephrogenesis, both during pregnancy as well as in preterm born infants. As the effects of many widely used drugs have not been unravelled yet, more research is needed to study the impact on renal development and long-term renal sequelae after drug treatment during nephrogenesis.

Introduction

The kidney is vulnerable to toxic effects of many drugs, as recently reviewed by Perazella in this journal (1). However, drug treatment can also have an impact on development of the kidney. As nephrogenesis ceases around 36 weeks of gestation in man (2), most developmental nephrotoxic effects can be expected during treatment of pregnant women. In addition, premature born neonates are exposed to extrauterine life before completion of nephrogenesis, and many of them will be treated with drugs during active renal development. In this review, we focus on the effects of drug treatment on the developing kidney, both prenatal as well as postnatal.

Renal development

Nephrogenesis is the highly complex process that leads to the formation of nephrons, the functioning units of the kidney (2). The ureteric bud reciprocally induces the metanephric mesenchyme, which leads to condensation of the mesenchyme. Via branching morphogenesis and several stages of immature nephrons (comma- and S-shaped bodies), mature nephrons with a fully developed and elongated tubular system are formed. This process starts around the 5th week and ceases around the 36th week of gestation in man (2), around which time about 1 million nephrons per kidney are present with a very wide inter-individual range (~250,000 to 2,000,000 nephrons per kidney) (3,4).

After term birth, no new nephrons are formed: growth of the kidney to adult size is solely based on hypertrophia. However, it has been consistently shown that intrauterine growth restriction leads to a reduced number of nephrons (5). There is data suggesting that premature birth, i.e. before completion of nephrogenesis, is also associated with a reduced nephron endowment (6,7). This may be due to extrauterine growth restriction, which is very common in premature infants (8) and which we have shown to lead to a reduced nephron number in an animal model(9). However, nephrotoxic medications used during the postnatal period in premature infants may also affect final nephron number. The rate of premature birth (i.e. before 37 weeks of gestation and therefore before completion of nephrogenesis) varies widely, with about 7.9% of all births in the Netherlands (10) and approximately 12.5% in the US (11), illustrating the large number of neonates that enter extrauterine life during active nephrogenesis.

Congenital anomalies of the kidney and urinary tract (CAKUT)

Maldevelopment of the kidney and/or the urinary tract can present in many forms (for a review see (12). Easily recognizable malformations can be detected during (routine) prenatal or postnatal ultrasound screening, and includes renal agenesis or aplasia (unilateral or bilateral) and multicystic dysplastic kidney (MCDK). Other frequently

found abnormalities in the urinary tract may present as a dilated renal pelvis and/or ureter, which may be based on urine flow obstruction [i.e. pelvic-ureter junction obstruction (PUJO) or posterior urethral valves (PUV)].

In contrast, renal dysplasia and hypoplasia are entities that may be more difficult to identify in vivo, whereas they do involve long-term risks. By definition, hypoplastic kidneys contain a reduced number of nephrons that are otherwise normally developed (12). Unfortunately, the number of nephrons cannot be determined in vivo yet. As a surrogate marker, renal size is used, which has shown some correlation with nephron number (3). However, renal size cannot be used to predict an individuals' nephron endowment and is therefore unsuitable for risk stratification. The normal range of nephron number varies almost tenfold, but a two-fold difference has been described to be highly relevant: Keller et al. have shown that a 50% reduction in nephron numbers (~700,000 vs. 1,400,000 in hypertensive and normotensive individuals, respectively) is associated with hypertension, even though kidney weight was similar (13). This shows that even in "normal-sized" kidneys, nephron numbers may vary widely as do the risks at long-term sequelae such as hypertension.

Finally, renal development may be altered without any obvious structural aberrations, i.e. by altered expression of (tubular) transporters. An important example in the light of drug effects is renal tubular dysgenesis (14). This is characterized by kidneys of normal size and appearance on ultrasound, but without any urine production or clearance. Renal biopsy shows the absence of proximal tubules, which can be explained by abnormal renal development due to underlying mutations in the renin-angiotensin system (RAS) or by drugs blocking the RAS.

Despite the fact that such congenital anomalies may be difficult to diagnose, they are important to acknowledge as they form the main cause of end stage renal disease in children (15).

Drug treatment during renal development

Two main, distinct categories of drugs can be expected to influence renal development. First, drugs with known nephrotoxic effects in mature kidneys (1) may also have toxic effects on development. Second, drugs that are not nephrotoxic in fully developed kidneys may disturb the fine balance of growth factors that are essential for renal development. Unfortunately, information on either toxic effects is lacking for many drugs, which may lead to the use of drugs that are potentially unsafe and avoidance of drugs that may be considered safe. Here, we will present a brief overview on maldevelopment of the kidney and focus on drugs that have been shown or hypothesized on basis of clinical, fundamental or experimental research to be of influence on kidney development (Table 1).

Table 1 Drugs shown to influence renal development

Drug	Impact of maternal treatment during pregnancy on offspring kidney development	Impact of treatment during postnatal kidney development
Aminoglycosides	tubular alterations (16) low nephron number (17-19)	tubular damage (21) low nephron number (19)
Cyclosporin A	low nephron number (22)	
Prostaglandin synthetase inhibitors	tubular alterations (21) similar nephron number (28)	glomerular and tubular injury (21) similar nephron number (21,26,27)
ACE inhibition/ARB	renal insufficiency (31)	atrophy of the renal papilla, tubular alterations (32) low nephron number (33)
Dexamethasone	altered tubular transporters (36,37) low nephron number (5) similar nephron number (38)	low nephron number (5,35)
Furosemide	renal concentrating defect (40)	
Anti-epileptic drugs	more congenital malformations, specifically MCDK (44)	
Mycophenolate mofetil	renal agenesis/ectopia (45,46)	
Adriamycin	bladder agenesis, hydronephrosis (48)	
Cyclophosphamide	hydro(uretero)nephrosis (49)	

Nephrotoxic drugs

Three categories will be discussed, i.e. aminoglycosides, calcineurin inhibitors, and prostaglandin synthetase inhibitors.

In the Netherlands, 62% of neonates born before 32 weeks are treated with aminoglycosides, as are 26% of neonates born before 37 weeks of gestation (10). However, gentamicin and other aminoglycosides are well-known nephrotoxic drugs that can lead to tubular alterations. Similar alterations are found in offspring of pregnant rats treated with an aminoglycoside (16). Such offspring has also been shown to have a lower nephron number and develop subsequent glomerulosclerosis with aging (17-19). In line with these findings, it has been shown that organ culture of early metanephroi in media supplemented with gentamicin also leads to impaired nephron formation (20).

Most newborn rats and mice are at an early stage of kidney development with only around 20% of mature nephrons present at birth and ongoing nephrogenesis until 7-10 days-of-age. Neonatal rats and mice are therefore comparable to premature

born humans regarding the stage of kidney development (Figure 1), and provide a suitable model to study the effects of drugs on (postnatal) nephrogenesis. Treating neonatal rats with aminoglycosides during this postnatal nephrogenesis has also been shown to lead to a reduced nephron endowment (19) and tubular damage (21). However, these drugs are still commonly used in premature neonates for the treatment of (suspected) infections and a large proportion of premature infants receive aminoglycosides during active nephrogenesis. Indeed, most Neonatal Intensive Care Units in the Netherlands have a first line treatment for (suspected) sepsis that includes administration of an aminoglycoside (either gentamicin or amikacin), whereas clinical relevant alternatives are available that may have less impact on renal development, such as cephalosporines and carbapenems.

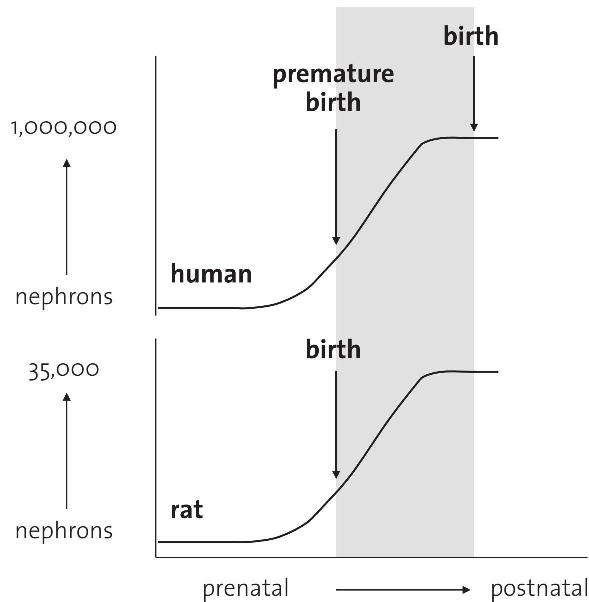
Secondly, a potentially nephrotoxic compound is the calcineurin inhibitor cyclosporin A. This is a lipophilic immunosuppressive drug and passes the placenta into the fetus. After treatment of pregnant rabbits with cyclosporin A, the offspring were shown to have a reduced nephron number (22) with systemic hypertension and progressive chronic renal insufficiency in adulthood (23). In mice deficient in calcineurin in the developing urinary tract, a defective peristalsis of the pyelo-ureteral junction is described (24). PUJO could therefore be expected in children from mothers treated with calcineurin inhibitors during pregnancy (e.g. after renal transplantation), but clinical data have as yet not been produced that substantiate this hypothesis.

A final example of nephrotoxic agents are the prostaglandin synthetase inhibitors, such as indomethacin and ibuprofen, that are used to treat a persistent patent ductus arteriosus, a frequently diagnosed condition in premature infants (11). Based on data from the Netherlands Perinatal Registry, about 1 in every 6 neonates born before 32 weeks of gestation is treated for a patent ductus arteriosus. These drugs inhibit the (renal) cyclo-oxygenase system and a well-known side effect of treatment in premature neonates is anuria or oliguria during the course of treatment, which occurs less often during treatment with ibuprofen (25). As a normal functioning cyclo-oxygenase system is essential for nephrogenesis, it can be expected that these drugs not only influence short term renal function, but also impair nephron formation (21,26,27). Indeed, harmful renal effects have been shown of acetylsalicyclic acid, another prostaglandin synthetase inhibitor (28).

Drugs disturbing renal developmental factors

First, an adequate functioning RAS is essential for normal renal development (29,30). Drugs interfering with this system [like angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB)] can therefore be expected to alter nephrogenesis, and indeed have been shown to do so (ACEi/ARB fetopathy) (31). Genetic knock-out mice of either angiotensin II type 1 receptor (AT 1 receptor) or angiotensinogen have both resulted in an atrophic renal papilla (29), underlining the role

Figure 1 Schematic representation of the timing of nephrogenesis in relation to normal birth in both humans and rats



of the RAS in renal development. Several clinical and frequently lethal sequelae have been described of RAS-blocking during development, including renal insufficiency, oligohydramnion with hypoplastic lungs and respiratory insufficiency and limb contractures (14). Kidney analysis of neonatal ACEi or ARB treated rats confirms the atrophy of the renal papilla together with tubular alterations such as fibrosis, atrophy and urinary concentration impairment (32), but also shows a reduction in nephron numbers (33).

Secondly, steroids are often prescribed to pregnant women with premature contractions to accelerate fetal lung maturation (34). However, maternal treatment with dexamethasone during pregnancy has also been shown to program kidney development (5,35) and have an effect on renal tubular transporters (36,37). This has resulted in a widely used model in the study for the developmental origins of health and disease (5). However, a recent study in monkeys showed no effect of prenatal dexamethasone exposure on nephron numbers (38), which underlines the need for human data to define the impact of steroid treatment during nephrogenesis on the kidney.

Thirdly, furosemide is a frequently used diuretic in premature neonates, even though chronic treatment of neonates has shifted towards other diuretics as furosemide increases hypercalciuria and is associated with nephrocalcinosis (39). Rats born after maternal treatment with the diuretic furosemide during pregnancy showed a renal concentrating defect that persisted after cessation of nephrogenesis (40). This may indicate that a different expression pattern of renal sodium transporters is induced by furosemide during nephrogenesis. However, furosemide has also been shown to reduce nephron formation in organ culture (41). This may be mediated by alterations in Pax2 expression (42), a highly important factor in kidney development.

Fourth, antiepileptic drugs during pregnancy are known teratogens (43) and have been described to increase the risk of congenital malformations in general, and of MCDK specifically (44). This may be due to increased BCL-2 levels, leading to defective apoptosis and thereby defective nephrogenesis (44).

A final but important drug class are immunosuppressive agents other than calcineurin inhibitors. Such agents have also been shown to have an impact on renal development, especially mycophenolate mofetil (45,46). The pathways involved have not been clarified yet, but a recent study has identified two factors involved, i.e. platelet-derived growth factor-B, potently inducing proliferation, and early growth response gene-1, a transcription factor (47). Other immunomodulatory drugs that have also been shown to influence renal development include adriamycin (48) and cyclophosphamide (49).

Perspectives

A wide range of drugs is administered to pregnant women and preterm neonates, during which renal development is ongoing. Highly prevalent diseases worldwide, such as HIV and malaria, require long-term treatments and bear the risk of renal maldevelopment. HIV as well as malaria infections during pregnancy are associated with intrauterine growth restriction (IUGR), which in itself leads to a reduced nephron endowment (5). The impact of such drugs on nephrogenesis is not yet fully clear. Antiretroviral drugs have been associated with nephropathy in children (50), which illustrates the possible influence on renal development. Of course, these side-effects must be put in perspective with the obvious benefits that are obtained by the use of such medications. Information on possible nephrotoxic effects should not be obtained or used to rule out the usage of certain drugs, but may be used to come to treatment schemes for pregnant women and newborns that provide an optimal balance between beneficial drug effects and toxicity.

Many drugs have been tested during (fetal) development and been described or considered to be safe. However, the spectrum of CAKUT is very broad (12). Specifically hypoplasia is a difficult diagnosis, as nephron number estimation is hardly performed and renal size is only a crude marker. That implicates that no drug, as far as the authors

are aware, has been proven to be definitively safe to use during kidney development. As the long-term effects of a low nephron endowment are becoming more and more clear (51), it is highly important to study the impact of drugs on renal development. There are many modifying effects of environmental insults, such as prematurity and IUGR (5,51), therefore, combinations of several drugs and/or factors in order to study the interactions should be included in future research as well.

Conclusions

Many renal toxic effects of drugs have been described, whereas the impact on renal development has received less attention. However, as renal maldevelopment may lead to CAKUT, including a reduced nephron number, long-term sequelae are likely to occur. This highlights the need for research into the effects of drug treatment during active nephrogenesis, both during pregnancy as well as in preterm born infants.

Acknowledgements

None of the authors has a conflict of interest to disclose. MFS and RRB are supported by a Kolff Junior Postdoc Grant from the Dutch Kidney Foundation.

References

1. Perazella MA. Renal vulnerability to drug toxicity. *Clin J Am Soc Nephrol* 2009;4(7):1275-1283.
2. Quaggin SE, Kreidberg J. Embryology of the kidney. In: Brenner and Rector's the kidney, 8th ed., edited by Brenner BM, Philadelphia, Saunders, Elsevier, 2008, pp 3-24
3. Nyengaard JR, Bendtsen TF. Glomerular number and size in relation to age, kidney weight, and body surface in normal man. *Anat Rec* 1992;232(2):194-201.
4. Hughson MD, Douglas-Denton R, Bertram JF, Hoy WE. Hypertension, glomerular number, and birth weight in African Americans and white subjects in the southeastern United States. *Kidney Int* 2006;69(4):671-678.
5. Schreuder M, Delemarre-van de Waal H, van Wijk A. Consequences of intrauterine growth restriction for the kidney. *Kidney Blood Press Res* 2006;29(2):108-125.
6. Rodriguez MM, Gomez AH, Abitbol CL, Chandar JJ, Duara S, Zilleruelo GE. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. *Pediatr Dev Pathol* 2004;7(1):17-25.
7. Rodriguez MM, Gomez A, Abitbol C, Chandar J, Montane B, Zilleruelo G. Comparative renal histomorphometry: a case study of oligonephropathy of prematurity. *Pediatr Nephrol* 2005;20(7):945-949.
8. De Curtis M, Rigo J: Extrauterine growth restriction in very-low-birthweight infants. *Acta Paediatr* 2004;93(12):1563-1568.
9. Schreuder MF, Nyengaard JR, Remmers F, van Wijk JA, Delemarre-van de Waal HA. Postnatal food restriction in the rat as a model for a low nephron endowment. *Am J Physiol Renal Physiol* 2006;291(5):F1104-F1107.
10. Stichting Perinatale Registratie Nederland: Perinatale Zorg in Nederland 2005. Utrecht, Stichting Perinatale Registratie Nederland, 2008
11. Eichenwald EC, Stark AR. Management and outcomes of very low birth weight. *N Engl J Med* 2008; 358(16):1700-1711.
12. Kerecuk L, Schreuder MF, Woolf AS. Renal tract malformations: perspectives for nephrologists. *Nat Clin Pract Nephrol* 2008;4(6):312-325.
13. Keller G, Zimmer G, Mall G, Ritz E, Amann K. Nephron number in patients with primary hypertension. *N Engl J Med* 2003;348(2):101-108.
14. Lacoste M, Cai Y, Guicharnaud L, Mounier F, Dumez Y, Bouvier R, Dijoud F, Gonzales M, Chatten J, Delezoide AL, Daniel L, Joubert M, Laurent N, Aziza J, Sellami T, Amar HB, Jarnet C, Frances AM, Daikha-Dahmane F, Coulomb A, Neuhaus TJ, Foliguet B, Chenal P, Marcorelles P, Gasc JM, Corvol P, Gubler MC. Renal tubular dysgenesis, a not uncommon autosomal recessive disorder leading to oligohydramnios: Role of the Renin-Angiotensin system. *J Am Soc Nephrol* 2006;17(8):2253-2263.
15. Lewis MA, Shaw J, Sinha M, Adalat S, Hussain F, Inward C. UK Renal Registry 11th Annual Report (December 2008): Chapter 13 Demography of the UK paediatric renal replacement therapy population. *Nephron Clin Pract* 2009;111 Suppl 1:c257-c267.
16. Mallie JP, Coulon G, Billerey C, Faucourt A, Morin JP. In utero aminoglycosides-induced nephrotoxicity in rat neonates. *Kidney Int* 1988;33(1):36-44.
17. Gilbert T, Lelievre-Pegorier M, Merlet-Benichou C. Long-term effects of mild oligonephronia induced in utero by gentamicin in the rat. *Pediatr Res* 1991;30(5):450-456.
18. Gilbert T, Nabarra B, Merlet-Benichou C. Light- and electron-microscopic analysis of the kidney in newborn rats exposed to gentamicin in utero. *Am J Pathol* 1988;130(1):33-43.
19. Gilbert T, Lelievre-Pegorier M, Malienou R, Meulemans A, Merlet-Benichou C. Effects of prenatal and postnatal exposure to gentamicin on renal differentiation in the rat. *Toxicology* 1987;43(3):301-313.
20. Gilbert T, Gaonach S, Moreau E, Merlet-Benichou C. Defect of nephrogenesis induced by gentamicin in rat metanephric organ culture. *Lab Invest* 1994;70(5):656-666.
21. Kent AL, Maxwell LE, Koina ME, Falk MC, Willenborg D, Dahlstrom JE. Renal glomeruli and tubular injury following indomethacin, ibuprofen, and gentamicin exposure in a neonatal rat model. *Pediatr Res* 2007;62(3):307-312.
22. Tendron A, Decramer S, Justrabo E, Gouyon JB, Semama DS, Gilbert T. Cyclosporin A administration during pregnancy induces a permanent nephron deficit in young rabbits. *J Am Soc Nephrol* 2003;14(12):3188-3196.

23. Tendron-Franzin A, Gouyon JB, Guignard JP, Decramer S, Justrabo E, Gilbert T, Semama DS. Long-term effects of in utero exposure to cyclosporin A on renal function in the rabbit. *J Am Soc Nephrol* 2004;15(10):2687-2693.
24. Chang CP, McDill BW, Neilson JR, Joist HE, Epstein JA, Crabtree GR, Chen F. Calcineurin is required in urinary tract mesenchyme for the development of the pyeloureteral peristaltic machinery. *J Clin Invest* 2004;113(7):1051-1058.
25. Ohlsson A, Walia R, Shah S. Ibuprofen for the treatment of patent ductus arteriosus in preterm and/or low birth weight infants. *Cochrane Database Syst Rev* 2008;(1) CD003481, 2008
26. Komhoff M, Wang JL, Cheng HF, Langenbach R, McKanna JA, Harris RC, Breyer MD. Cyclooxygenase-2-selective inhibitors impair glomerulogenesis and renal cortical development. *Kidney Int* 2000;57(2):414-422.
27. Kent AL, Douglas-Denton R, Shadbolt B, Dahlstrom JE, Maxwell LE, Koina ME, Falk MC, Willenborg D, Bertram JF. Indomethacin, ibuprofen and gentamicin administered during late stages of glomerulogenesis do not reduce glomerular number at 14 days of age in the neonatal rat. *Pediatr Nephrol* 2009;24(6):1143-1149.
28. Vieira-Filho LD, Lucena-Junior JM, Barreto IS, Angelim JL, Paixao AD. Repercussion of acetylsalicylic acid during fetal development on later renal hemodynamics of rats. *Fundam Clin Pharmacol* 2008;22(4):379-386.
29. Yosipiv IV, el Dahr SS. Activation of angiotensin-generating systems in the developing rat kidney. *Hypertension* 1996;27(2):281-286.
30. Guron G, Friberg P. An intact renin-angiotensin system is a prerequisite for normal renal development. *J Hypertens* 2000;18(2):123-137.
31. Sekine T, Miura K, Takahashi K, Igarashi T. Children's toxicology from bench to bed--Drug-induced renal injury (1): The toxic effects of ARB/ACEI on fetal kidney development. *J Toxicol Sci* 2009;34 Suppl 2:SP245-SP250.
32. Guron G, Marcussen N, Nilsson A, Sundelin B, Friberg P. Postnatal time frame for renal vulnerability to enalapril in rats. *J Am Soc Nephrol* 1999;10(7):1550-1560.
33. Woods LL, Rasch R: Perinatal ANG II programs adult blood pressure, glomerular number, and renal function in rats. *Am J Physiol* 1998;275(5 Pt 2):R1593-R1599.
34. Roberts D, Dalziel S. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst Rev* 2006;(3):CD004454.
35. de Vries WB, van den BP, Goldschmeding R, de Weger RA, Bal MP, van Bel F, van Oosterhout MF. Neonatal dexamethasone treatment in the rat leads to kidney damage in adulthood. *Pediatr Res* 2001;67(1):72-76.
36. Dagan A, Gattineni J, Cook V, Baum M. Prenatal programming of rat proximal tubule Na+/H+ exchanger by dexamethasone. *Am J Physiol Regul Integr Comp Physiol* 2007;292(3):R1230-R1235.
37. Dagan A, Kwon HM, Dwarakanath V, Baum M. Effect of renal denervation on prenatal programming of hypertension and renal tubular transporter abundance. *Am J Physiol Renal Physiol* 2008;295(1):F29-F34.
38. Bramlage CP, Schlumbohm C, Pryce CR, Mirza S, Schnell C, Amann K, Amstrong VW, Eitner F, Zapf A, Feldon J, Oellerich M, Fuchs E, Muller GA, Strutz F. Prenatal dexamethasone exposure does not alter blood pressure and nephron number in the young adult marmoset monkey. *Hypertension* 2009;54(5):1115-1122.
39. Schell-Feith EA, Kist-van Holthe JE, Conneman N, van Zwieten PH, Holscher HC, Zonderland HM, Brand R, van der Heijden BJ. Etiology of nephrocalcinosis in preterm neonates: association of nutritional intake and urinary parameters. *Kidney Int* 2000;58(5):2102-2110.
40. Mallie JP, Boudzoumou P. Functional renal maturation in rat neonates after prenatal exposure to furosemide. *Pediatr Nephrol* 1996;10(4):458-460.
41. Mallie JP, Gerard A, Gerard H. Does the in-utero exposure to furosemide delay the renal maturation? *Pediatr Pharmacol (New York)* 1985;5(2):131-138.
42. Cai Q, Dmitrieva NI, Ferraris JD, Brooks HL, van Balkom BW, Burg M. Pax2 expression occurs in renal medullary epithelial cells in vivo and in cell culture, is osmoregulated, and promotes osmotic tolerance. *Proc Natl Acad Sci U S A* 2005;102(2):503-508.
43. Harden CL, Meador KJ, Pennell PB, Hauser WA, Gronseth GS, French JA, Wiebe S, Thurman D, Koppel BS, Kaplan PW, Robinson JN, Hopp J, Ting TY, Gidal B, Hovinga CA, Wilner AN, Vazquez B, Holmes L, Krumholz A, Finnell R, Hirtz D, Le Guen C. Practice parameter update: management issues for women with epilepsy--focus on pregnancy (an evidence-based review): teratogenesis and perinatal outcomes: report of the Quality Standards Subcommittee and Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology and American Epilepsy Society. *Neurology* 2009;73(2):133-141.

44. Carta M, Cimador M, Giuffre M, Sergio M, Di Pace MR, De GE, Corsello G. Unilateral multicystic dysplastic kidney in infants exposed to antiepileptic drugs during pregnancy. *Pediatr Nephrol* 2007;22(7):1054-1057.
45. Tendron A, Gouyon JB, Decramer S. In utero exposure to immunosuppressive drugs: experimental and clinical studies. *Pediatr Nephrol* 2002;17(2):121-130.
46. Anderka MT, Lin AE, Abuelo DN, Mitchell AA, Rasmussen SA. Reviewing the evidence for mycophenolate mofetil as a new teratogen: case report and review of the literature. *Am J Med Genet A* 2009;149A(6):1241-1248.
47. Sabuda-Widemann D, Grabensee B, Schwandt C, Blume C. Mycophenolic acid inhibits the autocrine PDGF-B synthesis and PDGF-BB-induced mRNA expression of Egr-1 in rat mesangial cells. *Nephrol Dial Transplant* 2009;24(1):52-61.
48. Dawrant MJ, Giles S, Bannigan J, Puri P. Adriamycin produces a reproducible teratogenic model of vertebral, anal, cardiovascular, tracheal, esophageal, renal, and limb anomalies in the mouse. *J Pediatr Surg* 2007;42(10):1652-1658.
49. Park D, Kim S, Kang H, Oh J, Jang JY, Shin S, Kim TK, Choi YJ, Lee SH, Kim KY, Joo SS, Kim YB. Preventive effect of piperonyl butoxide on cyclophosphamide-induced teratogenesis in rats. *Birth Defects Res B Dev Reprod Toxicol* 2009;86(5):402-408.
50. Andiman WA, Chernoff MC, Mitchell C, Purswani M, Oleske J, Williams PL, Spiegel H, Gona P, Seage GR, III. Incidence of persistent renal dysfunction in human immunodeficiency virus-infected children: associations with the use of antiretrovirals, and other nephrotoxic medications and risk factors. *Pediatr Infect Dis J* 2009;28(7):619-625.
51. Luyckx VA, Brenner BM. The Clinical Importance of Nephron Mass. *J Am Soc Nephrol* 2010;21(6):898-910.

3

Adult renal size is not a suitable marker for nephron numbers: an individual patient data meta-analysis

Ruud RG Bueters, MSc
Nicole CAJ van de Kar, MD PhD
Michiel F Schreuder, MD PhD

Radboud University Nijmegen Medical Centre, Department of Pediatric Nephrology, Nijmegen, The Netherlands

Bueters RR, van de Kar NC, Schreuder MF. Adult renal size is not a suitable marker for nephron numbers: an individual patient data meta-analysis. Kidney Blood Press Res. 2013;37(6):540-6.

Abstract

Background
Renal size is often used as a marker for nephron numbers as estimation of glomerular numbers is not yet possible in vivo. However, the validity of an association between the two is questionable. As a proper marker for nephron number in an individual is needed in clinical practice, this study was designed to assess the association between renal size and nephron numbers.

Methods
An individual patient data meta-analysis was performed on data retrieved with a PubMed and Embase search. Only studies were included that described individual human data on kidney size and nephron numbers determined by stereology, the gold standard methodology to estimate nephron numbers. As renal size increases until the end of puberty, and nephron numbers decline after the age of 60 years, only data from individuals aged 18-60 years without renal disease were included.

Results
Six papers were identified that provided data on renal weight and nephron numbers from 114 individuals. Backward linear regression identified kidney weight and race as the only 2 significant factors explaining nephron numbers (R square 0.085, p=0.007). Controlling for race, there was a significant correlation between nephron number and kidney weight (r=0.231, r square=0.053, p=0.01).

Conclusion
These data indicate that only ~5% of the variation in nephron numbers is explained by differences in renal weight. Renal weight is closely correlated with renal volume, but in vivo renal volume estimation using any radiological modality is a poor predictor of true renal size. Therefore, renal size in adulthood should not be used as a marker for nephron numbers in an individual.

Introduction

Nephron numbers are important in determining renal function and blood pressure. A low nephron endowment leads to glomerular hyperfiltration (1) and is associated with hypertension (2), glomerular enlargement and glomerulosclerosis (3). As the number of nephrons is fixed during prenatal kidney development without the possibility of later nephron formation, several perinatal factors, such as intrauterine growth restriction and premature birth, have been shown to influence final nephron numbers (4) and lead to (pre)hypertension in young adults (5).

The gold standard method to determine nephron number is by stereology (6). Unfortunately, stereological nephron number estimation is currently only possible *ex vivo*, limiting the study of the impact of perinatal insults on kidney development to animal research. To circumvent this problem, renal size is often used as a marker for nephron number. Indeed, 2 studies have shown a statistically significant association between renal weight and nephron number (7,8), explaining up to 45% of the variation in nephron numbers. In contrast, the landmark study of Keller et al. (2) showed a two-fold difference in nephron numbers between individuals with and without hypertension, but no difference in renal weight.

This illustrates that it is debatable whether renal size or weight can be used to predict nephron endowment in an individual, but it is used that way in an increasing number of papers as a variation in nephron numbers may have clinical consequences.

In order to assess the association between renal weight and nephron numbers in humans, we performed an individual patient data meta-analysis that shows that only 5% of the variation in nephron number is explained by renal size after correction for one other significant factor (race).

Materials and methods

Search strategy

The PRISMA statement was used for this systematic review (9). A review protocol was absent before the start of this study.

A PubMed search was conducted for articles published from January 1966 onward that contained the keywords "nephron number" or "glomerular number" in combination with the keywords "stereology" or "stereological" (total hits 134, 4 June 2013, Figure 1). In addition, an EMBASE search was conducted with the same keywords (total hits 109, 4 June 2013). Two authors screened the title and/or abstract of these records, and potentially eligible papers were read in full. Disagreements between reviewers were resolved by consensus.

After selection, only 5 articles remained. In addition, the “related articles” function in PubMed was used from articles that were considered for inclusion. Also, the publications from two research groups that have the most experience in stereological analysis of the kidney were looked at by searching PubMed with the names of the respective senior researchers (JF Bertram and JR Nyengaard). Finally, reference lists from included publications were searched manually. These strategies provided 1 additional article providing individual data on nephron number and renal size and/or weight (Figure 1).

Selection of articles

All studies in English describing data in humans of both renal size and/or weight together with a stereological estimation of nephron number were considered. As both a young age (for the purpose of this study defined as the age of 18 years) as well as an older age (at which a decline in nephron number can be expected, i.e. 60 years (8) may influence the association between nephron number and kidney weight, the analysis was performed on individuals aged 18-60 years. To allow for a meta-analysis of individual patient data, such data needs to be presented per individual, rather than an average per group. Only studies using stereology were of interest, as stereology is a bias-free design-based method that provides a reliable estimation of nephron number without any assumption of size and shape (6). Title and/or abstract of all articles identified were screened, and relevant original studies were read in full. When several articles described (part of) the same cohort, individual data on nephron number, height and body weight were compared to guarantee that each individual was only included once in the analysis. In order to exclude the influence of renal disease on either nephron number or kidney weight, individuals were excluded that were known to have chronic renal failure and/or were treated with dialysis or received a renal transplant. In total, 6 articles were included in the meta-analysis. No risk of bias of individual studies or risk of publication bias was assessed.

Data abstraction

Per individual, the following data were collected: age, gender, race (Caucasian *vs.* African (American)), height, weight, body surface area (BSA), body mass index (BMI), kidney weight (measured with a scale at autopsy), and nephron number (estimated with stereology). As 4 papers (2,10-12) did not report BSA, but did report height and weight, BSA was calculated using Mosteller's formula (13): $BSA = ((height*weight)/3600)^{½}$, using height in cm and weight in kg. For 2 papers (7,8), information on height and BSA was available, which was used to calculate weight using an adaptation of Mosteller’s formula: $weight = ((BSA^{2})*3600)/height$.

Analysis

With these data an individual patient data meta-analysis was performed.

Bivariate correlation analyses between nephron numbers and the other variables (age, gender, race, height, weight, BSA, BMI, and kidney weight) were performed using Pearson's correlation analysis. Backward linear regression (criterion $F \geq 0.10$) was used with all collected data to identify the factors that were significantly associated with nephron number. Using these factors, the association between nephron number and each of these factors was determined after correction for the other significant factor(s) and presented as R or R square. Results are presented as mean (standard deviation, SD) unless otherwise stated. Differences between groups were analyzed

Figure 1 Flow diagram of in- and excluded papers for the individual patient data meta-analysis

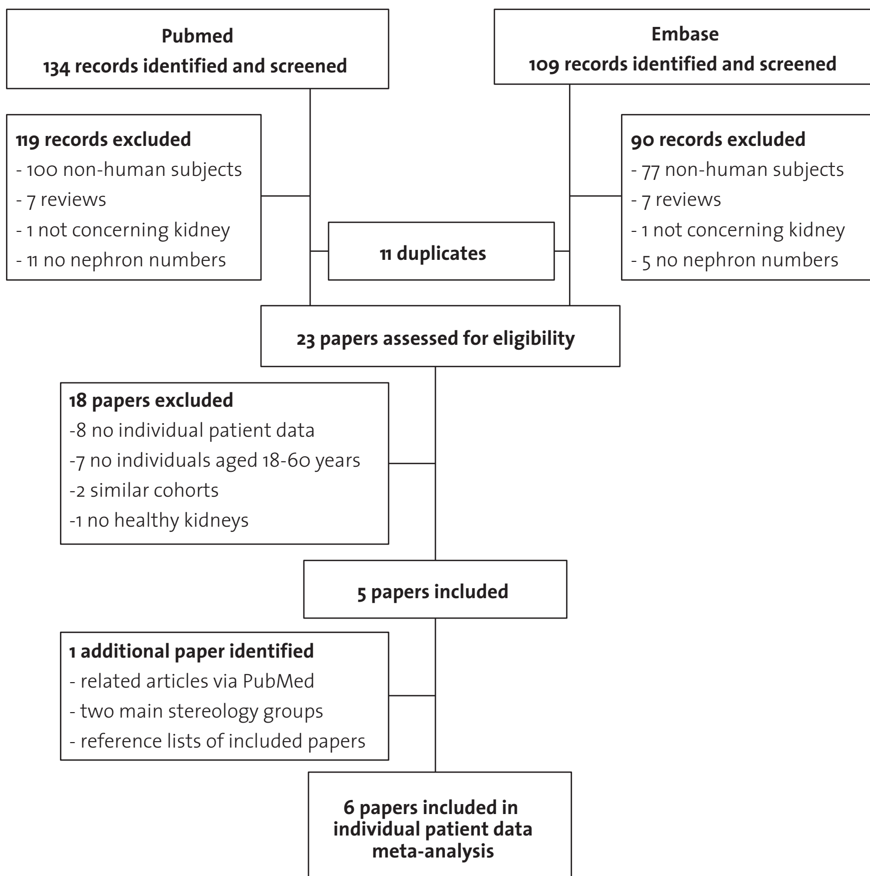


Table 1 Characteristics of included studies

		Nyengaard 1992 (8)	Bendtsen 1992 (7)	Keller 2003 (2)
Number of patients (n)		37	57[a]	20
Country of residence		Denmark	Denmark	Germany
Male:female (n:n)		19:18	32:25	18:2
Age (yr)		58.1 (19.8)	63.2 (14.7)	46.2 (6.9)
Age	< 18yr (n)	1	1	0
	18-60yr (n)	17	17	20
	≥ 60yr (n)	19	39	0
Race	Caucasian (n)	37	57	20
	African (American) (n)			
Height (cm)		166.4 (8.5)	166.7 (8.1)	176.9 (8.2)
Weight (kg)		63.4 (15.2)	68.1 (12.7)	88.8 (16.4)
BSA (m^2)		1.70 (0.23)	1.77 (0.19)	2.08 (0.20)
BMI (kg/m^2)		22.7 (4.3)	24.4 (3.7)	28.4 (5.2)
Kidney weight (g)		133.0 (35.2)	143.7 (37.6)	180.0 (39.7)
Nephron number (n * 1,000)		638.6 (202.3)	612.5 (190.4)	1,074.4 (422.4)

Data are presented as mean (standard deviation) or as numbers. Individuals with renal failure were excluded from analysis. BSA, Body surface area. BMI, Body mass index. [a], 7 of the 64 described patients were excluded on basis of reduced renal function that could influence the number of nephrons.

by one-way ANOVA. Comparison of two proportions of categorical data was done by the chi-square test. Statistical differences were considered significant if $p<0.05$ (two-tailed). SPSS (version 16.0.2) was used as statistical analysis package.

Results

From the 6 papers included (Figure 1), 197 individuals were identified. Seven patients were excluded on basis of chronic renal failure. Of the 190 individuals remaining, 10 (5.3%) were aged 17 years or younger, and 66 (34.7%) were aged 60 years or over, leaving 114 (60.0%) 18 to 60-years-old patients (Table 1).

Bivariate correlation analyses between nephron numbers and the other factors (age, gender, race, height, weight, BSA, BMI, and kidney weight) are presented in Table 2. Linear regression including all factors explained only 15.0% of the variation in nephron numbers (R square 0.150, p=0.02). Backward regression identified kidney weight and race as the only 2 significant factors explaining nephron numbers (R square 0.085, p=0.007). Every gram increase in kidney weight was associated with an increase in

	McNamara 2008 (10)	**McNamara 2009 (11)**	**Zimanyi 2009 (12)**	**Cumulative**	**Cumulative 18-60yr**
	28	24	24	190	114
	Senegal	Senegal	USA		
	14:14	24:0	24:0	131:59	91:23
	34.9 (19.7)	41.0 (16.3)	45.3 (10.0)	51.2 (18.9)	42.9 (10.7)
	8	0	0	10	
	16	21	23	114	114
	4	3	1	66	
			12	126	66
	28	24	12	64	48
	157.3 (23.1)	168.9 (5.0)	179.4 (6.5)	168.2 (12.9)	172.5 (8.5)
	64.3 (17.6)	69.7 (11.3)	94.1 (20.7)	72.3 (18.7)	78.1 (18.0)
	1.67 (0.33)	1.80 (0.16)	2.16 (0.25)	1.83 (0.28)	1.93 (0.25)
	25.9 (4.7)	24.4 (3.9)	29.2 (5.9)	25.3 (4.9)	26.1 (4.9)
	118.8 (35.6)	147.6 (34.6)	199.2 (36.1)	149.3 (43.7)	166.0 (40.8)
	925.5 (225.4)	1,053.4 (306.9)	895.3 (454.7)	803.7 (340.1)	921.5 (351.2)

Table 2 Correlations between nephron numbers and various parameters

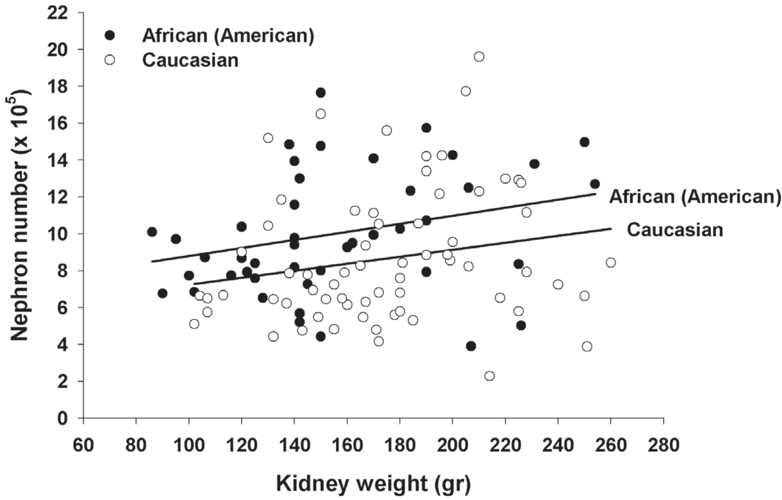
	r	p
Age	-0.076	0.2
Gender	0.216	0.01
Race	0.183	0.03
Height	0.120	0.1
Weight	0.025	0.4
BSA	0.059	0.3
BMI	-0.026	0.4
Kidney weight	0.171	0.03

BSA, Body surface area. BMI, Body mass index.

nephron number of 2,029 (95% confidence interval 424 - 3,634). Controlling for race, there was a significant correlation between nephron number and kidney weight ($r=0.231$, $p=0.01$), indicating that 5.3% of the variation in nephron numbers is explained

by variations in kidney weight. Figure 2 shows the linear regression between nephron number and kidney weight per race group. The mean number of nephrons showed a trend to be higher in African (American) group (Table 3), whereas kidney weight was higher in the Caucasian group.

Figure 2 Association between nephron number and kidney weight per race group



Linear regression between nephron number and kidney weight in Caucasians (open circles, y=1897x+535,012; r=0.200, p=0.1) and African (Americans) (filled circles, y=2185x+660,823; r=0.279, p=0.05) aged 18-60 years.

Table 3 Characteristics of included individuals per race group

	Caucasian	African (American)	p
Number of patients (n)	66	48	
Male:female (n:n)	51:15	40:8	0.4
Age (yr)	45.5 (9.6)	39.5 (11.2)	0.003
Height (cm)	173.7 (8.8)	171.0 (8.0)	0.1
Weight (kg)	81.6 (20.6)	73.3 (12.5)	0.01
BSA (m^2)	1.97 (0.27)	1.86 (0.19)	0.02
BMI (kg/m^2)	26.9 (5.7)	25.0 (3.4)	0.04
Kidney weight (g)	175.2 (38.2)	153.4 (41.4)	0.004
Nephron number (n * 1,000)	867.3 (362.7)	996.0 (323.8)	0.05

Data are presented as mean (standard deviation) or as numbers. Individuals with renal failure and age <18 years or >60 years were excluded from analysis.

Discussion

Based on this individual patient data meta-analysis, there is a significant association between renal weight and nephron numbers. However, renal weight in adulthood should not be used as a marker for nephron endowment, as variations in weight only explain about 5% of nephron numbers.

In clinical practice, renal size is estimated by ultrasound using the formula for an ellipsoid. Unfortunately, these renal size estimations are a poor predictor of true renal size and show an average underestimation of 19-25% and a poor repeatability (14-16). Estimations using CT scans seem to perform better (correlation coefficient 0.79, $p<0.01$) (17), but still explain only 62% of variation in renal size. Using optimal MRI settings improves this further (18), resulting in the greatest accuracy (at least in vitro) (15). As true renal size is a poor predictor of nephron endowment, and in vivo renal size estimation using any radiological modality is a poor predictor of true renal size, it is doubtful that renal size estimation is helpful in predicting nephron endowment. However, many papers still use renal size estimation as a marker for nephron numbers. Even though renal size is not a proper marker for nephron numbers in adults, there have been several studies that show an influence of renal size on functional markers. For instance, transplant survival is better in large kidneys when compared with the smaller kidneys (19-21). Furthermore, premature birth (i.e. before termination of nephrogenesis) has been shown in animal models to negatively influence nephrogenesis (4). In a cohort of 20-year-old individuals born very prematurely, kidneys were significantly smaller in length and volume than kidneys from term born controls with a normal birth weight (22). Such studies highlight that differences may be found, just as an association between renal weight and nephron numbers was described in some populations (7, 8), but this may be due to chance as this was not a consistent phenomenon.

As stated previously, estimation of nephron numbers is currently only possible ex vivo. However, it has recently been shown possible to estimate nephron numbers quite reliably by the use of a 9.4 Tesla MRI in embedded kidneys, which shows that techniques are progressing towards nephron number estimation in vivo (23).

Publication bias is a frequently observed phenomenon that prohibits proper meta-analysis, for which it may be vital to retrieve non-published data or cohorts. The association between renal size and nephron numbers has not been a subject of research, but is rather a by-product of studies describing nephron number of size variations in populations. We feel that this makes it unlikely that publication bias has been of influence on the results and therefore did not contact authors of potentially relevant studies.

This individual patient data meta-analysis has some limitations. First, only 6 cohorts were available that described adults in whom nephron numbers were estimated using

stereology. As stereology is the only gold standard and bias-free method to estimate nephron numbers (6), this criterion was essential to allow for pooling of the data. But even with stereology, there may be an inter-observer variability in estimating nephron numbers that has an impact on the current meta-analysis. Second, as stated previously, only adult data were included, which indicates that no conclusion on the merit of renal size in childhood can be provided. Third, most papers have used the right kidney predominantly (24). A study that did use kidneys from both sides (7) did not find any difference in nephron numbers between left and right (JR Nyengaard, personal communication), but differences between the sides in congenital anomalies are well noted (25). Any impact of such a potential difference between the sides cannot be excluded with the data available. Finally, nephrons are lost in the course of life, which was the reason to exclude individuals over 60 years of age from the current analysis. However, it may be that nephrons are lost at a higher rate before that age, which may have had an impact on the analysis. Keller et al. specifically describe that no large numbers of sclerotic glomeruli were found (2), which would argue against a potential influence of aging on the data, but other studies did not report such data on glomerulo-sclerosis. Based on a re-analysis of the data in individuals between the ages of 18 and 40 that showed similar outcomes (partial correlation between nephron number and kidney weight, controlled for race: $r=0.273$, $p=0.08$), we feel that early loss of nephrons does not have a significant effect on the results.

In conclusion, this individual patient data meta-analysis shows that renal weight, and thereby renal size estimations, in adulthood should not be used as a marker for nephron numbers.

References

1. Brenner BM, Lawler EV, Mackenzie HS. The hyperfiltration theory: a paradigm shift in nephrology. *Kidney Int* 1996;49(6): 1774-1777.
2. Keller G, Zimmer G, Mall G, Ritz E, Amann K. Nephron number in patients with primary hypertension. *N Engl J Med* 2003;348(2): 101-108.
3. Brenner BM. Hemodynamically mediated glomerular injury and the progressive nature of kidney disease. *Kidney Int* 1983;23(4): 647-655.
4. Schreuder MF, Nauta J. Prenatal programming of nephron number and blood pressure. *Kidney Int* 2007;72(3): 265-268.
5. Keijzer-Veen MG, Finken MJ, Nauta J, Dekker FW, Hille ET, Frölich M, Wit JM, van der Heijden AJ; Dutch POPS-19 Collaborative Study Group. Is blood pressure increased 19 years after intrauterine growth restriction and preterm birth? A prospective follow-up study in The Netherlands. *Pediatrics* 2005;116(3): 725-731.
6. Nyengaard JR. Stereologic methods and their application in kidney research. *J Am Soc Nephrol* 1999;10(5): 1100-1123.
7. Bendtsen TF, Nyengaard JR. The number of glomeruli in type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 1992;35(9): 844-850.
8. Nyengaard JR, Bendtsen TF. Glomerular number and size in relation to age, kidney weight, and body surface in normal man. *Anat Rec* 1992;232(2): 194-201.
9. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *PLoS Med* 2009;6(7): e1000100.
10. McNamara BJ, Diouf B, Hughson MD, Douglas-Denton RN, Hoy WE, Bertram JF. Renal pathology, glomerular number and volume in a West African urban community. *Nephrol Dial Transplant* 2008;23(8): 2576-2585.
11. McNamara BJ, Diouf B, Hughson MD, Hoy WE, Bertram JF. Associations between age, body size and nephron number with individual glomerular volumes in urban West African males. *Nephrol Dial Transplant* 2009;24(5):1500-1506.
12. Zimanyi MA, Hoy WE, Douglas-Denton RN, Hughson MD, Holden LM, Bertram JF. Nephron number and individual glomerular volumes in male Caucasian and African American subjects. *Nephrol Dial Transplant* 2009;24(8): 2428-2433.
13. Mosteller RD. Simplified calculation of body-surface area. *N Engl J Med* 1987;317(17): 1098
14. Bakker J, Olree M, Kaatee R, de Lange EE, Moons KG, Beutler JJ, Beek FJ. Renal volume measurements: accuracy and repeatability of US compared with that of MR imaging. *Radiology* 1999;211(3): 623-628.
15. Bakker J, Olree M, Kaatee R, de Lange EE, Beek FJ. In vitro measurement of kidney size: comparison of ultrasonography and MRI. *Ultrasound Med Biol* 1998;24(5): 683-688.
16. Partik BL, Stadler A, Schamp S, Koller A, Voracek M, Heinz G, Helbich TH. 3D versus 2D ultrasound: accuracy of volume measurement in human cadaver kidneys. *Invest Radiol* 2002;37(9): 489-495.
17. Hwang HS, Yoon HE, Park JH, Chun HJ, Park CW, Yang CW, Kim YS, Choi BS. Noninvasive and direct measures of kidney size in kidney donors. *Am J Kidney Dis* 2011;58(2): 266-271.
18. Zollner FG, Svarstad E, Munthe-Kaas AZ, Schad LR, Lundervold A, Rørvik J. Assessment of kidney volumes from MRI: acquisition and segmentation techniques. *AJR Am J Roentgenol* 2012;199(5): 1060-1069.
19. Goldberg RJ, Smits G, Wiseman AC. Long-term impact of donor-recipient size mismatching in deceased donor kidney transplantation and in expanded criteria donor recipients. *Transplantation* 2010;90(8): 867-874.
20. Saxena AB, Busque S, Arjane P, Myers BD, Tan JC. Preoperative renal volumes as a predictor of graft function in living donor transplantation. *Am J Kidney Dis* 2004;44(5): 877-885.
21. Lee JH, Won JH, Oh CK. Impact of the ratio of graft kidney volume to recipient body surface area on graft function after live donor kidney transplantation. *Clin Transplant* 2011;25(6): E647-E655.
22. Keijzer-Veen MG, Devos AS, Meradji M, Dekker FW, Nauta J, van der Heijden BJ. Reduced renal length and volume 20 years after very preterm birth. *Pediatr Nephrol* 2010;25(3): 499-507.

23. Heilmann M, Neudecker S, Wolf I, Gubhaju L, Sticht C, Schock-Kusch D, Kriz W, Bertram JF, Schad LR, Gretz N. Quantification of glomerular number and size distribution in normal rat kidneys using magnetic resonance imaging. *Nephrol Dial Transplant* 2012;27(1): 100-107.
24. Hughson MD, Gobe GC, Hoy WE, Manning RD Jr, Douglas-Denton R, Bertram JF. Associations of glomerular number and birth weight with clinicopathological features of African Americans and whites. *Am J Kidney Dis* 2008;52(1): 18-28.
25. Schreuder MF. Unilateral anomalies of kidney development: why is left not right? *Kidney Int* 2011;80(7): 740-745.

4

Antibiotics and renal branching morphogenesis: A comparison of toxicities

Ruud R.G. Bueters[1]
Lisanne J.A. Kusters[1]
Annelies Klaasen[1]
Lambertus P. van den Heuvel[1]
Michiel F. Schreuder[1]

[1]*Department of Pediatric Nephrology, Radboud university medical center, Nijmegen, the Netherlands.*

(**Bueters *et al.*** Published in Pediatric Research. 2014 Dec;76(6):508-14.)

Abstract

Background

Many premature born neonates receive antibiotic drugs to treat infections, which are applied during active nephrogenesis. We studied the impact of clinical concentrations of gentamicin and alternatives, ceftazidime and meropenem, on ureteric branching.

Methods

Mice metanephroi were dissected at embryonic day 13 and cultured in media with or without various concentrations of gentamicin, ceftazidime or meropenem. Zero and 24 hours kidney size were assessed by surface area measurements, and the ureteric tree was visualized by whole mount staining and confocal microscopy. Branching was evaluated by counting and gene expression levels of *Wt1*, *Sox9*, *Bmp7*, *Fgf8* and *Gdnf* were investigated.

Results

A concentration of 2000 μM ceftazidime impaired ureteric development. Additionally a 4.5 fold and a 2.5 fold down regulation was noted in *Fgf8 and Gdnf,* respectively. No adverse effects were noted after gentamicin or meropenem treatment. No relationship was noted between surface area expansion and ureteric bud formation, but surface area at explantation related to bud count after 24 hours of culture.

Conclusion

Ceftazidime, but not gentamicin or meropenem reduced ureteric branching in mice and suggest a role for *Fgf8* and *Gdnf* in its mechanism. Metanephros surface area measurements can be used to reduce intra- and inter litter variation.

Introduction

Kidney development, leading to the formation of nephrons, starts around the 5th week of gestation and terminates before term birth, around the 34th-36th week of gestation. Many factors have been described to disturb this developmental process, leading to long-term problems such as hypertension and chronic kidney disease (1).

One such disturbing factor may be the use of (nephrotoxic) drugs during kidney development, such as in pregnant women or neonates born before termination of nephrogenesis. Gentamicin, as well as other aminoglycosides, is widely used as part of the first line treatment of (suspected) bacterial infection in neonates to combat gram-negative infections. Based on data from the Netherlands Perinatal Registry, 62% of neonates born before 32 weeks, who can be considered the most vulnerable group, are treated with aminoglycosides (2). However, due to the fact that gentamicin is classified as a nephrotoxic drug, controversy remains on its safety as aminoglycosides have been shown to disturb kidney development and lead to a reduced nephron number in some experimental animals (3-6) and organ culture studies (7).

The aim of our research was to study the impact of antibiotic treatments on nephrogenesis in a model of early nephrogenesis. Gentamicin was studied as well as clinically relevant, alternative drug treatments to compare the toxic potentials of these drugs in a clinical dose range. As alternative treatments we chose a third generation cephalosporin, ceftazidime, and the carbapenem meropenem. These two drugs both have properties to deal with gram negative bacteria and have different mechanisms of action compared to gentamicin. Although beta-lactams have their own potencies to be nephrotoxic, ceftazidime can be given at a fairly high dose before proximal tubule damage is noticeable (8). As for meropenem, this drug is regarded as one of the safer carbapenems (9) due to its stability to renal dehydropeptidase-I (10), which was confirmed in a large clinical trial (11). Although these alternatives to gentamicin appear quite safe, information on the potential toxic effects on renal branching morphogenesis is lacking. We hypothesized that gentamicin would hamper early kidney development, and ceftazidime and meropenem would prove to be safe.

Materials and Methods

Drugs

Gentamicin (G1272) and ceftazidime hydrate (C3809) were obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands. Meropenem was obtained from Fresenius Kabi, Teramo, Italy.

Organ culture

Experiments were approved by the Animal Ethics Committee at the Radboud University Nijmegen. Embryonic day 13 time pregnant HSD:ICR female mice (Harlan, Horst, The Netherlands) were euthanized after arrival by cervical dislocation and kidneys were dissected from the embryos by means of two small needles. Intact isolated metanephroi were held on ice in pre-cooled Leibovitz medium (Gibco, Paisley, UK) until transfer to the organ culture system. Metanephroi were cultured on 0.4 μm pore size Millicell cell culture inserts (Millipore, Carrigtwohill, Ireland) placed in a six well plate containing DMEM/F-12 (1:1) medium (Gibco), supplemented with 10 mg/L insulin, 5,5 mg/L transferrin and 5 μg/L sodium selenite (Sigma-Aldrich, St. Louis, Missouri). Depending on treatment, drugs were added to the medium and metanephroi were incubated at 37°C and 5% CO_2 for 24 hours. For gentamicin, drug concentrations of 3, 30 and 300 μM were chosen. Ceftazidime and meropenem were both tested at 20, 200 and 2000 μM. Concentration levels were calculated as follows: The clinical pediatric dosing was used and corrected for plasma binding and volume of distribution as detailed in Table 1 (12-18). In addition, concentrations at a factor of 10 above and below were studied to take calculation uncertainties into account and to investigate a potential dose response relationship.

Table 1 Dose selection parameters

	Gentamicin	Ceftazidime	Meropenem
(Neonatal) dose (mg/kg)	4	50	20
Molecular weight	463.6	546.58	383.46
Plasma binding (%)	0	27	2
Volume of distribution (L/kg)	0.31	0.36	0.3

Data in this table were derived from multiple sources (26-32)

All metanephroi from one litter were randomly assigned to one of the treatment groups and a separate no-treatment group that consisted of metanephroi from the same litter was included for each drug. Per litter, one drug was investigated to rule out differences on basis of variability between litters. Multiple litters were studied per drug.

Metanephric growth

Growth of the explanted metanephroi was determined by measuring surface areas expansion in 24h. Photographs of the metanephroi were obtained with a Canon EOS 1000D camera attached to a Zeiss Axiovert 25 microscope at a total magnification of 12.5x. Background light was set on maximal intensity and a shutter speed of 40 ms

was used. The photographs were taken with a resolution of 10.1 megapixels and surface area size was analyzed with FIJI/ImageJA version 1.45i (19).

Ureteric tip imaging

Whole mount immunostaining of the metanephroi was performed to visualize the ureteric tree after 24 hours of culture. The metanephroi were fixed in ice-cold methanol for 10 minutes and washed with PBS for 15 minutes. Subsequently, the metanephroi were incubated in PBS containing 2% BSA for 12h to block non-specific binding. After washing with PBS containing 1% Triton X-100 (PBS-T), metanephroi were incubated with an antibody against calbindin-D28k (Sigma Aldrich), diluted 1:100 in PBS-T, for 24h. Again after washing with PBS-T, incubation with an Alexa 488 IgG antibody (Invitrogen, Eugene, Oregon) was performed at a dilution of 1:300 in PBS containing 2% BSA for 24h. All incubation and washing steps were performed at 4°C. After a final washing step in PBS-T of 15 minutes, the metanephroi were mounted on a slide in mounting medium (Dako, Carpinteria, California) and sealed using paraffin.

For each metanephros, ureteric branching was visualized by confocal laser scanning microscopy using a Leica TCS SP2 microscope. Optical sectioning was performed at a 4 μm interval at a magnification of 10x. Per metanephros, between 18 and 30 images were acquired with a resolution of 1024x1024 pixels. Subsequently, the amount of ureteric tips was counted with the multipoint tool in FIJI. A tip was defined as an end point of the whole branching structure that did not show any signs of branching.

Gene expression analysis

RNA was isolated from the metanephroi by combining the Trizol extraction method with the NucleoSpin RNA II isolation kit (Machery-Nagel, Düren, Germany). The methanephroi were suspended in Trizol (Invitrogen, Carlsbad, California) and incubated for approximately 30 minutes with occasional vortexing. After addition of chloroform (Merck), the samples were incubated on ice for 5 minutes and centrifuged at 14,000g, 4°C for 15 minutes. The aqueous phase was added 1:1 to 70% ethanol to adjust binding conditions and loaded on the Nucleospin column. Further purification was performed according to the manufacturers protocol. RNA concentration and quality was assessed with the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts.

Complementary DNA was generated on a Biozym MJ Research PTC-200 Peltier thermal cycler using random primers (Promega, Madison, Wisconsin), oligo dT (Promega) and M-MLV reverse transcriptase (Invitrogen). mRNA levels of *Wt1, Sox9, Bmp7, Fgf8, Gdnf, Casp3 and Casp9* were measured by quantitative PCR (qPCR) with *Actb* and *Hmbs* as internal standards.

qPCR was performed on a Biorad CFX96 using the gene expression mix and hydrolysis probes (Table 2) as ordered from Applied Biosystems (Applied Biosystems,

Pleasanton, California). Delta-delta CT values were investigated and an up or down regulation of factor 2 was considered biologically relevant.

Table 2 Applied Biosystems primer probe sets used in this study

Gene symbol	Primer-probe set
Actb	endogenous control
Hmbs	Mm01143545_m1
Fgf8	Mm00438921_m1
Gdnf	Mm00599849_m1
Sox9	Mm00448840_m1
Wt1	Mm00460570_m1
Bmp7	Mm00432102_m1
Casp3	Mm01195085_m1
Casp9	Mm00516563_m1

Statistics

Metanephric surface expansion at 24h and metanephric surface area at explantation were both investigated for correlation with ureteric bud tip development and tested with a Pearsons correlation coefficient.

Comparison of ureteric bud tip development for each treatment was investigated by one-way ANOVA followed with Dunnett as post hoc. For both analysis, statistical significance was investigated at the $\alpha = 0.05$ level.

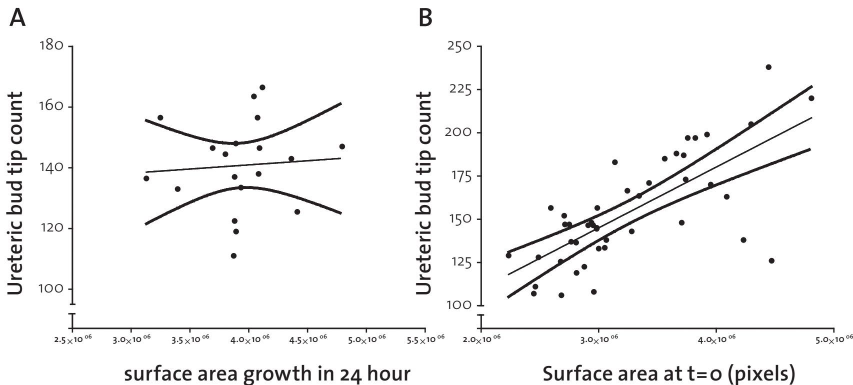
Results

Metanephric growth

First, we studied the usefulness of surface area growth as a good marker for renal development. Based on previous studies (7,20,21), a relationship between metanephric size expansion and ureteric tip count, a more direct measurement of renal development, was expected. However, as can be noted in Figure 1a, surface area expansion did not correlate with ureteric tip formation (R square= 0.005, p 0.77) and was therefore not used as a marker in our studies.

In addition, we noted a variation in metanephric size within a litter (up to 11%) and between our litters (±20%), based on differences in surface area measured directly after explantation. The impact of this variation on the ureteric bud tip count after 24 hours was studied. As can be seen in Figure 1b, a significant correlation was noted

Figure 1 Correlation (with 95% confidence interval) between surface area measurements and ureteric bud formation after 24 hours of organ culture (**A**) and at explantation (**B**)



Pearsons correlation coefficients were R^2=0.005228 and R^2=0.5082 for figure A and B respectively.

4

between metanephric size and ureteric bud development (R square= 0.5, P<0.01). Based on this finding, all ureteric tip counts were corrected for metanephric surface area at explantation.

Ureteric tip imaging

Ureteric tip development was studied in the three different antibiotic classes. A range of three doses was tested to evaluate the broad drug response and investigate a possible dose response relationship (Figure 2).

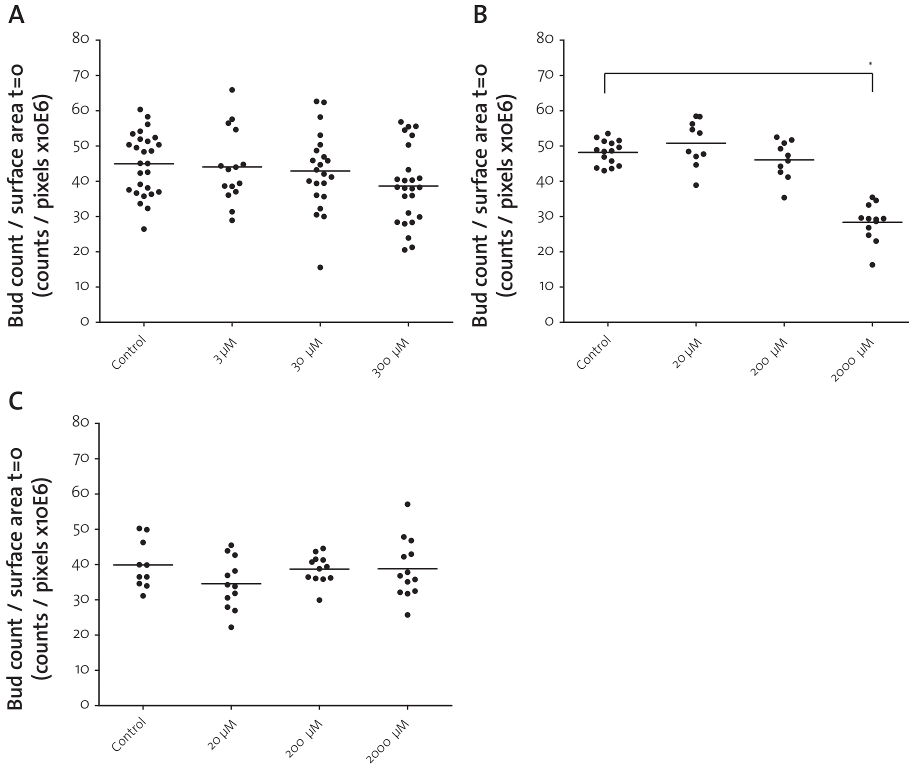
As can be noted in Figure 2b, only the 2000 μM ceftazidime concentration resulted in a statistically significant lower ureteric tip count after 24 hours of exposure (28,37 vs. 48,16, P<0.01). This effect was also clearly detectable visually (Figure 3). No effects on development compared to control were noted at the lower and the middle dose. In our experiments with gentamicin (Figure 2a) a trend was noticeable towards a lower ureteric tip count. Meropenem treatment (Figure 2c) did not show an effect on ureteric tip development compared to control at any of the concentrations studied.

Gene expression analysis

We studied a selection of targets in known nephrogenesis and apoptosis pathways by mRNA expression analysis (Figure 4). Robust CT levels indicated that the targets were present in our metanephric organ cultures.

Treatment with ceftazidime in a concentration of 2000 μM resulted in a 2.5 fold down regulation of *Gdnf* mRNA, a well known factor in the ureteric branching pathway

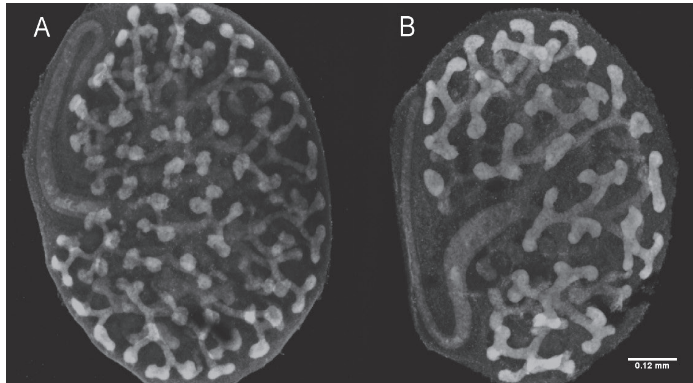
Figure 2 Quantitative analysis of ureteric buds in metanephroi cultured for 24hours in media with three antibiotic drugs, i.e. gentamicin (**A**), ceftazidime (**B**) and meropenem (**C**)



Individual data points as well as means per treatment group are presented. A low, clinical and high concentration were tested. Sample sizes of treatment groups were between 15 and 27; 10 and 15; and 10 to 13 for genatmicin, ceftazidime and meropenem respectively. * $p<0.01$.

(22). Additionally a 4.5 fold down regulation of the *Fgf8* mRNA was noted (Figure 4c). No changes in expression patterns were noted after treatment with 200 µM of ceftazidime. Gentamicin or meropenem treatment did not have an effect on the mRNA levels of our chosen targets. Furthermore, mRNA levels of Caspase 3 and Caspase 9 remained similar between controls and high dose treatments of gentamicin and ceftazidime.

Figure 3 Representative immunohistochemic staining of ureteric bud development in metanephroi cultured for 24 hours in media with 2000 μM ceftazidime (**B**) or vehicle control (**A**)

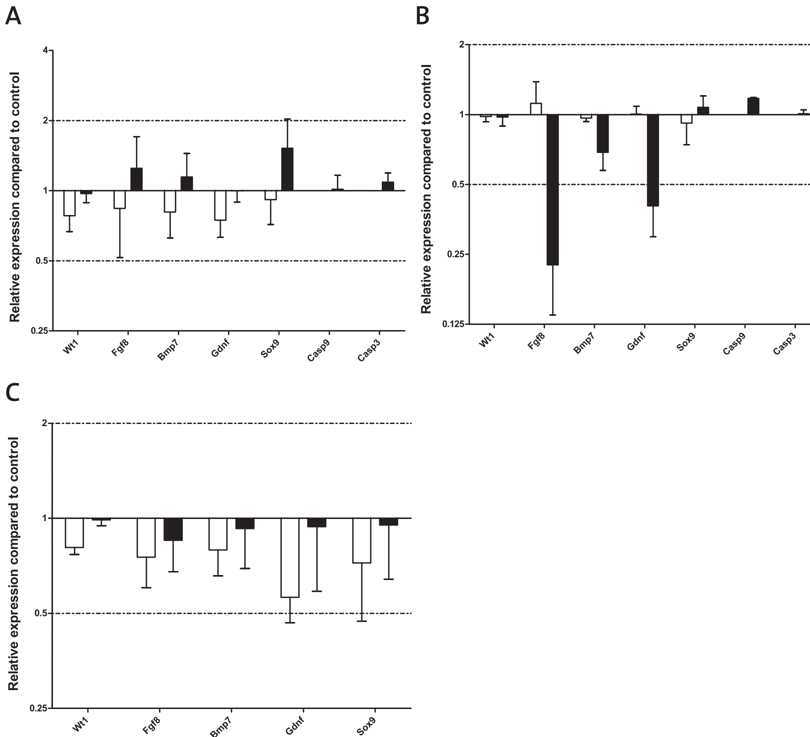


Discussion

We have shown that an upper clinical range concentration of ceftazidime reduces ureteric branching morphogenesis and suggest a role for *Fgf8* and *Gdnf* pathways in its toxicity. No adverse effects of gentamicin and meropenem within their postulated clinical dose range, were noted on ureteric branching or basic nephrogenesis pathways. Previous reports have used surface area expansion as a measurement for renal development (7,20,21). Our study shows no correlation between the surface expansion and a more direct kidney developmental marker such as ureteric bud count. This lack of correlation may be explained by flattening of the metanephros in organ culture and its partial loss of three dimensional structure as has been reported previously (23). However, the impact on actual measurements had not been studied. We show that surface area measurements in the first 24 hours after explantation are not a proper marker for renal development.

Although flattening of the organ rudiment during organ culture renders surface analysis unhelpful for quantification of kidney development, surface area at explantation can partially predict the amount of tips that will develop in metanephroi. We found a significant correlation between the surface area of explant size directly after dissection and the amount of ureter tips counted after 24 hours. One could expect such a correlation because kidney development is more pronounced *in utero* than in organ culture (24). Therefore, a more evolved (and therefore branched and larger) metanephros at time of dissection can be expected to have more end tips after 24 hours of growth. This data allows for correction of variation in gestational age. By

Figure 4 Gene expression of important nephrogenesis pathway targets in metanephroi cultured for 24 hours in media with three antibiotic drugs, i.e. gentamicin (**A**), ceftazidime (**B**) and meropenem (**C**)



Means with SEM are presented for two (A) or three (B+C) experiments, with exception of the caspase targets, which were done in two experiments. Concentrations tested were 30 (white bars) and 300 µM (black bars) for gentamicin, 200 (white bars) and 2000 µM (black bars) for both ceftazidime and meropenem. 6-9 metanephroi were pooled per experiment.

using our correlation to correct all ureteric tip counts for the measured surface area after explantation, variability of developmental stage will be more limited, and more precise comparisons between dose groups, but also between litters can be obtained. Our initial research question was whether clinical relevant levels of gentamicin could influence nephrogenesis and whether other classes of antibiotics might be safer.

The potential of gentamicine to influence nephrogenesis has already been established in rat. Studies were mostly performed by either maternal dosing (3-5) or postnatal dosing (3,25). In these studies no effect of gentamicin was noted on renal

development after early postnatal dosing during active nephrogenesis. However after maternal dosing gentamicin accumulation was confirmed in the fetal kidney. Additionally exposure to gentamicine in utero resulted in a reduction of glomerular numbers up to 20% and alterations in the tubular structure. However the dose administered to the pregnant females in these studies was 75 mg/kg and was almost 20 fold higher than the 4 mg/kg clinical dose we extrapolated the dose levels from in this study.

Our results indicate that 24 hours exposure of gentamicin did not result into impaired ureteric branching or altered expression of key pathways. Even though we did notice a trend towards reduced ureteric branching this difference was not statistically significant. This contradicts earlier findings of Gilbert et al who found reduced ureteric branching in rat organ cultures at a gentamicin concentration of 100 μM (7). The main difference between our approaches can be found in animal species (rat *vs*. mice in the current study) and in developmental stage. We believe the species difference is probably of secondary importance to the developmental stage as effects of in utero gentamicin exposure on renal development were reported in rats, mice and guinea pigs alike (3-7, 26). Although some differences in susceptibility may be present. Comparing Theiler and Witschi developmental stages (27), our E13 mice are approximately 12-24 hours more mature compared with the E14 rat embryos. It is well known that timing of insults during organ development is of great importance for the outcome and that dosing at a day later may completely abolish any effect (28). Taken these factors into account, gentamicin has the potential to disturb nephrogenesis, but the clinical dose range seemed to be safe when administered in the time frame and species that we studied.

From the data of our selected potential alternatives, we conclude that ceftazidime does not appear to be a good substitute for gentamicin. In the high dose group an impaired kidney development and down regulation in corresponding pathways was identified.

We hypothesize that ceftazidime affects the renal progenitor cell population causing down regulation of *Fgf8* and subsequent down regulation of *Gdnf* resulting in ureteric bud impairment. *Fgf8* is important in cell survival of renal progenitor cells, which are an important source of Gdnf production (29,30). In contrast no expression changes were noted in *Bmp7*, which is also localized to the progenitor cell population (31), suggesting no general progenitor cell loss with possibly cell loss in a specific sub population. This finding is supported with the normal expression levels of Caspase 3 and Caspase 9, markers of apoptotic insults and also important in the normal development of the renal tubular network (32). Alternatively, ceftazidime may directly affect both the mesenchyme and the ureteric bud. Although this high dose is 10 fold higher than our theoretical calculated clinical dose, the actual intracellular concentration in human cells is unknown. Based on our results, the safety of

ceftazidime for treatment of pregnant women should be reconsidered, as it may be harmful for early renal development of the fetus. In addition, ceftriaxone was earlier reported to have a negative effect on nephrogenesis in rats (33), which underlines the general potential harmful effects of maternal cephalosporine use on fetal nephrogenesis.

In contrast, meropenem did not show any negative effect on ureteric bud branching or mRNA levels of important development pathways, and may therefore be a potential useful substitution for gentamicin with regards to nephrogenesis.

Our studies have some limitations. First, due to the stage of development and the method of antibody staining, we are currently unable to evaluate ureteric bud staining beyond 24 hours of exposure. Therefore it might be that prolonged exposure to gentamicin or meropenem will eventually result in renal damage. Using genetically modified mice with intrinsic fluorescent protein expression in the ureteric bud may solve this problem and allow for such longer-term studies (24). Secondly, we have no data on drug uptake within the cells. Gentamicin has been shown to be detectable within embryonic kidney cells after 8 hours of exposure (7). Our ex vivo model is slightly more mature and of a different species which could have influence uptake. However we noted a dose related trend in our experiments and are therefore confident that we had uptake of gentamicin in our model. For ceftazidime and meropenem data on uptake in organ culture are not yet available, which may play a role in the absence of effects in the meropenem treated metanephroi. However, the reduced formation of ureteric bud tips in the high dose ceftazidime group suggests uptake of ceftazidime by the metanephros within hours. Furthermore, we investigated a selection of targets in the nephrogenesis pathways and studied gene expression rather than protein expression. An immunohistochemical approach was attempted, but has not provided additional insight so far. Besides the technical difficulties of studying protein expression levels in small tissues as metanephroi, we feel that the significant morphological change in these cultured metanephroi show the relevance of the altered gene expression levels. In order to study apoptosis we decided to study mRNA levels of 2 different caspases. Alternatively TUNEL could be used to the same extent and should be performed in future studies. Finally, at present we do not have data on different time points during development at which the kidney may be more vulnerable to specific drugs. This would be a very important future step in relating study findings to the human embryonic kidney development.

Our objective was to determine whether these antibiotic compounds could interfere with a basal developmental process as ureteric bud differentiation when administered in a clinical dose range. Based on our data we can conclude that short-term gentamicin or meropenem treatment at this stage of embryonic kidney development does not have an adverse impact on nephrogenesis. However, ceftazidime was shown to inhibit ureteric branching and the expression of *Fgf8* and *Gdnf*, two important

players in kidney development. Furthermore, metanephric surface area growth should not be used as a renal developmental predictor, but surface area at explantation can be used to correct for intra- and inter litter variation in developmental stages.

Acknowledgments

We would like to thank the Department of Pathology for providing the microscopy facilities and especially Irene Otte-Holler for her help with confocal imaging.

References

1. Schreuder M, Delemarre-van de Waal H, van Wijk A. Consequences of intrauterine growth restriction for the kidney. *Kidney Blood Press Res* 2006;29(2):108-125.
2. The Netherlands Perinatal Registry.[Registry Perinatal care in the Netherlands 2005]. Utrecht, 2008.
3. Gilbert T, Lelievre-Pegorier M, Malienou R, Meulemans A, Merlet-Benichou C. Effects of prenatal and postnatal exposure to gentamicin on renal differentiation in the rat. *Toxicology* 1987;43(3):301-313.
4. Gilbert T, Lelievre-Pegorier M, Merlet-Benichou C. Long-term effects of mild oligonephronia induced in utero by gentamicin in the rat. *Pediatr Res* 1991;30(5):450-456.
5. Gilbert T, Nabarra B, Merlet-Benichou C. Light- and electron-microscopic analysis of the kidney in newborn rats exposed to gentamicin in utero. *Am J Pathol* 1988;130(1):33-43.
6. Schwedler SB, Gilbert T, Moreau E, Striker LJ, Merlet-Benichou C, Striker GE. Nephrotoxin exposure in utero reduces glomerular number in sclerosis-prone but not sclerosis-resistant mice. *Kidney Int* 1999;56(5):1683-1690.
7. Gilbert T, Cibert C, Moreau E, Geraud G, Merlet-Benichou C. Early defect in branching morphogenesis of the ureteric bud in induced nephron deficit. *Kidney Int* 1996;50(3):783-795.
8. Tune BM. Nephrotoxicity of beta-lactam antibiotics: mechanisms and strategies for prevention. *Pediatr Nephrol* 1997;11(6):768-772.
9. Topham JC, Murgatroyd LB, Jones DV, Goonetilleke UR, Wright J. Safety evaluation of meropenem in animals: studies on the kidney. *J Antimicrob Chemother* 1989;24 Suppl A:287-306.
10. Bax RP, Bastain W, Featherstone A, Wilkinson DM, Hutchison M, Haworth SJ. The pharmacokinetics of meropenem in volunteers. *J Antimicrob Chemother* 1989;24 Suppl A:311-320.
11. Norrby SR, Newell PA, Faulkner KL, Lesky W. Safety profile of meropenem: international clinical experience based on the first 3125 patients treated with meropenem. *J Antimicrob Chemother* 1995;36 Suppl A:207-223.
12. Dutch Health Institute.[Meropenem.], 2013. (www.fk.cvz.nl/preparaatteksten/m/meropenem.asp)
13. Lam YW, Duroux MH, Gambertoglio JG, Barriere SL, Guglielmo BJ. Effect of protein binding on serum bactericidal activities of ceftazidime and cefoperazone in healthy volunteers. *Antimicrob Agents Chemother* 1988;32(3):298-302.
14. van den Anker JN, Schoemaker RC, Hop WC, van der Heijden BJ, Weber A, Sauer PJ, Neijens HJ, de Groot R. Ceftazidime pharmacokinetics in preterm infants: effects of renal function and gestational age. *Clin Pharmacol Ther* 1995;58(6):650-659.
15. Craig WA. The pharmacology of meropenem, a new carbapenem antibiotic. *Clin Infect Dis* 1997;24 Suppl 2:S266-275.
16. Meropenem: pediatric drug information. In: Basow D, ed. UpToDate. Waltham, MA; 2013.
17. Dutch Health Institute. [Gentamicin], 2013. (www.fk.cvz.nl/preparaatteksten/g/gentamicine%20 (systemisch).asp.)
18. Dutch Health Institute. [Ceftazidime], 2013. (www.fk.cvz.nl/preparaatteksten/c/ceftazidim.asp.)
19. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;9(7):671-675.
20. Chan SK, Riley PR, Price KL, McElduff F, Winyard PJ, Welham SJ, Woolf AS, Long DA. Corticosteroid-induced kidney dysmorphogenesis is associated with deregulated expression of known cystogenic molecules, as well as Indian hedgehog. *Am J Physiol Renal Physiol* 2010;298(2):F346-F356.
21. Sebinger DD, Unbekandt M, Ganeva VV, Ofenbauer A, Werner C, Davies JA. A novel, low-volume method for organ culture of embryonic kidneys that allows development of cortico-medullary anatomical organization. *PloS One* 2010;5(5):e10550.
22. Costantini F. GDNF/Ret signaling and renal branching morphogenesis: From mesenchymal signals to epithelial cell behaviors. *Organogenesis* 2010;6(4):252-262.
23. Rosines E, Johkura K, Zhang X, Schmidt HJ, Decambre M, Bush KT, Nigam SK. Constructing kidney-like tissues from cells based on programs for organ development: toward a method of in vitro tissue engineering of the kidney. *Tissue Eng Part A* 2010;16(8):2441-2455.

24. Srinivas S, Goldberg MR, Watanabe T, D'Agati V, al-Awqati Q, Costantini F. Expression of green fluorescent protein in the ureteric bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis. *Dev Genet* 1999;24(3-4):241-251.
25. Kent AL, Douglas-Denton R, Shadbolt B, Dahlstrom JE, Maxwell LE, Koina ME, Falk MC, Willenborg D, Bertram JF. Indomethacin, ibuprofen and gentamicin administered during late stages of glomerulogenesis do not reduce glomerular number at 14 days of age in the neonatal rat. *Pediatr Nephrol* 2009;24(6):1143-1149.
26. Lelievre-Pegorier M, Gilbert T, Sakly R, Meulemans A, Merlet-Benichou C. Effect of fetal exposure to gentamicin on kidneys of young guinea pigs. *Antimicrob Agents Chemother* 1987;31(1):88-92.
27. Richardson L, Venkataraman S, Stevenson P, Yang Y, Burton N, Rao J, Fisher M, Baldock RA, Davidson DR, Christiansen JH. EMAGE mouse embryo spatial gene expression database: 2010 update. *Nucleic Acids Res* 2010;38(Database issue):D703-709.
28. Ortiz LA, Quan A, Weinberg A, Baum M. Effect of prenatal dexamethasone on rat renal development. *Kidney Int* 2001;59(5):1663-1669.
29. Perantoni AO, Timofeeva O, Naillat F, Richman C, Pajni-Underwood S, Wilson C, Vainio S, Dove LF, Lewandoski M. Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. *Development* 2005;132(17):3859-3871.
30. Grieshammer U, Cebrian C, Ilagan R, Meyers E, Herzlinger D, Martin GR. FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. *Development* 2005;132(17):3847-3857.
31. Dudley AT, Godin RE, Robertson EJ. Interaction between FGF and BMP signaling pathways regulates development of metanephric mesenchyme. *Genes Dev* 1999;13(12):1601-1613.
32. Araki T, Hayashi M, Nakanishi K, Morishima N, Saruta T. Caspase-9 takes part in programmed cell death in developing mouse kidney. *Nephron Exp Nephrol* 2003;93(3):e117-e124.
33. Nathanson S, Moreau E, Merlet-Benichou C, Gilbert T. In utero and in vitro exposure to beta-lactams impair kidney development in the rat. *J Am Soc Nephrol* 2000;11(5):874-884.

5

Early postnatal gentamicin and ceftazidime treatment in Wistar rats: implications for kidney development

Ruud R.G. Bueters[1,2]
Annelies Klaasen[1,2]
Roger J.M. Brüggemann[3,4]
Lambertus P. van den Heuvel[1,2,5]
Michiel F. Schreuder[1,2]

[1]*Department of Pediatric Nephrology, Radboud University Medical Center, Nijmegen, the Netherlands.*
[2]*Radboud Institute for Molecular Life Science, Nijmegen, the Netherlands*
[3]*Department of pharmacy, Radboud University Medical Centre, Nijmegen, the Netherlands*
[4]*Radboud Institute for Health Science, Nijmegen, the Netherlands*
[5]*Department of Pediatrics, University Hospital Leuven, Leuven, Belgium.*

Abstract

Background

Up to two-thirds of premature born neonates are treated for bacterial infections with aminoglycosides such as gentamicin. Although acute toxicities are well described, there is uncertainty on developmental changes after treatment of premature born neonates. We studied the effect of gentamicin and ceftazidime on kidney development in the rat. Additionally we evaluated the modulating effect of extrauterine growth restriction.

Methods

Wistar rats were cross-fostered in normal food (NF) or food restricted (FR) litters at postnatal day (PND) 2 to simulate growth restriction and treated daily with placebo, 4 mg/kg of gentamicin or 50 mg/kg ceftazidime until PND 8. Gentamicin pharmacokinetics were studied in a separate group of animals. Kidneys were evaluated by qPCR on a set of developmental genes on PND 8, and by stereology on glomerular number and glomerular generation count at PND 35.

Results

Gentamicin plasma concentration and exposure were found to be in a human clinical range (mean C_{max} of 4.88 mg/L and Mean $AUC_{0\text{-}4h}$ of 10.71 mg.h/L for sexes combined) and did not affect any measured parameters. Ceftazidime reduced *Renin* expression by 1.7 fold ($p<0.01$). Extrauterine growth restriction resulted in a 22% body weight reduction by day 35 ($p<0.001$), 1.4-1.5 fold down regulation of *Renin, Oat1* and *Agtr1a* ($P<0.05$) expression and a 12% reduction in glomerular numbers (mean 30841 vs. 35187, $p<0.001$). Glomerular generation count was unaffected.

Conclusion

Our experiments showed that gentamicin at clinical levels did not disturb kidney development, ceftazidime can affect Renin expression, and extrauterine growth restriction impairs kidney development, but did not modulate potential drug toxicity.

Introduction

Antibiotics are routinely used to treat (suspected) bacterial infections in neonates on neonatal intensive care units. Gentamicin is a well-known aminoglycoside which is used as part of first line antibiotic treatment, but has a reputation for not being without harm. In neonates blood levels have to be carefully monitored because too high through concentrations may lead to oto- or nephrotoxicity (1, 2).

Although these acute toxicities are well known, there is much uncertainty on possible subtle developmental influences, which may play a role in long-term function. Gentamicin can enter cells by binding to the megalin receptor and is subsequently taken up via endosomes. In the cell it can either generate reactive oxygen species causing mitochondrial toxicity or induce the Bax BCl-2 apoptosis pathway (3). Disturbances of proliferation and apoptosis patterns can have major implications for renal development.

Indeed some studies have shown aminoglycosides to disturb kidney development *in vivo* or *ex vivo* (4-7), however other studies could not find an effect (8, 9). In the studies that did find a toxic effect, dose levels were high and one can argue that the level of acute toxicity had already been reached. In the other studies the kidneys were not affected by gentamicin treatment and dose levels were more similar to the clinical practice.

An alternative for gentamicin treatment in daily clinical practice may be found in ceftazidime, a drug from the cephalosporin drug class. Ceftazidime is regarded as a less potent drug regarding acute nephrotoxicity (10), but one of our earlier studies identified it as a possible kidney development disruptor (8). In that study ceftazidime affected *Gdnf* and *Fgf8*, two important genes involved in the ureteric bud growth process of kidney development. To follow-up on these findings and investigate the true potential for ceftazidime as an alternative drug, we included it in this study.

Most studies focus on the primary effects of drug treatment. This is a simplification from the clinical situation where a more complex interaction can be expected between drug treatment and other factors such as the gestational day at birth, malnourishment, growth restriction, and genetic background. It has been hypothesized that a multiple hit of these factors can influence organ development (11).

The aim of this study was to investigate the effect of gentamicin and ceftazidime at neonatal clinical dose levels on kidney development after early postnatal administration. Additionally we used an *in vivo* model of food restriction to add extrauterine growth restriction as a factor to the intervention to model for a multiple hit situation as frequently noted in neonatal clinical practice. We evaluated the number of glomeruli and other renal parameters as a measure for altered renal development. Secondary we measured the expression of a selection of important gene targets in kidney development. These could give an indication of a proposed mechanism behind any effects observed.

Material and methods

Main animal experiment

Timed-pregnant Wistar WU rats were ordered from Harlan (Horst, The Netherlands) and were delivered at the central animal laboratory of the Radboud University Nijmegen approximately one week before the expected date of birth. Animals were housed individually and checked at least twice a day for litters. The day that a litter was found complete was designated as PND 1. On PND 2 pups were cross-fostered to generate NF and FR litters of 12 and 20 pups, respectively. An additional NF and FR litter was formed to replace potential losses in the study population and maintain appropriate litter size. Starting on PND 2 animals were dosed with 0.9% NaCl (sham), 4 mg/kg gentamicin (Sigma-Aldrich, the Netherlands) or 50 mg/kg ceftazidime (Sigma-Aldrich, the Netherlands) intraperitonealy (ip) for 7 days adjusted for daily body weight. This resulted in 6 treatment groups (n=6 animals per group) in total (sham-NF and FR, gentamicin-NF and FR, and ceftazidime-NF- and FR) per gender. Body weight was determined daily up to day 35. Experiments were approved by the Animal Ethics Committee (protocol 2013-109) at the Radboud University Nijmegen.

Gentamicin pharmacokinetic experiment.

At PND 6, 15 pups/sex were injected once ip with gentamicin at 4 mg/kg. Blood was collected from 3 animals/sex/time point (5 time points in total) by exsanguination at 20 min, 40 min, 1, 2 and 4 hours post dose. Blood samples were collected into Li-heparin containing tubes and were cooled and centrifuged for 10 minutes at 5000*g* within 2 hours. Plasma was aspirated and stored at -80 °C until analysis for gentamicin concentration by the Cobas Integra 400 plus analyzer (Roche Diagnostics limited, Rotkreuz, Switzerland).

At PND 8, (at least) 3 animals/sex/group were sacrificed and kidneys were collected and weighed. The left kidney was frozen in liquid nitrogen for mRNA analysis and the right kidney was immersion-fixed in 10% formalin. At day 35 all remaining animals were sacrificed by exsanguination followed by perfusion-fixation with 10% formalin. Kidneys were collected, weighed and stored in 10% formalin and the right kidney was processed for stereology.

mRNA analysis

PND 8 left kidneys were pulverized in deep frozen state by a microdismembrator (Sartorius-Stedim, the Netherlands), processed according to methods earlier described (8), and one µg of RNA was used in a subsequent reverse transcriptase reaction. qPCR was performed on a Bio-Rad CFX96 using gene expression mix and hydrolysis probes (table 1) as ordered from Applied Biosystems (Pleasanton, California) or Biolegio (Nijmegen, the Netherlands). The panel of transcripts included important targets

in the developmental pathway, transporters in different tubular sections and major players in the renin-angiotensin system, which were selected based on the 'GenitoUrinary Development Molecular Anatomy Project' (GUDMAP) which analyzed expression patterns at different stages of development (12) or based on the importance of the targets in kidney function.

Delta Ct (ΔCt) values were reported, which were calculated by correcting the threshold cycle (Ct) of the gene of interest to the Ct of the housekeeping genes. Fold changes were calculated in case differences in ΔCt values were flagged for statistical significance.

Glomerular generation counting

Glomerular generations were determined according to the method described in the paper of Hinchliffe et al. (13). The direct method was used and 5 radials were counted per slide.

Stereology

Perfusion-fixed right kidneys were embedded in paraffin and sectioned exhaustively at 20 μm. Every 10th and 11th slide, starting at a random position (determined by a random number table), were taken and stained for haematoxylin-eosin. Subsequently, slides were scanned with the Olympus Olivia slidescanner and evaluation was performed with the Newcast module of the Visiopharm integrator system version 3.6.5.0 (Horzholm, Denmark).

Briefly, a sample and its adjacent section were aligned manually and a counting frame was superimposed. Subsequently, a region of interest was drawn manually and a randomly oriented counting grid was placed on the region with x and y step lengths of 2500 μm. Glomeruli were then counted in all section pairs and the total number of glomeruli per kidney (N(glom)) was estimated by the physical fractionator/disector principle. On average 158 glomeruli were counted per kidney and the total number of glomeruli was estimated using the following formula: $N(glom) = 1/SSF * 1/ASF * Ps/Pf * \sum Q^{-} /2$.

In this formula SSF is the section sample fraction and ASF is the area sample fraction (calculated as counting frame area divided by (x-step length * y- step length). The factor Ps/Pf was introduced to correct for slides with artificial edges; Ps is the number of counting frame corners that hit kidney tissue, Pf is the number of counting frame corners that hit evaluated kidney tissue. Finally, $\sum Q^{-}$ is the number of counted glomeruli and the factor ½ was introduced for counting both ways between the slide pairs.

Table 1 Applied Biosystems primer probe sets used in this study

Gene symbol	Common name	Primer-probe set
Reference		
Actb	Beta actin	Rn00667869_m1
Hmbs [a]	Hydroxymethylbilane synthase	5'-GGAGCCATGTCCGGTAACG-3' (forward) 5'-CCACTCGAATCACCCTCATCA-3' (reverse) 5'-CACAACCGCGGAAGAAAACGGCT-3'(probe-FAM)
Development		
Wnt4	Wingless-type MMTV integration site family, member 4	Rn00584577_m1
Gdnf	Glial cell line-derived neurotrophic facto	Rn00569510_m1
Bmp7	Bone morphogenetic protein 7	Rn01528889_m1
Fgf7	Fibroblast growth factor 7	Rn00573319_m1
Hgf	Hepatocyte growth factor	Rn00566673_m1
Lim	Lim homeobox 1	Rn00595845_m1
Ret	RET proto oncogene	Rn01463098_m1
Gfra1	GDNF receptor alpha 1	Rn00564156_m1
Renin-angiotensin system		
Renin	Renin	Rn00561847_m1
Agtr1a	Angiotensin receptior type 1	Rn00578456_m1
Agtr2 [a]	Angiotensin receptor2	5'-CCTTCCTGTATTGTTTCGTTGGA-3' (forward) 5'-TGGAGCCAAGTAATGGGAACTC-3' (reverse) 5'- CCGCTTCCAACAGAAGCTCCGTAGTG-3'(probe-FAM)
Tubular transporters		
Oat1	Organic anion transporter 1	Rn00568143_m1
Casr	Calcium sensing receptor	Rn00566496_m1
Enac	Epithelial sodium channel	Rn00566891_m1
Nkcc2	Na^+-K^+-Cl^- cotransporter	Rn00568143_m1
Nhe3	Sodium hydrogen exchanger 3	Rn00561944_m1
Nhe8	Sodium hydrogen exchanger 8	Rn01423020_m1
Ncc	Sodium-chloride symporter	Rn00571074_m1

[a] Ordered at Biolegio

Pharmacokinetic analysis

Non-compartmental pharmacokinetic parameters such as the time of maximum concentration (T_{max}), the maximum concentration in plasma (C_{max}) and the area under the plasma-concentration time curve up to the last measured concentration after 4 hours (AUC_{0-4h}) were analyzed by use of Phoenix 6.4 (Certara, Princeton, NJ). Based

on the curve shape, the AUC was calculated according to the linear up/log down trapezoidal method and no extrapolation of the area under the time-plasma curve from zero to infinity ($AUC_{0\text{-}inf}$) was performed.

Statistical analysis

Means with standard deviation (SD) are presented for all findings unless otherwise indicated. Parameters of interest were analyzed by univariate analysis using sex, nest type and treatment as fixed factors. Post hoc analysis was performed by Tukey. The time effect on body weight was analyzed between and within subjects with an analysis for repeated measures, using the Greenhouse-Geisser correction. The difference between left and right kidney weights was analyzed by ANCOVA. All analyses were performed by the use of SPSS version 20 (IBM, Armonk, NY) at the alpha 0.05 level.

Results

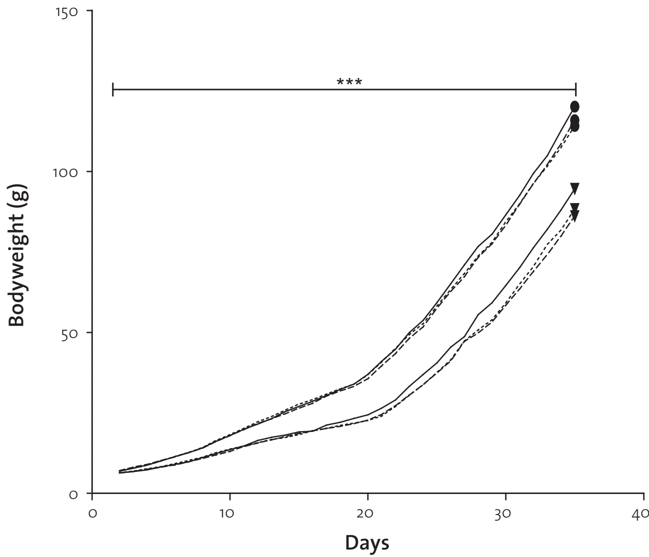
In vivo observations

Body weights of male and females were incidentally different on day 2 and day 30-35 ($p<0.001$), but were pooled for analysis. Mean body weight was 7 (SD 0.6)g for 'normal food' (NF) and 6.4 (SD 0.6)g for food restricted (FR) animals at crossfostering at postnatal day (PND) 2, corresponding to a 9% weight difference ($p<0.001$). The difference between NF and FR increased over the course of the study ($p<0.001$) to 22% (NF 14.11 (SD 1.1)g; FR 11.04 (SD 1.17)g) at PND 8 and 23% (116.7 (SD 12.4)g and 89.5 FR (SD 11.9)g) at PND 35 (figure 1). One male sham-FR animal died in the second week: we considered this to be related to the nature of the study (neonatal manipulations) but unrelated to the study interventions. No clinical signs were noted in the dosed rats. No effect of drug treatment was noted on body weight in the study period ($p=0.081$).

Pharmacokinetic analysis

A time concentration curve was constructed for both male and female rats dosed with a single dose of 4 mg/kg gentamicin on PND 6 (figure 2). The time of maximum concentration (T_{max}) was noted at the first time point of sampling (20 min after dosing). A mean concentration (C_{max}) of 4.91 mg/L and a mean exposure ($AUC_{0\text{-}4h}$) of 11.3 mg.h/L were noted for males, and a mean C_{max} and $AUC_{0\text{-}4h}$ of 4.85 mg/L and 10.2 mg.h/L, respectively, were noted for females. The $AUC_{0\text{-}inf}$ could not be calculated due to non-linearity in the elimination slope.

Figure 1 Body weights of study animals from postnatal day (PND) 2 up to PND 35



Lines of normal food (NF) and food restricted (FR) groups are indicated with dots and inversed triangles, respectively. Sham-dosed groups are indicated with a solid line, gentamicin groups with a striped line and ceftazidime groups with a dotted line. Body weight of FR animals was lower compared with the NF animals from day 2 onwards on all data points. Univariate analysis was performed with Tukey post-hoc. ***(P<0.001). n=15-16/group up to day 8; n=8-10/group for the remaining period.

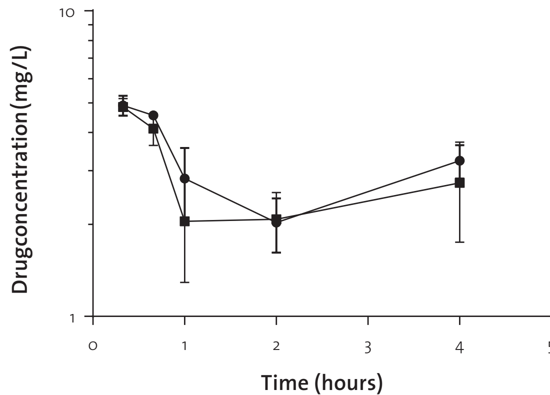
Gene expression

Gene expression analysis (figure 3) was performed on kidney tissue collected on PND 8 and analyzed by non-parametric tests. Gender did not influence the gene expression of any target and therefore the analyses between litter size and drug treatment were performed with a pooled set of data. Comparing FR with NF animals a 1.4-1.5 fold down-regulation (P<0.05) of *Renin, Oat1* and *Agtr1a* expression was noted. Additionally, drug treatment with ceftazidime resulted in a 1.7 fold reduction (P<0.01) in *Renin* compared with sham dosed animals.

Renal morphology

Left, right and total absolute kidney weights (table 2) were approximately 20% lower (p<0.001) in FR rats compared with NF rats and a difference between sexes was detected (p<0.001). When corrected for individual body weight, kidney weights were comparable between sexes and were not influenced by drug treatment. Overall left relative kidney weight (7.1 (SD 0.9) mg/g) was lower than right relative kidney weight

Figure 2 Time concentration curves in male (dot) and female (square) pups after a single interperitoneal administration of 4 mg/kg gentamicin on postnatal day 6.



Means and SEM are shown. C_{max} and $AUC_{0\text{-}4h}$ were 4.91 mg/L and 11.3 mg.h/L for males, and 4.85 mg/L and 10.2 mg.h/L for females. n=3/sex/time point.

(7.5 (SD 0.8) mg/g) (p=0.005). This difference was not explained by sex, treatment, or food restriction, or any interactions between these parameters.

Glomerular generation count (table 2) was performed and yielded on average 4.7 (SD 0.34) generations in the sham-NF animals. The kidneys of other groups had a similar number of generations (range 4.6-4.8) and no effect of food restriction (p=0.76) or drug treatment (p=0.56) was noted.

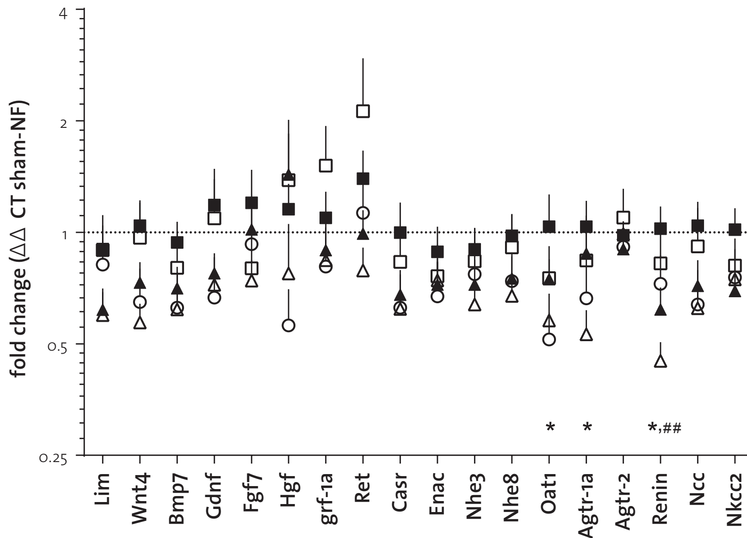
FR animals showed a decreased number of glomeruli compared with NF animals (means: 30841 (SD 4075) vs. 35187 (SD 4026), respectively; p<0.001) on PND 35 (table 2) and females showed to have lower glomerular numbers in general (31564 (SD 4698) vs. 34102 (SD 4131); p<0.001). After stratification for sex, the difference for litter size remained statistically significant in both sexes (p=0.029 and p=0.004 for males and females, respectively). Drug treatment did not influence glomerular numbers (p=0.47).

Table 2 Renal parameters at day 35

Treatment	Sham NF	Gentamicin NF	Ceftazidime NF	Sham FR	Gentamicin FR	Ceftazidime FR	Statistical significance
Body weight (g)	120.1 (10.1)	115.9 (14.3)	114.1 (13.2)	94.6 (13.6)	86.1 (12.0)	88.3 (9.6)	Litter size ** sex **
Left kidney weight (g)	0.88 (0.11)	0.81 (0.15)	0.76 (0.098)	0.62 (0.094)	0.62 (0.081)	0.65 (0.097)	Litter size ** sex **
Relative left kidney weight (mg/g body weight)	7.3 (0.7)	7.0 (1.1)	6.7 (0.6)	6.6 (0.8)	7.3 (1.3)	7.3 (0.7)	-
Right kidney weight (g)	0.90 (0.097)	0.84 (0.12)	0.83 (0.11)	0.69 (0.11)	0.68 (0.086)	0.69 (0.082)	Litter size ** sex **
Relative right kidney weight (mg/g body weight)	7.5 (0.3)	7.3 (0.5)	7.3 (0.4)	7.3 (0.8)	8.0 (1.3)	7.8 (0.4)	-
Mean kidney weight (g)	1.79 (0.18)	1.65 (0.25)	1.59 (0.20)	1.31 (0.19)	1.30 (0.15)	1.34 (0.16)	Litter size ** sex **
Mean Kidney weight (mg/g body weight	14.8 (0.7)	14.3 (1.5)	14.0 (1.0)	13.9 (2.5)	15.3 (2.5)	15.1 (0.8)	-
Glomerular generations (count)	4.7 (0.34)	4.6 (0.32)	4.8 (0.44)	4.7 (0.50)	4.6 (0.32)	4.7 (0.50)	-
Glomerular Number	35,332 (4,545)	35,206 (3,952)	35,023 (4,115)	28,994 (2,451)	32,561 (3,705)	30,783 (5,089)	Litter size ** sex *

Data are presented as mean (standard deviation). NF= normal food, FR= food restricted. Univariate regression analysis was performed on all parameters with Tukey post hoc for treatment. Left versus right kidney weight was assessed by ANCOVA. * $p<0.05$, ** $p<0.001$. n=8-10/group.

Figure 3 Relative renal gene expression compared to Sham-NF of relevant transporters, growth factors and receptors in kidney development measured on day 8



Mean Delta-Delta Ct values with SEM are plotted for each target gene. Shown are sham (dot), gentamicin (square) and ceftazidime (triangle) treatment. Normal food (NF) and food restricted (FR) are distinguished by closed and open symbols, respectively. Statistical analysis was performed by multivariate analysis with Tukey post-hoc for treatment: * $p<0.05$ FR vs. NF or ## $p<0.01$ ceftazidime vs. sham. n=5-8/group.

Discussion

The aim of our study was to evaluate the impact of two antibiotics, i.e. gentamicin and ceftazidime, on kidney development. Based on our data we conclude that gentamicin treatment did not affect kidney development in the studied clinical dose range. Gene expression of targets known to play a role in gentamicin toxicity such as the Na-H exchange transporters, *Casr* or members of the renin-angiotensin system were unaffected (14-16). Parameters considered as an end-point of kidney development such as glomerular numbers were not different from sham-dosed animals. These findings are in line with other previously performed *in vivo* studies (9). The classical tubular gentamicin damage was not observed and was not expected due to the low dose level.

A pharmacokinetic profile was generated for a single dose of gentamicin to compare our experimental set-up with the normal therapeutic level of neonates and a similar dose range was confirmed. Of Interest, the plasma level at the 4 hour time

point showed an increase. This may be explained by individual variation in exposure because all time points were sampled from different animals. This higher plasma level prohibited calculation of the $AUC_{0\text{-}inf}$ and limited our exposure information to 4 hours.

Nevertheless the profile we observed was similar to earlier reported pharmacokinetic studies in neonatal animals (17,18). In general gentamicin administration in juvenile rats has a slightly lower peak level as well as slower elimination rate compared with adult rats (17,18). In addition, neonatal rats have been described to have a lower rate of gentamicin accumulation in the kidneys compared with adult rats (19), which may have implications for the susceptibility to gentamicin toxicity.

The ceftazidime toxicity described by our group in an *ex vivo* model (8) was not confirmed in this *in vivo* study based on unaffected glomerular numbers. The nephrogenesis inhibiting effect may therefore be limited to the stage of very early kidney development, which holds more relevance to pregnant women than to preterm born neonates. The *Gdnf* and *Fgf8* genes, important in early nephrogenesis, were associated with these finding, but *Gdnf expression* did not change in the current study. We did note a 1.7 fold reduction in *Renin* expression, which plays an important role in the formation of kidney vasculature (20). The reduction was relatively small, however, and no direct morphological consequences were noted. Therefore the biological relevance remains unclear.

Our second aim was to evaluate whether extrauterine growth restriction could modulate the potential toxicity of drug treatment. Using the rat model of litter enlargement, we have previously shown that extrauterine growth restriction results in a reduction in nephron numbers (21). From the changes noted in body weight it is clear that a state of extrauterine growth restriction was induced, even though body weights were slightly different from the start of the study. Extrauterine growth restriction impaired kidney development evidenced by the 12% decrease in nephron numbers. Interestingly, the effect of extrauterine growth restriction was smaller in this study compared with the study of Schreuder et al, which reported a 25% difference (21). This variation in observed effect may be explained by the fact that the left kidney was sampled for stereology by Schreuder er al in contrast to the right kidney in the present study. It has been described before that the left kidney is more prone to congenital kidney and urinary tract abnormalities (22). Furthermore data from Simonetti et al. also showed that impaired intrauterine growth led to low birth weight and reduced left kidney weight in humans (23). We detected a difference in left *vs.* right kidney weight, but this was unrelated to our treatment or litter size, and is likely explained by normal anatomical left-right differences in rats.

Nevertheless, the observed decrease in right kidney nephron numbers did not affect relative right kidney weight compared with the NF animals. This shows that kidney weight is a poor predictor for nephron numbers, an observation that was made previously by our group by analyzing human data (24). In the end, although extrauterine

growth restriction itself impairs kidney development, we did not find evidence that it modulated a potential toxicity response of gentamicin or ceftazidime treatment.

There were some limitations to our experimental set-up. We only counted glomeruli in one kidney, while our results suggest that it would have been better to analyze both kidneys. Furthermore, we did not include a functional follow-up of the kidney in this study to investigate the clinical outcome of the observed effect, which could have led to a better clinical translation. Finally a pharmacokinetic profile of ceftazidime would have been helpful to put the ceftazidime related findings into perspective. Nevertheless these study results are important for better understanding of the clinical outcome. Both studies of Kent et al. and this study did not find evidence that gentamicin (with or without growth restriction) shows nephron developmental toxicity (9,25). Therefore it seems that there is no direct concern for developmental nephrotoxicity after gentamicin treatment in premature born neonates. We proposed ceftazidime would be a possible alternative for gentamicin in the clinic and based on the study findings it can be used without affecting kidney development. Additionally our study showed that the nutritional status is much more potent to disrupt nephrogenesis compared with clinically dosed antibiotics.

In conclusion, our experiments showed that (i) a clinical relevant dose of gentamicin or ceftazidime did not directly disturb the process of nephrogenesis, (ii) extrauterine growth restriction reduces nephron endowment, but did not modulate potential drug toxicity.

Based on these results there is no direct harm in using gentamicin or ceftazidime in neonatal practice concerning kidney development. Future experiments should focus on a functional follow-up and an additional challenge in later life to investigate any programming effects.

References

1. Rybak LP, Ramkumar V. Ototoxicity. *Kidney Int* 2007;72(8):931-935.
2. Lopez-Novoa JM, Quiros Y, Vicente L, Morales AI, Lopez-Hernandez FJ. New insights into the mechanism of aminoglycoside nephrotoxicity: an integrative point of view. *Kidney Int* 2011;79(1):33-45.
3. Servais H, Ortiz A, Devuyst O, Denamur S, Tulkens PM, Mingeot-Leclercq MP. Renal cell apoptosis induced by nephrotoxic drugs: cellular and molecular mechanisms and potential approaches to modulation. *Apoptosis* 2008;13(1):11-32.
4. Gilbert T, Cibert C, Moreau E, Geraud G, Merlet-Benichou C. Early defect in branching morphogenesis of the ureteric bud in induced nephron deficit. *Kidney Int* 1996;50(3):783-795.
5. Gilbert T, Lelievre-Pegorier M, Malienou R, Meulemans A, Merlet-Benichou C. Effects of prenatal and postnatal exposure to gentamicin on renal differentiation in the rat. *Toxicology* 1987;43(3):301-313
6. Gilbert T, Lelievre-Pegorier M, Merlet-Benichou C. Long-term effects of mild oligonephronia induced in utero by gentamicin in the rat. Pediatr Res 199130(5):450-456.
7. Schwedler SB, Gilbert T, Moreau E, Striker LJ, Merlet-Benichou C, Striker GE. Nephrotoxin exposure in utero reduces glomerular number in sclerosis-prone but not sclerosis-resistant mice. Kidney Int 1999;56(5): 1683-1690.
8. Bueters RR, Kusters LJ, Klaasen A, van den Heuvel LP, Schreuder MF. Antibiotics and renal branching morphogenesis: comparison of toxicities. Pediatr Res 2014;76(6):508-514.
9. Kent AL, Douglas-Denton R, Shadbolt B, Dahlstrom JE, Maxwell LE, Koina ME, Falk MC, Willenborg D, Bertram JF. Indomethacin, ibuprofen and gentamicin administered during late stages of glomerulogenesis do not reduce glomerular number at 14 days of age in the neonatal rat. *Pediatr Nephrol* 2009;24(6):1143-1149.
10. Tune BM. Nephrotoxicity of beta-lactam antibiotics: mechanisms and strategies for prevention. *Pediatr Nephrol* 1997;11(6):768-772.
11. Schreuder MF. Safety in glomerular numbers. *Pediatr Nephrol* 2012;27(10):1881-1887.
12. Richardson L, Venkataraman S, Stevenson P, Yang Y, Burton N, Rao J, Fisher M, Baldock RA, Davidson DR, Christiansen JH. EMAGE mouse embryo spatial gene expression database: 2010 update. *Nucleic Acids Res* 2010;38(Database issue):D703-D709.
13. Hinchliffe SA, Sargent PH, Chan YF, van Velzen D, Howard CV, Hutton JL, Rushton DI. "Medullary ray glomerular counting" as a method of assessment of human nephrogenesis. *Pathol Res Pract* 1992;188(6): 775-782.
14. Levi M, Cronin RE. Early selective effects of gentamicin on renal brush-border membrane Na-Pi cotransport and Na-H exchange. *Am J Physiol* 1990;258(5 Pt 2):F1379-F1387.
15. Valdivielso JM, Rivas-Cabanero L, Morales AI, Arevalo M, Lopez-Novoa JM, Perez-Barriocanal F. Increased renal glomerular endothelin-1 release in gentamicin-induced nephrotoxicity. *Int J Exp Pathol* 1999;80(5):265-270.
16. Duque I, Garcia-Escribano C, Rodriguez-Puyol M, Diez-Marques ML, Lopez-Novoa JM, Arribas I, Hernando L, Rodríguez-Puyol D. Effects of reactive oxygen species on cultured rat mesangial cells and isolated rat glomeruli. *Am J Physiol* 1992;263(3 Pt 2):F466-F473.
17. Milner RD, Milner GR, Lancaster D. Tissue gentamicin concentrations in the newborn and adult rat. *Pediatr Res* 1979;13(3):161-166.
18. Marre R, Tarara N, Louton T, Sack K. Age-dependent nephrotoxicity and the pharmacokinetics of gentamicin in rats. *Eur J Pediatr* 1980;133(1):25-29.
19. Nassberger L, DePierre JW. Uptake of gentamicin in foetal and newborn rats after a single intraperitoneal injection. *Pharmacol Toxicol* 1989;64(1):126-131.
20. Sequeira-Lopez ML, Nagalakshmi VK, Li M, Sigmund CD, Gomez RA. Vascular versus tubular renin: role in kidney development. Am J Physiol Regul Integr Comp Physiol 2015;309(6):R650-R657.
21. Schreuder MF, Nyengaard JR, Remmers F, van Wijk JA, Delemarre-van de Waal HA. Postnatal food restriction in the rat as a model for a low nephron endowment. *Am J Physiol Renal Physiol* 2006;291(5): F1104-F1107.

22. Schreuder MF. Unilateral anomalies of kidney development: why is left not right? *Kidney Int* 2011;80(7):740-745.
23. Simonetti GD, Mohaupt MG. Smaller left kidney in low birth weight children. *Kidney Int* 2012;81(4):418; author reply -9.
24. Bueters RR, van de Kar NC, Schreuder MF. Adult renal size is not a suitable marker for nephron numbers: an individual patient data meta-analysis. *Kidney Blood Press Res* 2013;37(6):540-546.
25. Kent AL, Maxwell LE, Koina ME, Falk MC, Willenborg D, Dahlstrom JE. Renal glomeruli and tubular injury following indomethacin, ibuprofen, and gentamicin exposure in a neonatal rat model. *Pediatr Res* 2007;62(3):307-312.

6

Effect of NSAIDs and diuretics on nephrogenesis in an ex vivo embryogenic kidney model

R.R.G. Bueters
A.Klaasen
L.P. van den Heuvel
M.F. Schreuder

Department of Pediatric Nephrology, Radboud University Nijmegen Medical Centre, the Netherlands.

(**Bueters *et al***. Published in Birth Defects Research B Developmental Reproduction Toxicology, 2013 Dec;98(6):486-92.)

Abstract

The kidney is one of the key organs in clearing foreign compounds. The effects of drugs on the developing kidney are relatively unknown. We studied the direct effect of furosemide, hydrochlorothiazide, ibuprofen and indomethacin on kidney development in an ex vivo embryonic kidney model. At embryonic day 13 metanephroi were dissected from mice and cultured in control media or media supplemented with various clinically relevant concentrations of drugs. The ureteric tree was visualized by whole mount staining and branching was evaluated by counting. Additionally gene expression levels of *Wt1*, *Sox9*, *Bmp7*, *Fgf8* and *Gdnf* were investigated.

No distinct differences were noted on either ureteric tip development or gene expression analysis for each drug after 24 hours of exposure. Even though short term exposure to clinically relevant concentrations seems not to disturb renal development, future research is needed to study prolonged or repeated exposures.

Keywords: NSAID, diuretics, kidney development, embryonic kidney model, nephrogenesis, toxicology

Introduction

The kidney is one of the key organs in clearing foreign compounds. Whether these are active toxins or (de)toxified metabolites, all drugs will pass through the kidney in one form or another when administered. Therefore a lot of research is being done on the mature kidney and how drugs are excreted, metabolized and of course which drugs are nephrotoxic (1).

Less emphasis is put on the effects of drugs on the kidney as a developmental organ. This is an important field of research because the kidney already has to deal with drugs in its immature form (2). For instance, unborn children of pregnant women who take drugs or children who are born preterm and not yet have a completely developed kidney. In this study we focus on two classes of drugs, taken by both groups.

First, non steroidal anti inflammatory drugs (NSAIDs) like ibuprofen may be taken by pregnant women as an over-the-counter drug for pain, even though NSAIDs are contra-indicated during pregnancy, especially the last trimester (3). Secondly indomethacin may be prescribed as a tocolytic (4). Finally born children with a patent ductus arteriosis are treated with ibuprofen or indomethacin, depending on the geographical area of treatment. It is well known that these drugs may cause renal toxicity in preterm children, such as acute renal failure (5-7), but these drugs are also classified as potentially nephrotoxic drugs for adult use (8).

Our second drug class of interest is the diuretics class. Pregnant women and preterm children may both be prescribed diuretics to control fluid homeostasis. A frequently used diuretic is furosemide. Although acute renal toxicity is not common, furosemide is associated with renal calcification in higher doses (cumulative dose >10 mg/kg) (9).

The acute toxic risks of these drug classes may be limited by strict dose monitoring, but it is currently unknown whether low concentrations of these drugs may disrupt normal kidney development. This may result in a predisposition for renal disease in later life (2).

There are several leads that these drugs may influence kidney development. First of all, two studies in rats showed abnormalities in kidney tubuli after 5 days of treatment with indomethacin or ibuprofen, but these changes were not confirmed at necropsy on day 14 and long-term sequelae were not studied (10,11). Secondly, Mallie et al showed that prenatal furosemide exposure in rats tended to a lower number of differentiated glomeruli at birth and a functional deficiency in electrolyte handling at birth, which was still present, but somewhat less pronounced, at day 12 (12,13).

Given these leads our hypothesis is that clinical and/or low doses of furosemide, indomethacin and ibuprofen can influence basic pathways of nephrogenesis and that hydrochlorothiazide might be an alternative for furosemide. Additionally we hypothesize that ibuprofen and indomethacin inhibit the cyclo oxygenase systems in our kidneys. We studied these hypotheses by determining the direct effect of these

drugs on ureteric tip development and several developmental gene expression markers in an ex vivo embryonic kidney model.

Materials and Methods

Drugs

Ibuprofen (I1892), indomethacin (I7378), furosemide (F4381) and hydrochlorothiazide (H2910) were all obtained from Sigma-Aldrich, The Netherlands.

Organ culture

Experiments were approved by the Animal Ethics Committee at the Radboud University Nijmegen. Embryonic day 13 time pregnant HSD:ICR female mice (Harlan®, Horst, The Netherlands) were euthanized immediately after arrival by cervical dislocation and kidneys were dissected from the embryos by means of two small needles. Intact isolated metanephroi were held on ice in pre-cooled Leibovitz medium (Gibco®, Invitrogen™, Paisley, UK) until transfer to the organ culture system. Metanephroi were cultured on 0.4 µm pore size Millicell® cell culture inserts (Millipore™, Carrigtwohill, Ireland) placed in a six well plate containing DMEM/F-12 (1:1) medium (Gibco®, Invitrogen™, Paisley, UK), supplemented with 10 mg/L insulin, 5.5 mg/L transferrin and 5 µg/L sodium selenite (Sigma-Aldrich®, St. Louis USA). Depending on treatment, drugs were added to the medium and metanephroi were incubated at 37°C and 5% CO_2 for 24 hours or 3 days (medium was refreshed once after 48 hours). The following concentrations were used: 0.2–2–20 µM ibuprofen, 10-100-1000 nM indomethacin, 70-700-7000 nM furosemide and 0.2-2-20 µM hydrochlorothiazide. Indomethacin and hydrochlorothiazide required methanol as a co-solvent, which had a final concentration of 0.1% in medium. A vehicle control group was included for each drug. Concentration levels were calculated as follows: The clinical human child dose was taken and corrected for plasma binding, and volume of distribution (14-19). A concentration at a factor 10 above and below was taken to deal with calculation uncertainties and to investigate a dose response relationship.

All metanephroi from one litter were randomly assigned to one of the different treatment groups. Per litter, one drug was investigated to limit differences on basis of inter-litter variability. Multiple litters were studied per drug.

Ureteric tip imaging

Whole mount immunostaining of the metanephroi was performed to visualize the ureteric tree after 24 hours of culture. The metanephroi were fixed in ice-cold methanol for 10 minutes and washed with PBS for 15 minutes. Subsequently, the metanephroi were incubated in PBS containing 2% BSA for 12h to block non-specific

binding. After washing with PBS containing 1% Triton X-100 (PBS-T), metanephroi were incubated with an antibody against calbindin-D28k (Sigma Aldrich®, St. Louis, USA), diluted 1:100 in PBS-T, for 24h. Again after washing with PBS-T, incubation with an Alexa 488 IgG antibody (Invitrogen, Eugene, USA) was performed at a dilution of 1:300 in PBS containing 2% BSA for 24h. All incubation and washing steps were performed at 4°C. After a final washing step in PBS-T of 15 minutes, the metanephroi were mounted on a slide in mounting medium (Dako, Carpinteria, USA) and sealed using paraffin.

For each metanephros, ureteric branching was visualized by confocal laser scanning microscopy using a Leica TCS SP2 microscope. Optical sectioning was performed at a 4 µm interval at a magnification of 200x. Per metanephros, between 15 and 30 images were acquired with a resolution of 1024x1024 pixels. Subsequently, the amount of ureteric tips was counted with the multipoint tool in FIJI. A tip was defined as an end point of the whole branching structure that did not show any signs of branching.

Ureteric bud count was corrected for area surface of the metanephroi at the time of explantation as described by Bueters et al (submitted). Metanephric surface area was measured from photographs with FIJI/ImageJA version 1.45i (http://imagej.nih.gov/ij/). Photographs of the metanephroi were obtained with a Canon EOS 1000D camera attached to a Zeiss Axiovert 25 microscope at a total magnification of 12.5x. Background light was set on maximal intensity and a shutter speed of 40 ms was used. The photographs were made at a resolution of 3888x2592 pixels.

Gene expression analysis

We selected five targets in pathways involved in nephrogenesis by using literature search and GUDMAP, i.e. *Wt-1, Sox-9, Bmp-7, Fgf-8 and Gdnf* (20-24).

RNA was isolated from the metanephroi by combining the Trizol extraction method with the NucleoSpin® RNA II isolation kit (Machery-Nagel, Düren, Germany). The methanephroi were suspended in Trizol (Invitrogen, Carlsbad, USA) and incubated for approximately 15 minutes with occasional vortexing. After addition of chloroform (Merck), the samples were incubated on ice for 5 minutes and centrifuged at 14,000g, 4°C for 15 minutes. The aqueous phase was added 1:1 to 70% ethanol to adjust binding conditions and loaded on the Nucleospin® column. Further purification was performed according to the manufacturers protocol. RNA concentration and quality was assessed with the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

Complementary DNA was generated on a Biozym MJ Research PTC-200 Peltier thermal cycler using random primers (Promega, Madison, USA), oligo dT (Promega, Madison, USA) and M-MLV reverse transcriptase (Invitrogen, Carlsbad, USA). mRNA levels were measured by quantitative PCR (qPCR) with *Actb* and *Hmbs* as internal

standards. Additionally, NSAID high dose treated samples were investigated for *Ptgs1* and *Ptgs2* expression as a marker for the influence on the cyclooxygenase (COX) system and therefore drug delivery in the organ culture system.

qPCR was performed on A biorad CFX96 using the gene expression mix and hydrolysis probes (Table 1) as ordered form Applied bioscience (Applied Biosystems, Pleasanton, USA). Delta-delta CT values were reported and investigated.

Data analysis

Comparison of ureteric bud tip development and gene expression for each treatment class was investigated by one-way ANOVA followed with Tukey's multiple comparisons as post hoc. For both assays, statistical significance was investigated at the $\alpha=0.05$ level.

Table 1 Applied Biosystems primer probe sets used in this study

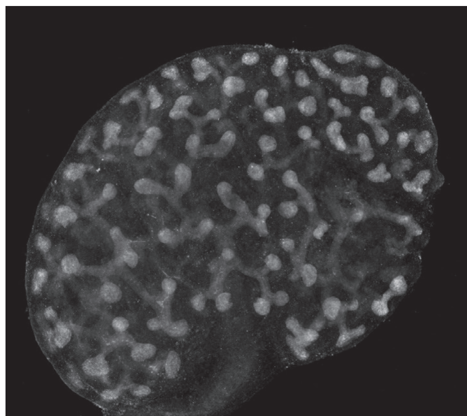
Gene symbol	Primer-probe set
Actb	endogenous control
Hmbs	Mm01143545_m1
Fgf8	Mm00438921_m1
Gdnf	Mm00599849_m1
Sox9	Mm00448840_m1
Wt1	Mm00460570_m1
Bmp7	Mm00432102_m1
Ptgs1	Mm00477214_m1
Ptgs2	Mm00478374_m1

Results

Metanephroi were successfully stained and suitable for analysis (figure 1).

We tested three doses of each drug on their ureteric tip development as shown in figure 2. The number of ureteric buds/surface area $_{t=0}$ were comparable between our treated and untreated groups after the 24 hour incubation period, with a slight statistical significant decrease in the 100 nM indomethacine treatment group (figure 2a). However no effect was observed at the 1000 nM concentration and a dose response relationship could not be established.

Figure 1 Representative immunohistochemic staining of ureteric bud development in metanephroi cultured for 24 hours (amplification 200x)

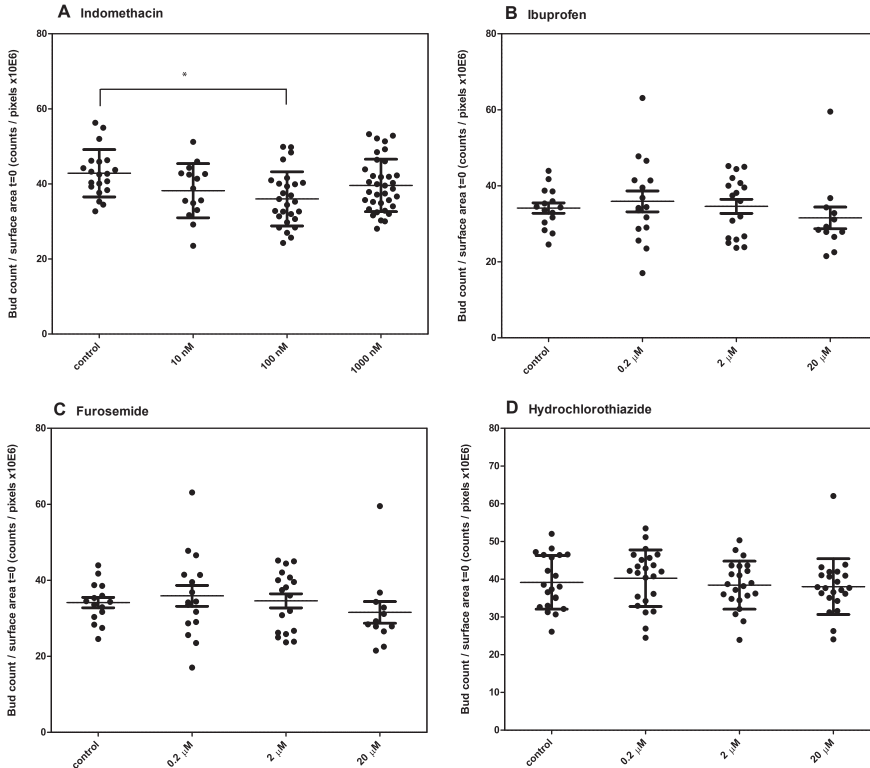


We also studied a selection of targets in known nephrogenesis pathways by mRNA expression analysis. The measured CT levels of all our assays indicated that all targets were present in our metanephric organ cultures, as expected. Subsequently, we compared our treatment groups with the non-treatment groups as shown in figure 3. Treatment with the clinical and high concentrations of each diuretic treatment (figure 3c & d) did not influence expression levels, which were similar to those of the control population.

The NSAID treatments (figure 3a & b) resulted in more alterations, especially in *Fgf8*. However this response was only noted in the clinical tested dose. The higher dose tested did not result into a similar response.

Furthermore, we tested if expression of the *Ptgs1* and *Ptgs2* genes was affected in the NSAID treatment (data not shown). We did not see consistent results in this

Figure 2 Quantitative analysis of ureteric buds in metanephroi cultured for 24hours in media with indomethacin (**A**), ibuprofen (**B**), furosemide (**C**) or hydrochlorothiazide (**D**)

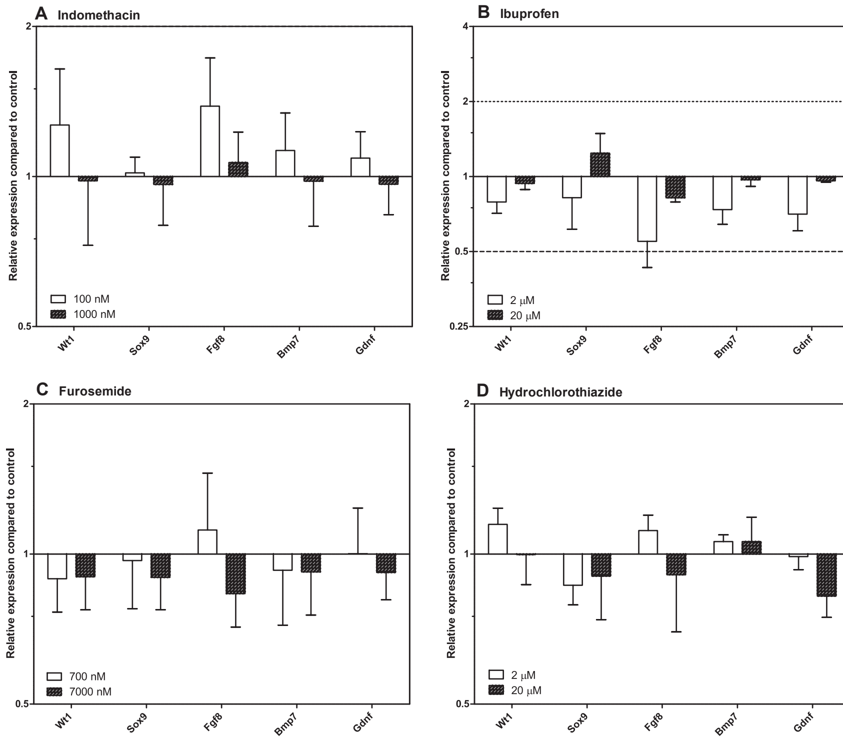


Individual data points as well as means ± sd. per treatment group are presented. Low, clinical and high concentrations were tested. * $p < 0.05$.

experiment. Both ibuprofen and indomethacin did show *Ptgs2* up-regulation in one out of three tested samples (respectively 2.3 and 2.2 times).

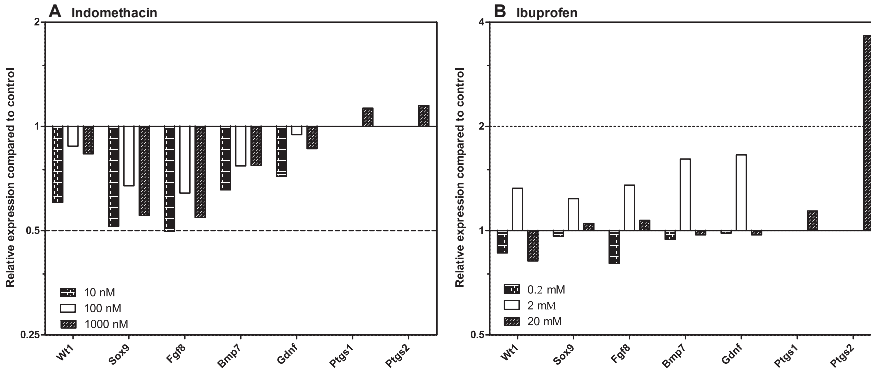
Finally a three-day exposure pilot was performed to investigate if lengthening the exposure time would make any difference on the noted response (figure 4a & b). A trend towards down regulation of *Sox9* and *Fgf8 (*crossing the 2 fold threshold in the lowest dose) was noted in indomethacin treated samples. Ibuprofen did not show any response on our selected targets. Additionally we noted a 3.7x up regulation in *Ptgs2* after 3 days of ibuprofen treatment, but no other effects were noted on either *Ptgs1* or *Ptgs2* expression after 3 days treatment of indomethacin.

Figure 3 Gene expression of important nephrogenesis pathway targets in metanephroi cultured for 24 hours in media with indomethacin (**A**), ibuprofen (**B**), furosemide (**C**) and hydrochlorothiazide (**D**)



Means ± SEM are presented for three experiments with clinical or high dose concentrations. 6-9 metanephroi were pooled per experiment.

Figure 4 Gene expression profile after 3-day culture pilot with ibuprofen or indomethacin (n=1)



6-8 metanephroi were pooled per condition.

Discussion

Our main question was whether clinical and low doses of NSAIDs or diuretics would disrupt nephrogenesis and which pathways would play a role in such effects. Based on the current experiments, no significant effect of our four tested drugs on either ureteric branching morphogenesis or any of the targeted nephrogenesis pathways was found.

These findings were unexpected as we hypothesized a response from the NSAID treatments because the COX systems are regarded important in kidney development (25-27). Indeed, a study by Kent et al hinted at increased tubular growth after administering low dose ibuprofen and indomethacin to rat pups (11). Although small differences were noted in *Fgf8* expression between ibuprofen and indomethacin versus controls, we did not see any response of our investigated genes involved in tubular formation (*Gdnf, Sox9*). Additionally, we did not see any dose dependent response in *Fgf8* expression, therefore the biological relevance of this small difference is questionable. Furthermore, we feel that all results under a 2-fold difference are more likely to occur due to sample and assay variation.

We could not support the rat study of Kent et al, which may be due to several factors. Most importantly, our model consists of a very rudimentary kidney organ and it could very well be that it behaves differently to the NSAID stimuli than the more mature kidney, which Kent used in his study (11). Furthermore, our study is limited in the number of genes tested, and therefore it could be that other genes are responsible. Finally there may be a species difference to NSAID treatment.

We investigated whether the metanephroi were fully exposed to the study drugs because we used a different exposure route compared to the *in vivo* situation. Our ex vivo culture model relies on diffusion to insert drugs into the target tissue because blood vessels are not yet developed. This may occasionally hinder drug delivery, depending on the particle type (28). However the noted up-regulation in the *Ptgs2* target in both the high concentration treated indomethacin and ibuprofen group after 24 hours show that uptake of these drugs by the tissue is likely.

Besides the drug availability, we investigated if a longer (3-day) period of exposure influences the broad drug response. Even though we noted a trend towards down regulation in *Sox9* and *Fgf8* of our target genes after indomethacin treatment, the differences compared to controls were small. We did not observe any significant changes in ibuprofen treated samples. Therefore, the drug response in this pilot experiment was similar to the one after 24 hours of incubation. Currently we are unable to confirm these findings with ureteric branching experiments. After approximately 36 hours of culture the kidney tissue becomes too dense for our antibody to fully penetrate. Analyses at later time points are therefore unreliable and this is a limitation of our current model.

Both diuretic treatments did not show an influence on nephrogenesis. Mallie et al showed differences in glomeruli differentiation after exposure to furosemide, which made us expect alterations in the ureteric bud count (13). Comparing our studies, our kidney model is similar in development to one of the two used dosing periods. However we used a lower dose that is within normal clinical ranges and a shorter exposure time, which may well explain the difference in our findings.

It is likely that the metanephroi are able to cope with the less optimal situation in our experiments, explaining the lack of toxicity. However, as we investigated only a few routes of possible nephrogenesis disruption, it is possible that toxicity is mediated via other pathways than the ones investigated in this study. Additional studies may be advisable where microarray techniques are used on multiple time points of development.

A potential limitation of our study could be found in the dosing that was based on exposure levels in neonates, whereas maternal dosing may be more relevant for this (early) stage of kidney development. Therefore, we also calculated the target exposure levels using adult pharmacokinetic data (14, 29, 30) and these are in the same order of magnitude as our tested doses (for instance indomethacin 30 nM). As these compounds all pass the placenta without restriction (30-33), we are confident that our dosing, and therefore results are applicable to *in utero* exposure as well.

In summary we did not find significant differences in kidney development of mouse metanephroi after 24 hour of culture with a clinical or low dose of indomethacin, ibuprofen, furosemide or hydrochlorothiazide. However, it is too early to conclude that pregnant women and preterm children can safely be treated with these drugs with

regards to kidney development. Even though short term exposure to clinically relevant concentrations seems not to disturb renal development, future research is needed to study prolonged or repeated exposures.

References

1. Werner M, Costa MJ, Mitchell LG, Nayar R. Nephrotoxicity of xenobiotics. *Clin Chim Acta* 1995;237(1-2):107-154.
2. Schreuder MF, Bueters RR, Huigen MC, Russel FG, Masereeuw R, van den Heuvel LP. Effect of drugs on renal development. *Clin J Am Soc Nephrol* 2011;6(1):212-217.
3. FDA. http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm125225.htm ; last accessed 7 march 2013.
4. Abramovici A, Cantu J, Jenkins SM. Tocolytic therapy for acute preterm labor. *Obstet Gynecol Clin North Am* 2012;39(1):77-87.
5. Cuzzolin L, Fanos V, Pinna B, di Marzio M, Perin M, Tramontozzi P, Tonetto P, Cataldi L. Postnatal renal function in preterm newborns: a role of diseases, drugs and therapeutic interventions. *Pediatr Nephrol* 2006;21(7):931-938.
6. Shaffer CL, Gal P, Ransom JL, Carlos RQ, Smith MS, Davey AM, Dimaguila MA, Brown YL, Schall SA. Effect of age and birth weight on indomethacin pharmacodynamics in neonates treated for patent ductus arteriosus. *Crit Care Med* 2002;30(2):343-348.
7. Akima S, Kent A, Reynolds GJ, Gallagher M, Falk MC. Indomethacin and renal impairment in neonates. *Pediatr Nephrol* 2004;19(5):490-493.
8. Musu M, Finco G, Antonucci R, Polati E, Sanna D, Evangelista M, Ribuffo D, Schweiger V, Fanos V. Acute nephrotoxicity of NSAID from the foetus to the adult. *Eur Rev Med Pharmacol Sci* 2011;15(12):1461-1472.
9. Gimpel C, Krause A, Franck P, Krueger M, von Schnakenburg C. Exposure to furosemide as the strongest risk factor for nephrocalcinosis in preterm infants. *Pediatr Int* 2010;52(1):51-56.
10. Kent AL, Douglas-Denton R, Shadbolt B, Dahlstrom JE, Maxwell LE, Koina ME, Falk MC, Willenborg D, Bertram JF. Indomethacin, ibuprofen and gentamicin administered during late stages of glomerulogenesis do not reduce glomerular number at 14 days of age in the neonatal rat. *Pediatr Nephrol* 2009;24(6):1143-1149.
11. Kent AL, Maxwell LE, Koina ME, Falk MC, Willenborg D, Dahlstrom JE. Renal glomeruli and tubular injury following indomethacin, ibuprofen, and gentamicin exposure in a neonatal rat model. *Pediatr Res* 2007;62(3):307-312.
12. Mallie JP, Boudzoumou P. Functional renal maturation in rat neonates after prenatal exposure to furosemide. *Pediatr Nephrol* 1996;10(4):458-460.
13. Mallie JP, Gerard A, Gerard H. Does the in-utero exposure to furosemide delay the renal maturation? *Pediatr Pharmacol* (New York) 1985;5(2):131-138.
14. zorgverzekeringen Cv. Farmacotherapeutisch Kompas. A. van Gameren; 2013.
15. Peterson RG, Simmons MA, Rumack BH, Levine RL, Brooks JG. Pharmacology of furosemide in the premature newborn infant. *J Pediatr* 1980;97(1):139-143.
16. Weninger M, Pollak A, Salzer-Muhar U, Vergesslich KA, Salzer HR. Pharmacokinetics of intra-arterial indomethacin treatment for patent ductus arteriosus. *Eur J Pediatr* 1989;149(2):138-140.
17. Aranda JV, Varvarigou A, Beharry K, Bansal R, Bardin C, Modanlou H, Papageorgiou A, Chemtob S. Pharmacokinetics and protein binding of intravenous ibuprofen in the premature newborn infant. *Acta Paediatr* 1997;86(3):289-293.
18. Hirt D, Van Overmeire B, Treluyer JM, Langhendries JP, Marguglio A, Eisinger MJ, Schepens P, Urien S. An optimized ibuprofen dosing scheme for preterm neonates with patent ductus arteriosus, based on a population pharmacokinetic and pharmacodynamic study. *Br J Clin Pharmacol* 2008;65(5):629-636.
19. FDA. http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/020186s026lbl.pdf; last accessed 7 march 2013.
20. Costantini F. GDNF/Ret signaling and renal branching morphogenesis: From mesenchymal signals to epithelial cell behaviors. *Organogenesis* 2010;6(4):252-262.
21 Reginensi A, Clarkson M, Neirijnck Y, Lu B, Ohyama T, Groves AK, Sock E, Wegner M, Costantini F, Chaboissier MC, Schedl A. SOX9 controls epithelial branching by activating RET effector genes during kidney development. *Hum Mol Genet* 2011;20(6):1143-1153.
22. Blank U, Brown A, Adams DC, Karolak MJ, Oxburgh L. BMP7 promotes proliferation of nephron progenitor cells via a JNK-dependent mechanism. *Development* 2009;136(21):3557-3566.

23. Davies JA, Little MH, Aronow B, Armstrong J, Brennan J, Lloyd-MacGilp S, Armit C, Harding S, Piu X, Roochun Y, Haggarty B, Houghton D, Davidson D, Baldock R. Access and use of the GUDMAP database of genitourinary development. *Methods Mol Biol* 2012;886:185-201.
24. Grieshammer U, Cebrian C, Ilagan R, Meyers E, Herzlinger D, Martin GR. FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. *Development* 2005;132(17):3847-3857.
25. Yamamoto E, Izawa T, Kuwamura M, Yamate J. Immunohistochemical Expressions of Main PGE(2) Biosynthesis-related Enzymes and PGE(2) Receptor in Rat Nephrogenesis. *J Toxicol Pathol* 2011;24(4):257-261.
26. Avner ED, Sweeney WE, Jr., Piesco NP, Ellis D. Growth factor requirements of organogenesis in serum-free metanephric organ culture. *In Vitro Cell Dev Biol* 1985;21(5):297-304.
27. Komhoff M, Wang JL, Cheng HF, Langenbach R, McKanna JA, Harris RC, Breyer MD. Cyclooxygenase-2-selective inhibitors impair glomerulogenesis and renal cortical development. *Kidney Int* 2000;57(2):414-422.
28. Lee WC, Berry R, Hohenstein P, Davies J. siRNA as a tool for investigating organogenesis: The pitfalls and the promises. *Organogenesis* 2008;4(3):176-181.
29. Alvan G, Orme M, Bertilsson L, Ekstrand R, Palmer L. Pharmacokinetics of indomethacin. *Clin Pharmacol Ther* 1975;18(3):364-373.
30. Ibuprofen: Drug information. In: UpToDate, Basow D, editor. UpToDate: Waltham, MA; 2013.
31. Indomethacin: Drug information. In: UpToDate, Basow D, editor. UpToDate: Waltham, MA; 2013.
32. Furosemide: Drug information. In: UpToDate, Basow D, editor. UpToDate: Waltham, MA; 2013.
33. Hydrochlorothiazide:drug information. In: UpToDate, Basow D, editor. UpToDate: Waltham,MA; 2013.

7

Impact of early postnatal NSAID treatment on nephrogenesis in Wistar rats

Ruud.R.G. Bueters[1]
Annelies Klaasen[1]
Nuria Maicas[2]
Sandrine Florquin[3]
Lambertus P. van den Heuvel[1]
Michiel.F. Schreuder[1]

[1]*Department of Pediatric Nephrology, Radboud University Medical Center, Nijmegen, the Netherlands.*
[2]*Department of Experimental Nephrology , Radboud University Medical Center, Nijmegen, the Netherlands*
[3]*Department of Pathology, Radboud University Medical Center, Nijmegen, the Netherlands*

(**Bueters *et al***. Published in Birth Defects Research B Developmental Reproduction Toxicology, 2015 Oct;104(5):218-26.)

Abstract

Background

Prematurely born children with patent ductus arteriosus are treated with ibuprofen or indomethacin, which may inhibit kidney development. We determined whether clinical doses affected kidney development and function, with or without extrauterine growth retardation.

Methods

Wistar rats were crossfostered in normal food (NF) or food restricted (FR) litters at postnatal day (PND) 2. On PND 3-4 three doses of 0.9% NaCl, 0.1 mg/kg indomethacin or 10 mg/kg ibuprofen were administered via intraperitoneal injection with 12h intervals. Kidneys were evaluated for apoptosis, proliferation, and gene expression at PND 8; stereological assessment of nephron number at PND 35; clinical pathology and neutrophil gelatinase-associated lipocalin (NGAL) at 4 and 9 months. Blood pressure was measured at the ages of 4, 6 and 9 months.

Results

NF and FR bodyweight differed from PND 3 onwards, ranging from 16.5g at weaning ($p<0.001$) to 39g at necropsy ($p=0.019$). Kidney proliferation/apoptosis ratio's were 7:1 and 3:1 ($p=0.001$). Different expression of *Wnt4* (0.7x), *Oat1* (1.3x), *Nphs1* (1.7x) and *Aqp4* (1.3x) was noted, but the biological relevance was doubted. Nephron numbers were decreased by 12% ($p=0.109$) in the ibuprofen-NF group and 7.5% ($p=0.237$) in FR groups. This coincided with a tendency to increased NGAL at 9 months. No differences were noted in electrolytes, creatinine or urea clearence. No valid blood pressure results could be obtained.

Conclusion

A clinical Ibuprofen dose showed potential to inhibit kidney development in neonatal rats. FR did not modulate these effects.

Introduction

Nephrons are the functioning units of the kidney and form from the 5th gestational week onwards (1). The process of nephrogenesis in humans ends around the 34th to 36th week of gestation. At that time a little under 1 million nephrons have been formed per kidney with a very large (i.e. 10-fold) interindividual range (2). Environmental influences during nephrogenesis, including growth restriction and medications, have a significant impact on final nephron numbers and may therefore explain a significant part of the range (3).

Prematurely born children may suffer from patent ductus arteriosus and non-steroidal anti-inflammatory drugs (NSAIDs) are often used to treat this condition. Data from the Netherlands has shown that about 1 in 6 neonates born before 32 weeks is treated with these drugs (Perinatal registry, Utrecht, the Netherlands). Ibuprofen and indomethacin are the most commonly used drugs for this indication, and both of these drugs have adverse effects on the kidney. These effects may range from a small reduction in glomerular filtration rate (GFR) to acute renal failure (4,5) which is more often noted in the less mature neonates (6,7).

As emphasis is put on direct adverse effects of the drugs, the long-term effects on the developing organs are less studied. Kent et al. studied the effect of both NSAID treatments on glomerulogenesis. They did not found any clear differences between treatments on glomerular numbers, but suggested that tubular development was altered (8). A baboon study, performed by Sutherland et al., identified a shortened nephrogenic zone after treatment with ibuprofen (9).

Besides drug toxicity, other stressors play a role in the vulnerability of the developing kidney. For instance, being born preterm may on its own disturb kidney formation as nephron formation does continue after birth, but with an accelerated maturation (10). Rodriguez et al. described that being born before 32 weeks is likely to result in a lower nephron number (11). Additionally, preterm born neonates have a sub-optimal intake of nutrients and suffer from extra-uterine growth restriction (12), which has been shown to reduce nephron formation in an animal model (13) and leads to a decreased neonatal glomerular filtration rate (14). Whether the combination of growth restriction with drug use has an additional deleterious effect on nephrogenesis remains to be determined.

Our goal was to determine whether clinical neonatal indomethacin or ibuprofen doses affected kidney development and function over short- and long-term periods. Additionally, we hypothesized that developmental effects may be modulated by the combination with extrauterine growth retardation as an additional stressor.

Material and methods

Animal experiments

Timed-pregnant Wistar WU rats were ordered from Charles River laboratories (Sulzfeld, Germany) and were delivered at the central animal laboratory Nijmegen approximately one week before delivery. Animals were housed individually and checked at least twice a day for litters. The day that a litter was found complete was designated as postnatal day (PND) 1, all dams had littered within 2 days. On PND 2 pups were cross-fostered to generate control (normal food: NF) and food restricted (FR) litters of 12 and 20 pups, respectively. All litters consisted of an equal number of males and females and the presence of siblings in a litter was avoided as much as possible. An additional NF and FR litter was formed to replace potential losses in the study population. Starting on PND 3, three doses of 0.9% NaCl (sham), 0.1 mg/kg indomethacin (Liometacen, Chiesie, Parma, Italy) or 10 mg/kg ibuprofen (Pedea, Orphan Europe, United Kingdom) were administered intraperitoneally with 12-hour intervals. PND3 was chosen as a starting point of the treatment because until PND8 the kidney is still developing in the rat, and PND12 is considered to be equivalent to term birth in humans (15). This indicates that a newborn rat can be considered similar to a very preterm born human, which mimics the situation of a preterm child with ongoing nephrogenesis, the clinical target population for the investigated treatment indication. In order to minimize mortality, either by too long or invasive handling or side effects of the drugs, we decided not to dose in very young pups.

The dose levels and the treatment interval were chosen to be similar to clinical practice. Ibuprofen and indomethacin are administered parentally in the clinic and we selected the intraperitoneal route to approach the clinical route of administration. This resulted in 6 treatment groups in total (sham-normal food (NF) and food restricted (FR), indomethacin-NF and FR, ibuprofen-NF- and FR) per sex. Body weight was determined daily up to weaning (day 28) and weekly thereafter till 9 months of age. After day 35 only males were kept in study. One FR litter of 20 pups were lost to follow-up as the individual markings (by water-resistant tattoo pen) vanished overnight due to a leaking water bottle on PND6. Due to the loss of follow-up in the initial study cohort, a second cohort was run as a side arm of another study for completion of the day 35 analyses. In that second cohort study the littering and crossfostering conditions were similar to the conditions described above. All other endpoints were determined from the results of the first cohort only. Experiments were approved by the Animal Ethics Committee at the Radboud University Nijmegen.

At day 8, 3-4 animals/sex/group were sacrificed and kidneys were collected. No samples were collected from FR males because animals were lost from follow-up as described above and long-term follow-up was prioritized. The left kidney was frozen

in liquid nitrogen for mRNA analysis and the right kidney was fixed in 10% formalin. At day 35, 3-4 animals/sex/group were sacrificed by exsanguinations followed by perfusion with 10% formalin. Kidneys were stored in 10% formalin and the right kidney was processed for stereology.

The remaining animals were followed until 9 months of age and were investigated for electrolyte balance, creatinine clearance and blood pressure at the age of 4, 6 and 9 months.

Day 8 mRNA analysis

Left kidneys were pulverized in deep frozen state by a microdismembrator (Sartorius-Stedim, the Netherlands). Isolation of RNA was performed by combining the TRIzol extraction method (Invitrogen, Carlsbad, California) with the Nucleospin RNA II isolation kit (Machery-Nagel, Düren, Germany). The Trizol extraction manufacturer´s protocol was followed up to the isopropanol separation step. Subsequently, the aqueous phase was added 1:1 to 70% ethanol and loaded on the nucleospin column. Further isolation and purification was performed according to the nucleospin manufacturer´s protocol. RNA concentration and quality were assessed with the nanodrop 2000x spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts).

One µg of RNA was used in a subsequent reverse transcriptase reaction using random primers (Promega, Madison, Wisconsin), oligo dT (Promega) and M-MLV reverse transcriptase (Invitrogen). mRNA levels of target genes were measured by quantitative PCR (qPCR) with Actb and Hmbs as internal standards. qPCR was performed on a Bio-Rad CFX96 using gene expression mix and hydrolysis probes (table 1) as ordered from Applied Biosystems (Pleasanton, California) or Biolegio (Nijmegen, Netherlands). Delta-Delta Ct (ΔΔCt) values were reported which were calculated by correcting the threshold cycle (Ct) of the gene of interest to the Ct of the housekeeping genes and normalizing to the Sham-NF group. Statistical evaluation was performed on the Delta Ct (ΔCt) values. Only fold changes >2 were considered biologically relevant.

Immunohistochemistry

Day 8 right kidneys were fixed in formalin and subsequently processed for immuno-histochemistry. Antigen retrieval was performed by citric acid and endogenous peroxidase activity was blocked. Additionally, slides were preincubated with goat serum and primary antibodies for Ki67 (1:500) (Abcam, Cambridge, UK) and Caspase-3 (1:500)(BD Pharmingen, Breda, Netherlands) were used as markers of cellular proliferation and apoptosis, respectively. As secondary antibody, 1:200 goat anti-rabbit biotinylated antibody (Vector laboratories, Amsterdam, Netherlands) was used and signal was amplified with avidin-HRP. PowerDAB (Immunologic, California, USA) was used for detection.

Stereology

Perfusion-fixed day 35 right kidneys were embedded in paraffin and sectioned exhaustively at 20 µm. Every 10th and 11th slide, starting at a random position (determined by a random number table), were taken and stained for haematoxylin-eosin. Subsequently, slides were scanned with the Olympus Olivia slidescanner and evaluation was performed with the Newcast module of the Visiopharm integrator system version 3.6.5.0 (Horzholm, Denmark).

A sample and its adjacent section were aligned manually and a counting frame was superimposed. Subsequently, a region of interest was drawn manually and a randomly oriented counting grid was placed on the region with x and y step lengths of 2500 µm. Glomeruli were then counted in all section pairs.

The total number of glomeruli per kidney (N(glom)) was estimated by the physical fractionator/disector principle. On average 152 glomeruli were counted per kidney and the total number of glomeruli was estimated using the following formula: $N(glom) = 1/SSF * 1/ASF * Ps/Pf * \sum Q^{-} /2$.

In this formula SSF is the section sample fraction and ASF is the area sample fraction (calculated as counting frame area divided by (x-step length * y- step length). The factor Ps/Pf was introduced to correct for slides with artificial edges; Ps is the number of counting frame corners that hit kidney tissue, Pf is the number of counting frame corners that hit evaluated kidney tissue. Finally, $\sum Q^{-}$ is the number of counted glomeruli and the factor ½ was introduced for counting both ways between the slide pairs.

Glomerular generation counting

Glomerular generations were determined according to the method described in the paper of Hinchliffe et al. (16). The direct method was used and 5 radials were counted per slide.

Histopathology

Animals were sacrificed after 9 months and the right kidney was immersion-fixed in 10% formalin.

Samples were processed and 2 slides for each animal were stained for haematoxylin-eosin or periodic acid-Schiff. These slides were evaluated for glomerular and tubular abnormalities by an experienced renal pathologist that was blinded to treatment groups and sex, with exception of the NF-sham group in order to establish a reference.

Blood pressure measurements

Three males per group were surgically implanted with telemetry transmitters PAC40 (Data Sciences International, St. Paul, Minnesota) with insertion of the catheter in the

abdominal aorta before the age of 4 months. Animals received buprenorphine and carprofen just before and on the first three days after surgery and were anesthesised with isoflurane 2.5% during the procedure. Surgical procedure was performed according to DSI surgical protocol for aortic cannulation. Animals were given at least two weeks to recover.

Telemetry signals were measured at 4, 6 and 9 months. All tracings were collected by Dataquest A.R.T. version 3.11 (Data Sciences International). Thirty-second measurements were collected every 6 minutes for 24 hours.

Glomerular and tubular function

Animals were housed in metabolic cages for 24 hours at 4 and 9 months. Urine was collected for 24 hours and on exiting the metabolic cage a blood sample was collected from the tail vein. Blood was centrifuged at 3000 RPM and plasma was directly taken to the clinical laboratory for analysis of sodium, potassium, magnesium, phosphate, urea and creatinine. Urine samples were measured for volume, urea, creatinine and osmolality. Creatinine and urea clearances were calculated and normalized for body weight.

NGAL measurement

Urine samples collected at 4 and 9 months were analyzed for the presence of neutrophil gelatinase-associated lipocalin (NGAL) protein levels using the rat lipocalin 2 ELISA kit (R&D Systems, Abingdon, UK)with detection limit of (78.1) pg/ml.

Statistical analysis

Mean (standard deviation, SD) are presented for all findings unless otherwise indicated. Parameters of interest were analyzed by univariate analysis using food status and treatment as fixed factors and Tukey as post-hoc analysis. Observations of both sexes were pooled as no differences between sexes were noted (except for absolute kidney weight). One-way ANOVA was used followed by Dunnett post hoc if a direct comparison to the sham-NF was made. All analyses were performed by the use of SPSS version 20 (IBM, Armonk, NY) at the alpha 0.05 level.

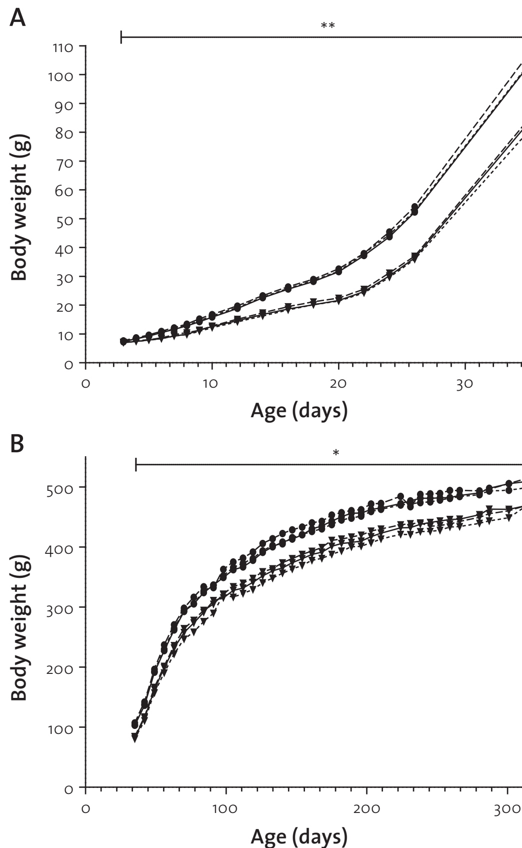
Results

In life phase

Mortality was noted in the first week of the study. In total, five animals, belonging to different litter sizes (3 NF, 2 FR) and different treatments (2 sham, 2 indomethacin, 1 untreated) were found dead. The mortality was considered to be related to the nature of the study (neonatal manipulations) but unrelated to specific drug treatment. Other than mortality, no abnormal clinical observations were noted over the study period.

As shown in figure 1a and 1b, body weights were significantly different between NF and FR animals on every day from day 3 onwards until the end of study. The body weight difference increased over the first three study months and was around 16.5g at the time of weaning (sham-NF 52.2g ; sham-FR 36.4g (both sexes); p<0.001)and 33g at wk 12 (Sham-NF 324g; Sham-FR 291g (males); p<0.05) . For the remainder of the study period the body weight difference remained between 35-50g and was 39g at necropsy

Figure 1 (**A**) Body weights of study animals from PND 3 up to PND 35 . (**B**) Body weights of study animals from PND 35 up to end of study (9 months)



Values of normal food (NF) animals are plotted with dots and food restricted (FR) with triangles. Sham-dosed groups are indicated with a solid line, indomethacin groups with a striped line and ibuprofen groups with a dotted line. Body weight of FR animals was lower compared with the NF animals from day 3 onwards on all data points. Univariate analysis was performed with Tukey post-hoc. **(P<0.001), *(P<0.05).

(sham-NF 509.4g ; sham-FR 470.0g (males); p=0.019). After stratification for food status, no drug-related changes were noted. We did note a statistically significant increase in body weight on day 10 and 12 of indomethacin-treated vs. sham-treated NF animals (sham-NF 18.8g, indomethacin-NF 19.7g ; p=0.045). However, the finding was incidental and no clear trend was observed. Similarly, a slight difference between indomethacin and ibuprofen-treated FR animals was noted at week 11, but no difference with sham-dosed animals was present.

Day 8 investigations

Based on the potential effects on nephron formation and tubular function, 22 transporters, growth factors and receptors were selected (table 1), and sham-NF normalized Ct measurements of the various treatments were reported in figure 2. Overall, the different treatment groups were close to the Sham NF- reference group. Statistically significant changes were noted between FR and NF animals for *Wnt4* (0.7 fold change, p<0.05), *Oat1* (1.3 fold change, p<0.05), *Nphs1* (1.7 fold change, p<0.001) and *Aqp4* (1.3 fold change, p<0.05). Even though statistical significance was observed, the fold changes were small (<2 fold) and therefore it is doubtful if these findings have any biological relevance.

Additionally, we investigated the balance between proliferation and apoptosis within the kidneys at day 8 (figure 3) and noticed a statistically significant difference between NF and FR animals (F=13.0, p=0.001). In NF animals, the ratio between proliferating and apoptotic cells was 7.8:1 (780 (SD 210) proliferating- and 100 (SD 20) apoptotic cells/mm^2), whereas a ratio of only 3.5:1 (420 (SD 170) proliferating- and 120 (SD 40) apoptotic cells/mm^2) was noted in FR animals. There were no clear focused areas of either proliferating or apoptotic cells present in the kidney sections. No sex differences were noted (F=0.1, p=0.796) and drug treatment (F=0.9, p=0.479) had no effect on the balance between proliferation and apoptosis.

Day 35 investigations

Kidney weight was significantly higher in NF animals compared with FR animals (F=29.2, p<0.001) (table 2). Furthermore a difference was noted between males and females (F 15.2, p<0.001). When corrected for body weight, both observed differences were no longer statistically significant (F=3.6, p=0.067 and F=0.8 p=0.375).

Glomerular numbers were estimated by stereology and were on average 34,492 (SD 3,707) in the Sham-NF group. Treatment with ibuprofen showed a trend towards a 12% lower number of nephrons (sham-NF 34,492 ; ibuprofen-NF 30,329 ; p=0.109) in the NF group, but not within the FR group. Indomethacin did not show treatment-related effects. The application of food restriction (sham-FR) resulted in a 7.5% decrease in glomerular number compared to the sham-NF group (sham-NF 34,492 ; sham-FR 31,879 ; p=0.237). Pooling of the sham and drug-treated FR groups led to a

Table 1 Applied Biosystems primer probe sets used in this study.

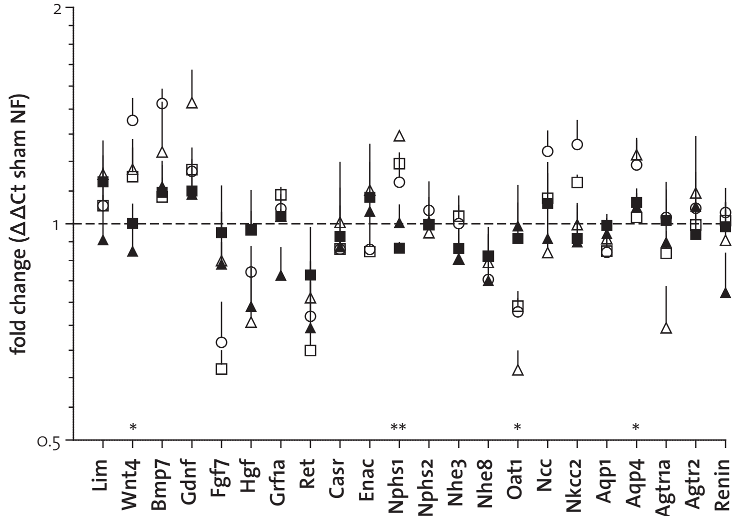
Gene symbol	Primer-probe set
Actb	Rn00667869_m1
Agtr1a	Rn00578456_m1
Agtr2 [a]	5'-CCTTCCTGTATTGTTTCGTTGGA-3' (forward) 5'-TGGAGCCAAGTAATGGGAACTC-3' (reverse) 5'- CCGCTTCCAACAGAAGCTCCGTAGTG-3'(probe-FAM)
Aqp1	Rn00562834_m1
Aqp4	Rn00563196_m1
Bmp7	Rn01528889_m1
Casr	Rn00566496_m1
Enac	Rn00566891_m1
Fgf7	Rn00573319_m1
Gdnf	Rn00569510_m1
Grf1a	Rn00564156_m1
Hgf	Rn00566673_m1
Hmbs [a]	5'-GGAGCCATGTCCGGTAACG-3' (forward) 5'-CCACTCGAATCACCCTCATCA-3' (reverse) 5'-CACAACCGCGGAAGAAAACGGCT-3'(probe-FAM)
Lim	Rn00595845_m1
Ncc	Rn00571074_m1
Nhe3	Rn00561944_m1
Nhe8	Rn01423020_m1
Nkcc2	Rn00568143_m1
Nphs1	RN00575235_m1
Nphs2	Rn00709834_m1
Oat1	Rn00568143_m1
Renin	Rn00561847_m1
Ret	Rn01463098_m1
Wnt4	Rn00584577_m1

[a] Ordered at Biolegio.

10% decrease when compared to sham-NF. All observed decreases were trends, no differences between sexes were noted ($F=2.0$, $p=0.175$). Additionally, food restriction did not modify the effects noted in the ibuprofen group.

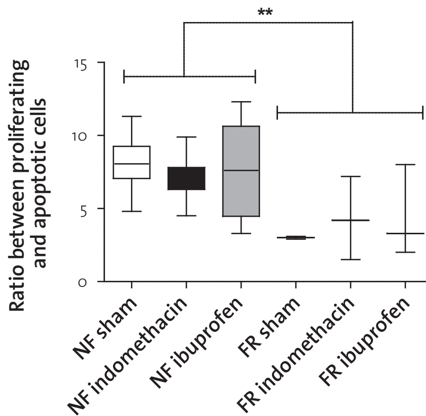
Finally, glomerular generations were counted. No differences between treatment or sexes were noted ($F=0.4$, $p=0.836$). The means of all experimental groups were around 4.6 - 4.7 generations and no abnormalities were noted. No correlation was present between glomerular generation count and the number of glomeruli (Pearson correlation $p=0.463$).

Figure 2 Renal gene expression of relevant transporters, growth factors and receptors in kidney development measured on day 8



Relative fold changes from Sham-NF (dotted line) with SEM are plotted for each target gene. Shown are sham (dot), indomethacin (square) and ibuprofen (triangle) treatment. Normal food (NF) and food restricted (FR) are distinguished by filled and cleared symbols. Univariate analysis was performed with Tukey post-hoc. FR vs NF * $p<0.05$, ** $P<0.001$.

Figure 3 The balance between proliferating and apoptotic cells in day 8 kidneys



Mean ratio's with 25%-75% percentiles and min-max values per treatment group are shown. Food restriction and normal food are indicated with NF and FR. Statistical analysis was performed by univariate analysis ** $p=0.001$.

7

Table 2 Renal parameters at day 35.

Treatment	**Sham-NF**	**Indomethacin-NF**	**Ibuprofen-NF**	**Sham-FR**	**Indomethacin-FR**	**Ibuprofen-FR**	Statistical significance
Mean kidney weight (g)	0.67 (0.06)	0.70 (0.08)	0.68 (0.07)	0.56 (0.06)	0.57 (0.04)	0.57 (0.07)	FR vs. NF $p<0.001$
Kidney / body weight [a] (ratio x100)	0.72 (0.06)	0.73 (0.06)	0.72 (0.06)	0.74 (0.07)	0.77 (0.05)	0.78 (0.06)	
Glomerular generations (count)[b]	4.7 (0.18)	4.6 (0.12)	4.7 (0.30)	4.6 (0.07)	4.7 (0.14)	4.6 (0.08)	
Glomerular number	34,492 (3,707)	32,459 (4,142)	30,329 (2,733)	31,879 (2,983)	30,451 (3,514)	30,706 (1,222)	

[a] Body weight extrapolated from last known body weight and group growth curve.
Data are presented as mean (standard deviation), except for [b] mean (standard error) and analysed with univariate analysis with Tukey post-hoc.

Long-term follow-up in male rats

Plasma and urine profiles were determined at 4 and 9 months of the study. No differences were seen in electrolytes between treatments and creatinine and urea clearance were not affected (table 3a and 3b). Additionally, urine NGAL was measured as a biomarker of tubular damage. No effects were noted at the 4 months time point. At 9 months of age, a trend towards an increase in NGAL was noted in all FR groups (Mean FR 1.26 (SD 0.69) µg/ml; NF 0.90 (SD0.52) µg/ml; $p=0.083$). Additionally a trend was noted towards an increase in the ibuprofen-NF group at the end of the study period, but individual variation was high (Mean ibuprofen-NF 1.23 (SD 0.84) µg/ml; sham-NF 0.735 (SD 0.12) µg/ml ; $p=0.237$) (table 3 and 4).

Blood pressure was evaluated by telemetry and waveforms had the expected shape. However, the amplitude of some signals was low and not physiological. Additionally, signal amplitude further deteriorated in the long-term follow-up of the study. Altogether, the collected data was not suitable for a valid evaluation at the end of the study period.

At the study end, mean kidney to body weight ratio was 5.9 mg/g (range 5.3-6.2 mg/g) in the sham-NF group and similar to the other experimental groups. Histopathological investigation revealed minor changes within the normal biological variation (data not shown).

Table 3 Clinical biochemistry parameters at 4 months of age

	Sham-NF	Ibuprofen-NF	Indomethacin-NF	Sham-FR	Ibuprofen-FR	Indomethacin-FR
Body weight (g)	388 (20.2)	388.8 (10.1)	404.6 (16.1)	352.6 (14.0)	343.2 (18.7)	360.4 (15.5)
Plasma						
Sodium (mmol/l)	141 (1.1)	141 (1.3)	141 (1.8)	140 (1.3)	141 (1.3)	141 (0.5)
Potassium (mmol/l)	4.8 (0.6)	4.6 (0.4)	4.5 (0.3)	4.7 (0.3)	4.5 (0.2)	4.5 (0.1)
Magnesium (mmol/l)	0.82 (0.07)	0.80 (0.06)	0.79 (0.03)	0.79 (0.04)	0.78 (0.02)	0.78 (0.04)
Phospate (mmol/l)	2.17 (0.24)	2.01 (0.21)	1.90 (0.18)	1.92 (0.11)	1.87 (0.17)	1.94 (0.17)
Urea (mmol/l)	5.4 (0.9)	5.5 (0.6)	5.2 (0.5)	5.4 (0.5)	5.2 (0.6)	5.2 (0.4)
Creatinine (μmol/l)	24.8 (5.4)	22.6 (2.4)	22.4 (0.5)	22.6 (2.4)	22.3 (2.6)	23 (2.2)
Urine						
Creatinine (mmol/l)	9.3 (1.3)	8.6 (1.5)	8.2 (0.8)	8.2 (1.5)	9.2 (1.4)	7.5 (0.9)
Urea (mmol/l)	1,113 (101)	1,051 (178)	1,021 (141)	1,032 (162)	1,190 (94)	972 (124)
Osmolality (mosmol/kg)	2,099 (219)	1,975 (284)	1,890 (274)	1,939 (328)	2,256 (164)	1,873 (259)
Volume (ml)	9.6 (1.7)	10.6 (1.3)	11.9 (1.4)	10.1 (3.0)	8.7 (2.4)	11.7 (2.7)
Creatinine clearance (ml/min/kg)	0.66 (0.16)	0.73 (0.18)	0.74 (0.02)	0.69 (0.08)	0.71 (0.16)	0.73 (0.11)
Urea clearance (ml/min/kg)	0.36 (0.09)	0.36 (0.09)	0.41 (0.04)	0.37 (0.08)	0.40 (0.08)	0.42 (0.09)
NGAL (μg/ml)	0.678 (0.086)	0.881 (0.366)	0.616 (0.154)	0.722 (0.248)	0.716 (0.202)	0.841 (0.577)

Data are presented as mean (standard deviation) and statistical analysis was performed with univariate analysis with Tukey post-hoc. No statistically significant findings were noted.

Table 4 Clinical biochemistry parameters at 9 months of age

	Sham-NF	Ibuprofen-NF	Indomethacin-NF	Sham-FR	Ibuprofen-FR	Indomethacin-FR
Body weight (g)	483.2 (24.7)	489.4 (17.2)	498.3 (13.9)	473.2 (30.2)	441.0 (26.7)	452.2 (8.7)
Plasma						
Sodium (mmol/l)	142 (1.0)	142 (0.0)	142 (1.5)	143 (1.8)	141 (1.5)	141 (0.9)
Potassium (mmol/l)	4.6 (0.2)	4.3 (0.3)	4.6 (0.4)	4.3 (0.3)	4.8 (0.5)	4.8 (0.5)
Magnesium (mmol/l)	0.80 (0.04)	0.80 (0.02)	0.79 (0.03)	0.80 (0.04)	0.82 (0.03)	0.85 (0.04)
Phospate (mmol/l)	1.70 (0.12)	1.65 (0.08)	1.79 (0.04)	1.68 (0.09)	1.69 (0.06)	1.66 (0.1)
Urea (mmol/l)	5.6 (1.0)	5.6 (0.2)	4.8 (0.5)	5.2 (0.5)	5.6 (0.8)	6.1 (0.5)
Creatinine (µmol/l)	24.6 (2.6)	25.6 (3.6)	24.8 (2.1)	28.0 (2.7)	23.5 (1.6)	26.0 (3.9)
Urine						
Creatinine (mmol/l)	11.3 (0.5)	12.2 (1.2)	12.3 (2.0)	11.7 (2.1)	12.5 (1.1)	11.9 (1.9)
Urea (mmol/l)	1,251 (79)	1,290 (76)	1,198 (260)	1,107 (259)	1,373 (76)	1,246 (126)
Osmolality (mosmol/kg)	2,82 (75)	2,230 (302)	1,885 (636)	1,991 (463)	2,531 (156)	2,251 (215)
Volume (ml/24h)	9.3 (1.8)	8.2 (0.8)	8.3 (1.2)	7.6 (1.1)	7.8 (1.7)	8.6 (2.7)
Creatinine clearance (ml/min/kg)	0.62 (0.14)	0.56 (0.08)	0.57 (0.13)	0.47 (0.13)	0.65 (0.15)	0.59 (0.14)
Urea clearance (ml/min/kg)	0.30 (0.08)	0.27 (0.01)	0.29 (0.12)	0.25 (0.1)	0.30 (0.04)	0.26 (0.06)
NGAL (µg/ml)	0.735 (0.117)	1.228 (0.841)	0.716 (0.114)	1.420 (0.675)	0.932 (0.186)	1.402 (0.986)

Data are presented as mean (standard deviation) and statistical analysis was performed with univariate analysis with Tukey post-hoc. No statistical significance was noted.

Discussion

Our goal was to determine whether indomethacin or ibuprofen doses similar to those used clinically, affected kidney development and function on short- and long-term, and whether those effects might be modulated by the addition of extrauterine growth retardation as a stressor. To study these effects rats were dosed within a period of ongoing nephrogenesis and followed up for a large period of their life span to investigate accumulative wear and tear to the kidney as postulated in Brenner's hyperfiltration theory (17). This theory states that an early reduction in glomerular numbers increases workload of the other glomeruli which eventually leads to glomerulosclerosis.

Based on our results, a clinical dose of 10 mg/kg ibuprofen, when administered in a dose regime mimicking the clinical practice, tended to lead to a decreased number of glomeruli in animals with a normal feeding regimen. Additionally, we noted a tendency to a slight increase in NGAL levels in the urine of these animals in our long-term follow-up. However, we did not find any functional or morphological impairments of concern. In contrast to our ibuprofen findings, we did not find any signs of toxicity in the indomethacin-dosed groups.

Groups with FR were studied to determine the interaction of extrauterine growth retardation on drug effects. Although we noted effects of FR comprising of lower body weight, kidney weight, altered proliferation/apoptosis balance and a lower glomerular number on the animals, we did not see a clear modulating effect on drug toxicity. In the case of ibuprofen, glomerular numbers in NF and FR groups were similar.

Our findings on ibuprofen treatment were somewhat contrasting with the findings published earlier by Kent et al. (8). In their studies, no differences in glomerular numbers were found, but an increase in kidney weight was described, possibly linked to edema formation. We did not find any evidence of the edema but our time interval between the last ibuprofen treatment and necropsy for stereology was longer (1 month vs. 1 week). Therefore, it could be that any edema present during treatment in the kidney has already recovered. In another study, tubular alterations were detected with light microscopy, and glomerular changes (primarily foot process effacement) were noted by EM-microscopy not only at a dose of 10 mg/kg but also at 0.1 mg/kg indomethacin (in a lesser degree) in neonatal rats (18). These glomerular findings could be the start of the cascade leading eventually to the reduction in glomeruli we observed at our time point of necropsy and should be investigated in a follow-up study. In our study, we did not detect any tubular alterations at day 35, but these could have been recovered as well. However, our analysis of tubular transporters by mRNA expression on day 8 did not show either indications of altered function or toxicity between ibuprofen and control-treated kidneys.

In our long-term follow-up period we noted a trend toward a slight increase in NGAL levels in the urine after ibuprofen treatment. Its presence in the urine can have multiple causes. Due to its low molecular weight and positive charge NGAL undergoes normal glomerular filtration and is afterwards reabsorbed by the proximal tubules. Therefore, higher levels of NGAL in the urine could indicate proximal tubular damage (19). Additionally, the thick ascending limb and the collecting ducts may be sources of NGAL (20). Although NGAL is mostly known for its involvement as a stress protein in acute kidney injury responses, levels of this biomarker can also be higher in patients with chronic kidney disease (21). The observed trend may therefore be indicative for a possible developing chronic kidney injury in these animals. Recently, higher urine levels of NGAL were also associated with an increased prevalence of higher systolic blood pressure and cardiovascular events (22). The source cell type of the NGAL elevations in our study was unclear because kidney lesions were absent at the light microscopic examination at the end of the study period. With the absence of any histopathological correlation and the lack of changes in any serum or urine renal functional parameter, the relevance of this observed trend is doubted. But if these trends hold true, alterations of the kidney were still subtle and did not yet lead to a functional or morphological deficit of the kidney. In humans Rodriguez et al showed that glomerulogenesis does not continue in pre-term neonates after 40 days and the kidney is therefore less developed compared with term neonates (11). Concurrently, a prehypertensive state was associated with children born preterm (23). Therefore subtle alterations may render the subject more prone to the development of renal disease. To study such an option, future studies should be undertaken that are more directed at this specific effect. Our latest time point was 9 months, which approximately corresponds with a human age of 25 years (15) and could therefore be too early. Furthermore, performing similar experiments in Sprague Dawley rats may also be of interest, as they are known to be more prone to chronic kidney disease (24).

Our second goal was to study the modulating effects of growth restriction during nephrogenesis on the effects of ibuprofen or indomethacin on renal development. The litter enlargement model described by Huizinga *et al (25)* was chosen because of its described effects on glomerular count (13) and the similarity in timing of the growth restriction to the human situation of prematurely born neonates (15). In line with previous experiments (25), differences in body weight between NF and FR animals were found in the present study and is therefore similar to other models of early postnatal food restriction (26). As the total growth patterns are quite parallel and no significant loss of pups was noted in the FR litters, the mother can be considered to distribute her milk evenly between pups although it is difficult to have a good standardization of specific caloric, protein and nutrient (such as vitamin A) intake during FR. In addition to lower body weights, we noted a histological change in FR animals with fewer proliferating cells in the kidney. This histological difference may be linked

to the impaired growth during kidney formation, which has been shown to have a distinct impact on the kidney (13).

The FR model showed a slight decrease in glomeruli and was not as evident as would be expected from earlier work where higher control values were reported (13). We are currently uncertain what the underlying cause is for this discrepancy.

At the long-term follow-up, NGAL levels of male FR animals were in line with those noted after ibuprofen treatment alone, which contributes to an association between a lower glomerular number and the NGAL increase. Similarly, no functional or morphological deficits were noted after clinical pathology and histopathological examination at the end of treatment.

We postulated that additional stressors might be required for low doses of drugs to influence renal development. It is evident that our food restriction model works as an additional stressor to the pups. However, this stressor did not seem to modulate the effect of either indomethacin or ibuprofen in our study because the values of the glomerular counts in the drug-treated FR groups were not different from the sham-FR group. Additionally, the ibuprofen-NF group was similar to the ibuprofen-FR group and there was no added or synergetic effect. At this moment in time not much is understood of the underlying working mechanism of either FR or ibuprofen regarding the decrease in glomerular numbers. Possible hypotheses are a common pathway with a limiting effect on the kidney, a partially antagonistic effect of both stressors or protection of one of the stressors against the other. These hypotheses should however be tested in subsequent studies.

The debate remains whether ibuprofen or indomethacin is safer to use for closing the patent ductus arteriosis. Evidence is accumulating that both compounds have impact on the glomeruli. Akinola et al. showed hypoplastic glomeruli after repeated dosing of ibuprofen at 22 mg/kg/day for 5 days (27). However, we already showed trends at a clinical dose level. Although patent ductus arteriosis has to be treated, care should be taken for the co-administration of other potential nephrotoxic drugs such as aminoglycosides. Long-term renal and eventually cardiovascular health should be kept in mind when treating newborns.

In summary, 10 mg/kg ibuprofen when administered in a clinical dose regime to neonatal rats tended to reduce glomerular numbers by 12% at day 35 and resulted in a trend towards an increased urine NGAL in individual male animals after a 9 month follow-up, without overt functional or morphological renal alterations. Postnatal food restriction resulted in a 7.5% reduction in glomerular numbers, a reduced body weight and altered proliferation/apoptosis balance and also a trend towards increased NGAL over time. No added or synergistic effects of FR on the effects of ibuprofen or indomethacin treatment were noted. Taken all these findings into account, ibuprofen showed potential to cause a subtle reduction in kidney glomerular count that may render the treated person more prone to later renal disease. As an alternative NSAID,

indomethacin did not show any effects in this study and may be less toxic with regard to nephrogenesis, even though in clinical practice more side-effects are seen. Additional studies should be performed to confirm the findings in this study, elucidate the underlying mechanism of ibuprofen toxicity regarding nephrogenesis and explore whether effects of ibuprofen treatment on the kidney may be mitigated.

References

1. Quaggin SE, Kreidberg J. Embryology of the kidney. In: Brenner BM, eds. Brenner and Rector's the kidney. 8th ed. Philadelphia : Saunders, Elsevier, 2008:3-24.
2. Bertram JF, Douglas-Denton RN, Diouf B, Hughson MD, Hoy WE. Human nephron number: implications for health and disease. *Pediatr.Nephrol* 2011;26(9):1529-1533.
3. Schreuder MF. Safety in glomerular numbers. *Pediatr Nephrol* 2012;27(10):1881-1887.
4. Akima S, Kent A, Reynolds GJ, Gallagher M, Falk MC. Indomethacin and renal impairment in neonates. *Pediatr Nephrol* 2004;19(5):490-493.
5. Shaffer CL, Gal P, Ransom JL, Carlos RQ, Smith MS, Davey AM, Dimaguila MA, Brown YL, Schall SA. Effect of age and birth weight on indomethacin pharmacodynamics in neonates treated for patent ductus arteriosus. *Crit Care Med* 2002;30(2):343-348.
6. Ohlsson A, Walia R, Shah SS. Ibuprofen for the treatment of patent ductus arteriosus in preterm and/or low birth weight infants. *Cochrane Database Syst Rev* 2013;(4):CD003481.
7. Bagnoli F, Rossetti A, Messina G, Mori A, Casucci M, Tomasini B. Treatment of patent ductus arteriosus (PDA) using ibuprofen: renal side-effects in VLBW and ELBW newborns. *J Matern Fetal Neonatal Med* 2013;26(4):423-429.
8. Kent AL, Douglas-Denton R, Shadbolt B, Dahlstrom JE, Maxwell LE, Koina ME, Falk MC, Willenborg D, Bertram JF. Indomethacin, ibuprofen and gentamicin administered during late stages of glomerulogenesis do not reduce glomerular number at 14 days of age in the neonatal rat. *Pediatr Nephrol* 2009;24(6):1143-1149.
9. Sutherland MR, Yoder BA, McCurnin D, Seidner S, Gubhaju L, Clyman RI, Black MJ. Effects of ibuprofen treatment on the developing preterm baboon kidney. *Am J Physiol Renal Physiol* 2012;302(10):F1286-1292
10. Sutherland MR, Gubhaju L, Moore L, Kent AL, Dahlstrom JE, Horne RS, Hoy WE, Bertram JF, Black MJ. Accelerated maturation and abnormal morphology in the preterm neonatal kidney. *J Am Soc Nephrol* 2011;22(7):1365-1374.
11. Rodriguez MM, Gomez AH, Abitbol CL, Chandar JJ, Duara S, Zilleruelo GE. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. *Pediatr Dev Pathol* 2004;7(1):17-25.
12. De Curtis M, Rigo J. Extrauterine growth restriction in very-low-birthweight infants. *Acta Paediatr* 2004;93(12):1563-1568.
13. Schreuder MF, Nyengaard JR, Remmers F, van Wijk JA, Delemarre-van de Waal HA. Postnatal food restriction in the rat as a model for a low nephron endowment. *Am J Physiol Renal Physiol* 2006;291(5):F1104-1107.
14. Bacchetta J, Harambat J, Dubourg L, Guy B, Liutkus A, Canterino I, Kassai B, Putet G, Cochat P. Both extrauterine and intrauterine growth restriction impair renal function in children born very preterm. *Kidney Int* 2009;76(4):445-452.
15. Sengupta P. The laboratory rat: Relating its age with human's. *Int J Prev. Med* 2013;4(6):624-630.
16. Hinchliffe SA, Sargent PH, Chan YF, van Velzen D, Howard CV, Hutton JL, Rushton DI. "Medullary ray glomerular counting" as a method of assessment of human nephrogenesis. *Pathol Res Pract* 1992;188(6):775-782.
17. Brenner BM, Lawler EV, Mackenzie HS. The hyperfiltration theory: A paradigm shift in nephrology. *Kidney Int* 1996;49(6):1774-1777.
18. Kent AL, Maxwell LE, Koina ME, Falk MC, Willenborg D, Dahlstrom JE. Renal glomeruli and tubular injury following indomethacin, ibuprofen, and gentamicin exposure in a neonatal rat model. *Pediatr Res* 2007;62(3):307-312.
19. Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, Yang J, Barasch J, Devarajan P. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 2003;14(10):2534-2543.
20. Paragas N, Qiu A, Zhang Q, Samstein B, Deng SX, Schmidt-Ott KM, Viltard M, Yu W, Forster CS, Gong G, Liu Y, Kulkarni R, Mori K, Kalandadze A, Ratner AJ, Devarajan P, Landry DW, D'Agati V, Lin CS, Barasch J. The Ngal reporter mouse detects the response of the kidney to injury in real time. *Nat Med* 2011;17(2):216-222.
21. Bolignano D, Lacquaniti A, Coppolino G, Donato V, Campo S, Fazio MR, Nicocia G, Buemi M. Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. *Clin J Am Soc Nephrol* 2009;4(2):337-344.

22. Hasegawa M, Ishii J, Kitagawa F, Takahashi K, Hayashi H, Koide S, Tomita M, Takahashi H, Ozaki Y, Yuzawa Y. Urinary neutrophil gelatinase-associated lipocalin as a predictor of cardiovascular events in patients with chronic kidney disease. *Heart Vessels* 2015;30(1):81-88.
23. Keijzer-Veen MG, Finken MJ, Nauta J, Dekker FW, Hille ET, Fröhlich M, Wit JM, van der Heijden AJ. Is blood pressure increased 19 years after intrauterine growth restriction and preterm birth? A prospective follow-up study in the Netherlands. *Pediatrics* 2005;116(3):725-731.
24. Goldstein RS, Tarloff JB, Hook JB. Age-related nephropathy in laboratory rats. *FASEB J* 1988;2(7):2241-2251.
25. Huizinga CT, Engelbregt MJ, Rekers-Mombarg LT, Vaessen SF, Delemarre-van de Waal HA, Fodor M. Ligation of the uterine artery and early postnatal food restriction - animal models for growth retardation. *Horm Res* 2004;62(5):233-240.
26. De Oliveira JC, Grassiolli S, Gravena C, de Mathias PC. Early postnatal low-protein nutrition, metabolic programming and the autonomic nervous system in adult life. *Nutr Metab (Lond)* 2012;9(1):80.
27. Akinola O, Noronha C, Oremosu A, Kusemiju O, Okanlawon OA. The effect of the cyclooxygenase blockers, ibuprofen on the development of glomeruli in Sprague-Dawley rats. *Niger Postgrad Med J* 2003;10(1):46-50.

8

The effects of early postnatal diuretics treatment on kidney development and long-term kidney function in Wistar rats

Ruud R.G. Bueters[1]
Annelies Klaasen[1]
Nuria Maicas[2]
Sandrine Florquin[3]
Lambertus P. van den Heuvel[1]
Michiel F. Schreuder[1]

[1]*Department of Pediatric Nephrology, Radboud University Medical Center, Nijmegen, the Netherlands.*
[2]*Department of Experimental Nephrology, Radboud University Medical Center, Nijmegen, the Netherlands*
[3]*Department of Pathology, Radboud University Medical Center, Nijmegen, the Netherlands*

Adapted from (**Bueters *et al***. Published in Nephron: Experimental Nephrology and Genetics:2016 Jan;132(2):110-8.)

Abstract

Background

Diuretics are administered to neonates to control fluid balance. We studied whether clinical doses affected kidney development and function and whether extrauterine growth retardation (EUGR) could be a modulator.

Methods

Wistar rats were cross-fostered in normal food (NF) or food restricted (FR) litters at postnatal day (PND) 2 and treated daily with 0.9% NaCl, 5 mg/kg furosemide or 5 mg/kg hydrochlorothiazide up to PND 8. Kidneys were evaluated on proliferation, apoptosis and a set of mRNA target genes at PND 8, glomerular- and glomerular generation count at PND 35, clinical pathology parameters at 3- and 9 months, NGAL at PND 8, 3 and 6 months, monthly blood pressure from 3 months onward and histopathology at study end.

Results

Treatment with furosemide or hydrochlorothiazide did not have relevant effects on measured parameters. Extrauterine growth retardation resulted in lower body weight from day 3 onwards (-29% at weaning; $p<0.001$. -10% at necropsy; $p<0.001$), less glomerular generations (4.4 ± 0.32 vs. 5.0 ± 0.423; $p=0.025$, males only), decreased glomerular numbers (27,861 ± 3,468 vs. 30,527 ± 4,096; $p=0.026$), higher creatinine clearance (0.84 ± 0.1 ml/min/kg vs. 0.77 ± 0.09 ml/min/kg; $p=0.047$) at 3 months and lower plasma creatinine (25.7 ± 1.8 µmol/l vs. 27.5 ± 2.8 µmol/l; $p=0.043$) at 9 months.

Conclusion

Furosemide and hydrochlorothiazide did not influence kidney development or function when administered in a clinically relevant dose to rat pups at a stage of ongoing nephrogenesis. EUGR led to impaired kidney development but did not modify furosemide or hydrochlorothiazide findings.

Introduction

From a historical perspective neonatal medicine has been mainly reliant on the translation of scientific data from the adult population. This perspective is changing because more evidence shows that neonates are different in ways of drug handling (1). The neonate population also has another important aspect regarding the practice of medicine, which is the interaction of treatments with the still ongoing development of many organs**.** This is especially important if born preterm. It has been hypothesized that low birth weight or early life damage may have impact on the kidney and its functions in later life (2,3). Already it has been shown that environmental influences during nephrogenesis, including growth restriction and medications, have a significant impact on final nephron numbers (4). Nephrons are formed from the 5th gestational week onwards and formation ends around the 34th to 36th week of gestation in humans (5). At that moment a little under 1 million nephrons have been formed per kidney, but there is a very large interindividual range (i.e. 10-fold) (6). In rodents the timing of nephrogenesis is different and is completed approximately 7 days after birth (7).

Diuretics are the most common drugs administered to control fluid balance in neonates, especially in children with chronic lung disease after very premature birth (8)**.** But there is a risk of kidney toxicity. Although acute kidney toxicity is not expected or seen, higher dose levels of furosemide are associated with renal calcification in children (9). Regarding kidney development, the described effects are more controversial. In the past, effects of furosemide on kidney development have been studied *in vivo*. Mallie et al. showed that prenatal furosemide exposure in rats tended to lower the number of differentiated glomeruli at birth and disturbed electrolyte handling (10,11). This effect was still present after a 12-day follow-up. In contrast, a recent study by our group did not identify effects on early kidney development after exposing metanephroi *in vitro* to more clinically relevant dose levels (12).

Our goal was to determine if a clinical neonatal dose of furosemide or hydrochlorothiazide (HCTZ) impaired kidney development and function on short and/or long-term. Hydrochlorothiazide was selected as a second drug because it has not been associated with nephrotoxicity. While the nephrotoxic potential of both furosemide and hydrochlorothiazide itself is low, they are often co-administered with other drugs and may thereby play a role in multi-hit toxicity. Additionally, we hypothesized that the potential effects of these drugs on kidney development may also be modulated by additional stressors such as extrauterine growth retardation**.**

In this study we investigated these hypotheses by inducing postnatal food restriction by means of litter enlargement to model extrauterine growth retardation in the rat. Either furosemide or hydrochlorothiazide were administered for 7 days to investigate interactions on development, and vehicle and control treatments were included. Early effects on proliferation/apoptosis, a set of mRNA target genes,

glomerular- and glomerular generation count on the kidney were evaluated. Furthermore, clinical pathology parameters, NGAL, blood pressure and renal histopathology were evaluated in a long-term follow-up. Finally, body weight was measured to monitor overall animal health and determine the extent of extrauterine growth retardation.

Material and methods

Animal experiments

All experiments were approved by the Animal Ethics Committee at the Radboud University Nijmegen.

Timed-pregnant Wistar WU rats (Charles River laboratories, Sulzfeld, Germany) were delivered at the central animal laboratory Nijmegen approximately one week before delivery. Dams were housed individually and checked for litters at least twice daily. The day a full litter was found was labeled as postnatal day (PND) 1. Pups were cross-fostered on PND2 to generate control (NF, 12 pups per litter) and food restricted (FR, 20 pups per litter) animals. Additional NF and FR litters were formed in order to replace potential losses in the study litters. Rats had ad libitum access to water and food (Ssniff R/M-H diet, Soest, Germany) with a sodium, potassium and chloride content of 0.24%, 0.91% and 1.00% respectively. Animals received a daily intraperitoneal dose of 0.9% NaCl (sham), 5 mg/kg furosemide (Lasix, Aventis Pharma BV, Gouda, Netherlands) or 5 mg/kg hydrochlorothiazide (H2910) (Sigma-Aldrich, the Netherlands) for 7 days starting on PND 2. In total, 6 treatment groups per gender were formed (sham-NF and FR, furosemide-NF and FR, HCTZ-NF and FR).

Body weight was measured daily up to weaning (day 28) and weekly thereafter till 9 months of age. After day 35 only males were kept in the study as they are generally more prone to develop hypertension (13). Some mortality was noted in the first weeks of the study (5 animals: 2 male NF (sham and furosemide), 2 female FR (sham and furosemide), 1 non-treated NF animal). This was considered to be related to the nature of the study (neonatal manipulations) but unrelated to the study interventions.

At day 8, (at least) 3 animals/sex/group were sacrificed and kidneys were collected. The left kidney was frozen in liquid nitrogen for mRNA analysis and the right kidney was fixed in 10% formalin.

At day 35, (at least) 3 animals/sex/group were sacrificed by exsanguinations followed by perfusion with 10% formalin. Kidneys were stored in 10% formalin and the right kidneys were processed for stereology.

The remaining (male) animals were followed until 9 months of age and were sampled for electrolyte concentrations and creatinine clearance at the age of 3 and 9 months. Blood pressure was recorded monthly from 3 months of age onwards.

mRNA analysis

Day 8 left kidneys were pulverized by a microdismembrator (Sartorius-Stedim, the Netherlands) in deep frozen state. Isolation of RNA was performed by combining the TRIzol extraction method (Invitrogen, Carlsbad, California) with the Nucleospin RNA II isolation kit (Machery-Nagel, Düren, Germany). The manufacturer´s protocol for Trizol extraction was followed up to the isopropanol separation step. Subsequently, the aqueous phase was added 1:1 to 70% ethanol and loaded on the nucleospin column. Further purification was performed according to the nucleospin manufacturer´s protocol. RNA concentration and quality were assessed with the nanodrop 2000x spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts).

One μg of RNA was used in a subsequent reverse transcriptase reaction using random primers (Promega, Madison, Wisconsin), oligo dT (Promega) and M-MLV reverse transcriptase (Invitrogen). mRNA levels were measured by quantitative PCR (qPCR) on a Bio-Rad CFX96 using gene expression mix and hydrolysis probes (table 1) as ordered from Applied Biosystems (Pleasanton, California) or Biolegio (Nijmegen, Netherlands), with *Actb* and *Hmbs* as internal standards. Delta Ct (ΔCt) values were reported which were calculated by correcting the threshold cycle (Ct) of the gene of interest to the Ct of the housekeeping genes. Fold changes were calculated in case delta Ct values were statistically significant.

Immunohistochemistry

Day 8 right kidneys were fixed in formalin and subsequently processed for immuno-histochemistry. Citric acid was used for antigen retrieval and endogenous peroxidase activity was blocked. Additionally, slides were preincubated with goat serum and primary antibodies for Ki67 (1:500) (Abcam, Cambridge, UK) and Caspase-3 (1:500)(BD Pharmingen, Breda, The Netherlands) were used as markers of cellular proliferation and apoptosis, respectively. As secondary antibody, 1:200 goat anti-rabbit biotinylated antibody (Vector laboratories, Amsterdam, The Netherlands) was used and signal was amplified with avidin-HRP. PowerDAB (Immunologic, California, USA) was used for detection.

Stereology

Perfusion-fixed day 35 right kidneys were embedded in paraffin and sectioned exhaustively at 20 μm. Every 10th and 11th slide, starting at a random position (determined by a random number table), were taken and stained for haematoxy-lin-eosin. Slides were scanned on a Olympus Olivia slidescanner and evaluation was performed with the Newcast module of the Visiopharm integrator system software package (version 3.6.5.0, Horzholm, Denmark).

A sample and its adjacent section were aligned manually and a counting frame was superimposed. Subsequently, a region of interest was drawn manually and a

Table 1 Applied Biosystems primer probe sets used in this study.

Gene symbol	Primer-probe set
Actb	Rn00667869_m1
Hmbs [a]	5'-GGAGCCATGTCCGGTAACG-3' (forward) 5'-CCACTCGAATCACCCTCATCA-3' (reverse) 5'-CACAACCGCGGAAGAAAACGGCT-3'(probe-FAM)
Wnt4	Rn00584577_m1
Gdnf	Rn00569510_m1
Bmp7	Rn01528889_m1
Fgf7	Rn00573319_m1
Hgf	Rn00566673_m1
Lim	Rn00595845_m1
Ret	Rn01463098_m1
Renin	Rn00561847_m1
Grf1a	Rn00564156_m1
Agtr1a	Rn00578456_m1
Agtr2 [a]	5'-CCTTCCTGTATTGTTTCGTTGGA-3' (forward) 5'-TGGAGCCAAGTAATGGGAACTC-3' (reverse) 5'- CCGCTTCCAACAGAAGCTCCGTAGTG-3'(probe-FAM)
Oat1	Rn00568143_m1
Casr	Rn00566496_m1
Enac	Rn00566891_m1
Nkcc2	Rn00568143_m1
Nhe3	Rn00561944_m1
Nhe8	Rn01423020_m1
Ncc	Rn00571074_m1

[a] Ordered at Biolegio.

randomly oriented counting grid was placed on the region with x and y step lengths of 2500 μm. All section pairs were then scored for presence of glomeruli.

The total number of glomeruli per kidney (N(glom)) was estimated by the physical fractionator/disector principle. On average 141 glomeruli were counted per kidney and the total number of glomeruli was estimated according to the following formula: $N(glom) = 1/SSF * 1/ASF * Ps/Pf * \sum Q^- /2$.

In this formula SSF is the section sample fraction and ASF is the area sample fraction (calculated as counting frame area divided by (x-step length * y- step length). The factor Ps/Pf was introduced to correct for slides with artificial edges; Ps is the number of counting frame corners that hit kidney tissue, Pf is the number of counting frame corners that hit evaluated kidney tissue. Finally, $\sum Q^-$ is the number of counted glomeruli and the factor ½ was introduced for counting both ways between the slide pairs.

Glomerular generation counting

Glomerular generations were determined with "the direct method" as described in the paper of Hinchliffe et al. (14). Five radials were counted per slide.

Histopathology

Animals (male) were sacrificed after 9 months and the right kidney was immersion-fixed in 10% formalin.

Samples were processed and stained for haematoxylin-eosin or periodic acid-Schiff (2 slides/animal). These slides were evaluated for glomerular and tubular abnormalities by an experienced renal pathologist (SF) that was blinded to treatment groups, with exception of the sham-NF group in order to establish a reference.

Blood pressure measurements

Blood pressure was measured non-invasively in all animals (male) from 3 months onwards by volume pressure recording on the CODA apparatus (Kent Scientific Corporation, Torrington, Conneticut) with software version 1.0. Prior to the measurements the room was heated to be above the ambient temperature of 22 °C. Additionally, animals were kept warm by the heating plate of the apparatus during the experiment and the tails were covered by a cloth to further stimulate thermo-regulation of the animals. Before recording, animals were acclimatized for 10 minutes in the restrainers and subsequently twenty blood pressure measurements were collected. The first ten measurements were used to train the animal and were discarded. The last 10 measurements were evaluated by the software and if in acceptable boundaries regarding blood flow, they were averaged per animal. Pulse pressure was calculated manually from the output data of the apparatus. Blood pressure was measured on three subsequent days in all animals in each month and an average of these three days was reported. This resulted in a robust average for each animal for each time point except for the 9-months time point. At 9-months representative results could only be obtained in 2/3 of the animals and these were reported.

Glomerular and tubular function

Animals were housed in urine collection cages for 24 hours at 3 and 9 months of age. Urine was collected for a period of 24 hours and on exiting the urine collection cage a blood sample was collected from the tail vein. Blood was centrifuged at 3000 RPM and plasma was directly taken to the clinical laboratory for analysis of sodium, potassium, magnesium, phosphate, urea and creatinine. Urine samples were measured for volume, urea, creatinine and osmolality or stored for possible future analysis. Creatinine and urea clearances were calculated and normalized for body weight.

NGAL measurement

Urine samples collected at 3 and 6 months, and plasma collected at day 8 were analyzed for the presence of neutrophil gelatinase-associated lipocalin (NGAL) protein levels as a marker of tubular damage using the rat lipocalin 2 ELISA kit (R&D Systems, Abingdon, UK) with detection limit of 78.1 pg/ml.

Statistical analysis

Mean (standard deviation, SD) are presented for all findings unless otherwise indicated. Parameters of interest were analyzed by univariate analysis using sex, litter size and treatment as fixed factors. Post hoc analysis was performed by Tukey. The time effect on blood pressure was analyzed between and within subjects with a multivariate analysis for repeated measures, using the Greenhouse-Geisser correction. Gene expression data was analyzed by the Kruskal Wallis test for non-parametric data. Interaction between glomerular generation and glomerular numbers was analyzed with linear regression. All analyses were performed by the use of SPSS version 20 (IBM, Armonk, NY) at the alpha 0.05 level.

Results

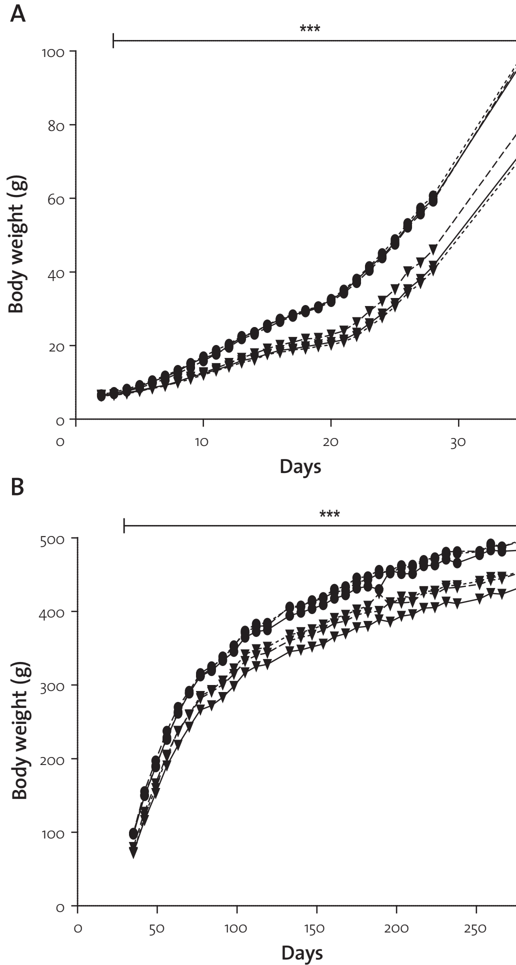
Body weight

Body weights were evaluated in the study to follow-up on general animal health and to evaluate the effect of postnatal food-restriction.

Body weights of male and females were only different incidentally (Day 2, 3 and 35) and were therefore pooled for analysis. Mean body weight was 6.4 (SD 0.5 and 0.6)g for NF and FR, respectively) for both normal food (NF) and food restricted (FR) animals at cross-fostering on PND 2.

From day 3 onwards (figure 1a) body weight of FR animals was lower compared with NF animals: on day 3 NF mean 6.9 (SD 0.7)g, FR mean 6.7 (SD 0.6)g, $p<0.001$; on the day of weaning NF mean 59.8 (SD 3.3)g and FR mean 42.2 (SD 5.3)g, difference 29%, $p<0.001$. Relative body weight difference declined after weaning. However, an absolute body weight difference of 30-40 grams was observed for the remainder of the study ($p<0.001$) (figure 1b). No drug treatment-related effects were detected on body weight, except for an incidental difference between furosemide and hydrochlorothiazide on day 10 within NF animals ($p=0.037$). At day 189, by chance the heaviest and lightest animal of the sham-NF and the hydrochlorothiazide-FR groups, respectively, were excluded from the routine body weight measurement due to urine sampling. This resulted in the observed abnormality in the body weight evolution graph.

Figure 1 Body weight evolution of all study animals is shown from PND 2 up to PND 35 (**A**). Body weight evolution of study animals (male) is shown from PND 35 up to end of study (9 months) (**B**).



Evolution of normal food (NF) animals are indicated with dots and food restricted (FR) with triangles. Sham-dosed groups are indicated with a solid line, furosemide groups with a striped line and hydrochlorothiazide groups with a dotted line. n= 19-20/group up to PND 8; n=11-14/group up to PND 35; n=4-6/group for the remainder of the study. Body weight of FR animals was lower compared with the NF animals from day 3 onwards on all days of measurement. Drug treatments within food regimes were well within each other's standard deviation (not shown for clarity). Univariate analysis was performed with Tukey post-hoc. ***$p<0.001$.

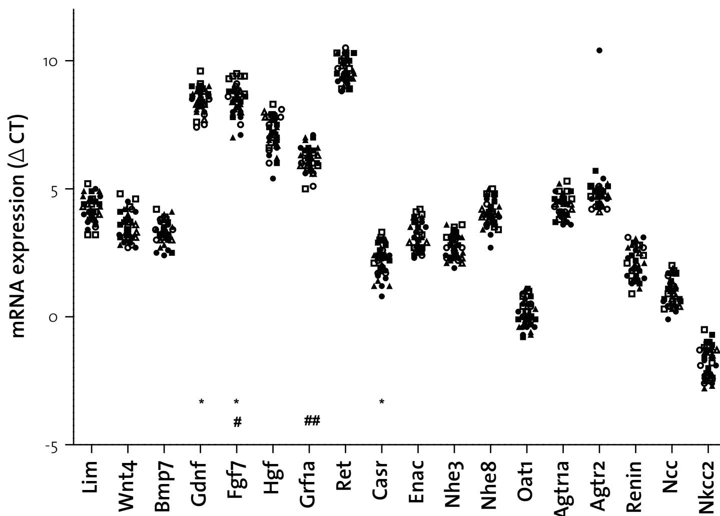
8

Day 8 investigations (gene expression, proliferation/apoptosis ratio)

To determine direct effects of treatment on kidney development gene expression and proliferation/apoptosis and NGAL were examined on PND 8.

Gene expression analysis on kidney tissue, collected on day 8 (figure 2), showed a 0.7 fold change of *Fgf7* in FR animals (p=0.009) and 0.68 fold change in furosemide treated animals versus sham (p=0.031). *Casr* and *Gdnf* genes were down-regulated in furosemide treated animals with fold changes of 0.67 (p=0.04) and 0.72 (p=0.02), respectively and *Grfa1* was up-regulated in FR animals compared with the NF animals (1.39 fold, p=0.002). All gene expression analyses were performed on pooled samples of both genders as no gender effect was noted. Gene expression of the loop diuretic sensitive *Nkcc2* and the thiazide sensitive *Ncc* transporters did not change.

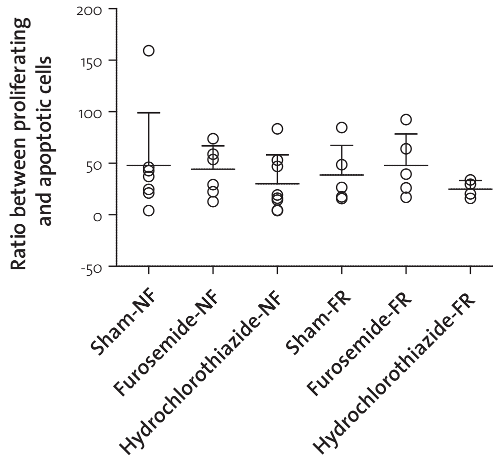
Figure 2 Renal gene expression of relevant transporters, growth factors and receptors in kidney development measured on day 8



Delta Ct values from all individual animals are plotted for each target gene. Shown are sham (dot), furosemide (square) and hydrochlorothiazide (triangle) treatment. Normal food (NF) and food restricted (FR) are distinguished by closed and open symbols (n=5-8 per group). Statistical analysis was performed with Kruskal-Wallis one-way ANOVA. * $p<0.05$ furosemide vs. sham; # $p<0.05$, ## $p<0.01$ FR vs. NF.

No difference was noted on the ratio between proliferating and apoptotic cells in kidney tissue on day 8 of the study (figure 3). Means for males and females were 47.1 (482 (SD 358) proliferating- and 13 (SD 8) apoptotic cells/mm^2, and 29.8 (430 (SD 244) proliferating and 14 (SD 4) apoptotic cells/mm^2, respectively (f=2.852, p=0.10). Similarly,

Figure 3 The balance between proliferating and apoptotic cells in day 8 kidneys



Mean ratio's with standard deviation per treatment group are shown (n=5-8/group). Normal feeding and food restriction regimes are indicated with NF and FR, respectively. Statistical analysis was performed by univariate analysis and no statistically significant differences were noted.

no differences were noted between NF and FR treated groups (means: 40.3 (516 (SD 378) proliferating and 15 (SD 6) apoptotic cells/mm^2) vs. 38.0 (380 (SD 158) proliferating and 12 (SD 6) apoptotic cells/mm^2), respectively; f=0.107, p=0.75). No influence of the drug treatment (mean ratio's: sham 44.0 (357 (SD 192) proliferating and 11 (SD 6) apoptotic cells/mm^2), furosemide 45.8 (621 (SD 380) proliferating and 16 (SD 8) apoptotic cells/mm^2) and HCTZ 28.5 (420 (SD 300) proliferating and 15 (SD 3) apoptotic cells/mm^2); f=1.032, p=0.37) and no interactions between the different variables were detected.

Ngal (table 4) was investigated in plasma as a marker for acute kidney damage. Plasma values were on average 0.168 (SD 0.031) and 0.149 (SD.0.028) µg/ml in NF and FR, respectively, and did not differ significantly (f=3.880, p=0.58). Additionally no changes were noted between the different drug treatments and the sham control.

Day 35 investigations (kidney weight, glomerular number and glomerular generation count)

On Day 35, the impact of the treatments on kidney organogenesis was assessed. To investigate whether the kidney developed normally, kidney weight, glomerular number and glomerular generation count were determined.

Mean kidney weight (table 2) in FR animals was lower compared with NF animals and a difference between the sexes was detected (males 0.64 (SD 0.07) g, females

0.57 (SD 0.08) g; f=12.745, p=0.001). Corrected for body weight (x100) these were 0.72 (SD 0.07) and 0.73 (SD 0.09). No differences were noted for the treatments studied (sham 0.61 (SD 0.09) g, furosemide 0.61 (SD 0.07) g, HCTZ 0.58 (SD 0.1) g; f=0.274, p=0.762) and interactions between the variables sex, litter size and treatment were not present. When corrected for body weight, kidney weight in FR animals was higher compared with NF animals (0.77 vs. 0.69; f=15.511, p<0.001) and an interaction between treatment and litter size was detected (f=4.812, p=0.015). The higher kidney weight in the FR group could be attributed to the slightly higher kidney weights in the HCTZ-FR group.

Glomerular generation count (table 2) was performed and yielded on average 4.6 (SD 0.66) generations in the sham-NF dosed animals. FR animals had on average less glomerular generations (4.4 (SD 0.39)) compared with the NF animals (4.7 (SD 0.41)) (f=5,666, p=0.025). This decrease was dependent on sex (f=4,260, p=0.049) and was detected in males, but not females (male NF 5.0 (SD 0.23); FR 4.4 (SD 0.32), female NF 4.5 (SD 0.41); FR 4.5 (SD 0.44)). Overall analysis did not show a difference between sexes (male 4.7 (SD 0.40), female 4.5 (SD 0.42); f=1.861, p=0.18) or drug treatments (sham 4.5 (SD 0.61), furosemide and HCTZ 4.6 (SD 0.31 and 0.27, respectively); f=0.553, p=0.58).

Table 2 Renal parameters

Treatment	**Sham NF**	**Furosemide NF**	**Hydrochlorothiazide NF**
Day 35			
Body Weight (g)	94.0 (4.47)	93.5 (9.76)	96.7 (8.78)
Right kidney weight (g)	0.67 (0.05)	0.65 (0.05)	0.63 (0.12)
Right kidney / body weight (ratio x100)	0.71 (0.04)	0.70 (0.05)	0.65 (0.08)
Glomerular generations (count)	4.6 (0.66)	4.8 (0.20)	4.7 (0.28)
Glomerular Number	29,454 (4,022)	30,640 (4,272)	31,488 (4,366)
End of experiment			
Body weight (g)	483 (27.4)	496 (8.7)	498 (23.9)
Right kidney weight (g)	2.98 (0.13)	3.13 (0.07)	3.14 (0.12)
Right kidney / body weight (ratio x100)	0.62 (0.02)	0.63 (0.01)	0.63 (0.02)

Data are presented as mean (standard deviation). PND 35: n=5-8 / group; end of experiment n= 4-6/group.

FR animals showed a decreased amount of glomeruli compared with NF animals (means: 27861 (SD 3468) vs. 30527 (SD 4096); f=5.492, p=0.026) on day 35 (table 2). Treatment (f=0.626, p=0.54) or sex (f=1.938, p=0.17) did not influence the glomerular number. However, an interaction was detected between treatment, sex and litter size (f=3.922, p=0.031). In contrast to all other groups, average glomerular number was comparable between male sham-FR (29,394) and male sham-NF (29,454) animals.

Glomerular generation count did not correlate with the total number of glomeruli (Pearson's correlation 0.046) indicating that glomerular generation counts are not a suitable predictor for glomerular numbers.

Long-term follow-up (clinical chemistry, NGAL, blood pressure and histopathology)

Long-term endpoints were included in the study to investigate any delayed effects of the food restriction and drug treatments.

Clinical biochemistry analysis at 3 months of age (table 3a) showed a higher creatinine clearance in FR animals compared with the NF animals (NF 0.77 (SD.0.09) ml/min/kg, FR 0.84 (SD 0.1) ml/min/kg; f=4.374, p=0.047). Additionally, an interaction was detected between treatment and nest type (f= 6.443, p=0.006) indicating

Sham FR	Furosemide FR	Hydrochlorothiazide FR	Statistical significance
72.9 (4.97)	78.3 (9.12)	67.5 (9.55)	NF vs. FR p<0.001 M vs. F p=0.005
0.54 (0.06)	0.57 (0.08)	0.55 (0.07)	FR vs. NF p<0.001 M vs. F p=0.001
0.74 (0.06)	0.73 (0.09)	0.81 (0.05)	FR vs. NF p<0.001 Attributed to HCTZ (p=0.015)
4.3 (0.59)	4.4 (0.23)	4.6 (0.26)	FR vs. NF (p=0.025) only in males
29,648 (4,446)	26,394 (3,433)	27,540 (1,961)	FR vs. NF (p=0.026)
435 (12.7)	455 (21.7)	456 (20.4)	FR vs. NF (p<0.001)
2.75 (0.10)	2.96 (0.12)	2.7 (0.43)	FR vs. NF (p=0.002)
0.63 (0.03)	0.65 (0.02)	0.59 (0.10)	

Table 3A Clinical biochemistry parameters at 3 months of age

	Sham-NF	Furosemide-NF	Hydrochlorothiazide-NF	Sham-FR	Furosemide-FR	Hydrochlorothiazide-FR
Body weight (g)	324 (7)	329 (6)	329 (9)	278 (11)	292 (10)	297 (18)
Plasma						
Sodium (mmol/l)	141 (2)	142 (1)	143 (3)	142 (1)	141 (1)	142 (1)
Potassium (mmol/l)	4.4 (0.2)	4.3 (0.3)	4.3 (0.3)	4.2 (0.2)	4.5 (0.2)	4.6 (0.6)
Magnesium (mmol/l)**	0.82 (0.03)	0.76 (0.03)	0.74 (0.03)	0.79 (0.04)	0.79 (0.01)	0.81 (0.04)
Phospate (mmol/l)	2.44 (0.14)	2.35 (0.17)	2.43 (0.2)	2.39 (0.12)	2.43 (0.11)	2.54 (0.1)
Urea (mmol/l)	4.5 (0.8)	4.5 (0.6)	4.5 (0.4)	4.1 (0.7)	4.4 (0.8)	4.6 (0.7)
Creatinine (μmol/l)	22.8 (3.1)	24.5 (3.4)	23.8 (3.4)	22.2 (2.6)	21.7 (0.8)	23.7 (4.8)
Urine (24h collection)						
Volume (ml)	8.5 (1.8)	9.6 (2.3)	7.6 (0.8)	7.6 (0.9)	8.3 (1.1)	8.9 (2.2)
Creatinine (mmol/l)	10.6 (1.6)	9.4 (1.7)	10.5 (0.5)	9.4 (1.2)	9.9 (0.9)	9.8 (2.2)
Urea (mmol/l)	1,257 (103)	1,134 (189)	1,208 (88)	1,163 (112)	1,219 (133)	1,191 (197)
Osmolality (mosmol/kg)	2,154 (466)	2,135 (360)	2,079 (340)	2,210 (243)	2,381 (370)	2,207 (391)
Creatinine clearance (ml/min/kg)*	0.84 (0.09)	0.76 (0.08)	0.71 (0.06)	0.80 (0.10)	0.88 (0.05)	0.85 (0.14)
Urea clearance (ml/min/kg)	0.51 (0.09)	0.51 (0.03)	0.44 (0.05)	0.54 (0.11)	0.56 (0.13)	0.53 (0.10)

Data are presented as mean (standard deviation) and statistical analysis was performed with univariate analysis with Tukey post-hoc. * different between NF and FR (p=0.047). ** HCTZ modifies the average NF values (p=0.006). n=4-6 /group

Table 3B Clinical biochemistry parameters at 9 months of age.

	Sham-NF	Furosemide-NF	Hydrochlorothiazide-NF	Sham-FR	Furosemide-FR	Hydrochlorothiazide-FR
Body weight (g)	479 (24)	489 (13)	490 (21)	427 (11)	447 (20)	450 (18)
Plasma						
Sodium (mmol/l)	142 (1)	142 (1)	141 (1)	142 (1)	142 (1)	142 (1)
Potassium (mmol/l)	4.5 (0.3)	4.8 (0.2)	4.4 (0.1)	4.6 (0.1)	4.6 (0.1)	4.6 (0.2)
Magnesium (mmol/l)	0.77 (0.02)	0.75 (0.03)	0.76 (0.03)	0.77 (0.03)	0.78 (0.03)	0.77 (0.02)
Phospate (mmol/l)	1.82 (0.08)	1.80 (0.06)	1.80 (0.05)	1.78 (0.06)	1.81 (0.12)	1.82 (0.11)
Urea (mmol/l)	5 (0.9)	4.8 (0.8)	4.8 (0.7)	4.7 (0.8)	5.3 (1.2)	4.7 (0.9)
Creatinine (μmol/l)*	29.0 (1.2)	26.8 (1.0)	26.8 (4.7)	26.8 (1.2)	25.2 (1.8)	25.2 (2.1)
Urine (24h collection)						
Volume (ml)	8 (1.8)	10 (1.0)	9 (1.2)	9 (3.4)	11 (2.7)	8 (1.5)
Creatinine (mmol/l)	13.8 (1.2)	13.7 (1.3)	13.4 (0.8)	12.7 (3.0)	11.8 (2.3)	13.8 (1.5)
Urea (mmol/l)	1,322 (29)	1,338 (99)	1,262 (184)	1,363 (202)	1,274 (206)	1,410 (82)
Osmolality (mosmol/kg)	2265 (48)	2375 (257)	2038 (431)	2430 (386)	2263 (360)	2504 (193)
Creatinine clearance (ml/min/kg)	0.53 (0.09)	0.71 (0.05)	0.64 (0.18)	0.62 (0.08)	0.79 (0.07)	0.68 (0.12)
Urea clearance (ml/min/kg)	0.30 (0.04)	0.40 (0.05)	0.33 (0.07)	0.41 (0.12)	0.43 (0.10)	0.39 (0.08)

Data are presented as mean (standard deviation) and statistical analysis was performed with univariate analysis with Tukey post-hoc. * different between NF and FR ($p=0.043$). n=4-6 /group.

plasma magnesium concentration in the HCTZ-treated groups to be lower in NF animals (0.74 (0.03) mmol/l) compared with FR animals (0.81 (0.04) mmol/l). No other changes were detected.

At 9 months of age (table 3b) plasma creatinine was lower in FR animals compared with the NF animals (NF 27.5 (SD 2.8) μmol/l, FR 25.7 (SD 1.8) μmol/l; f=4.551, p=0.043), but this did not result in differences changes in creatinine clearance. No other differences were noted.

NGAL levels (table 4) in urine reduced by 25% between 3 and 6 months of age in the long-term follow-up period. This effect was similar within all the different treatment groups. No differences were noted between feeding regimes and/or drug treatment.

Blood pressure was measured monthly from 3 months of age onwards (figure 4) and analyzed over the course of the study. Diastolic blood pressure (DBP) and mean blood pressure (MBP) declined over the course of the study within NF (f=3.728, p=0.018) and FR groups (f=3.161, p=0.027). No effects were noted on systolic blood pressure (SBP) or pulse pressure (PP).

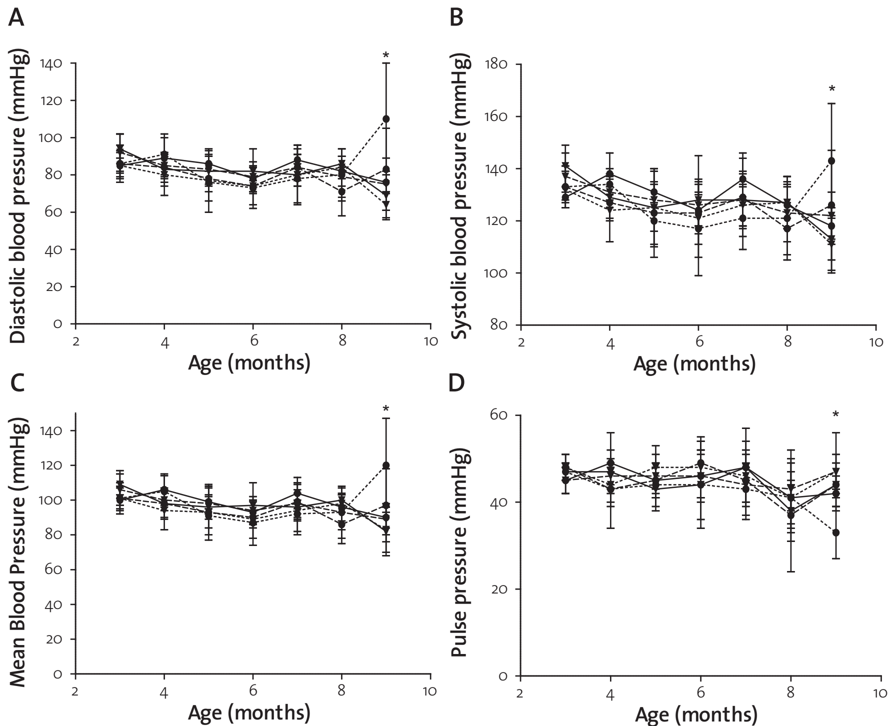
No differences were detected between the treatment groups on the individual time points except for the measurement at the age of 9 months. DBP, SBP and MBP were all increased in NF animals and PP was slightly reduced compared with FR animals (DBP (NF 88.6 (SD 23.0) mmHg, FR 69.1 (SD 11.4) mmHg; f=7.688, p=0.016); SBP (NF 128.6 (SD 18.3) mmHg, FR 114.9 (SD.10.7) mmHg; f=4.647, p=0.050); MBP (NF 101.4 (SD 21.1) mmHg, FR 84.8 (SD 11.4) mmHg; f=5.770, p=0.032); PP (NF 40.3 (SD 6.2) mmHg, FR 45.8 (SD 5.8) mmHg; f=5.334, p=0.038)).

Table 4 NGAL measurements.

	Sham-NF	**Furosemide-NF**	**Hydrochlorothiazide-NF**
PND 8 plasma (μg/ml)	0.161 (0.027)	0.187 (0.031)	0.155 (0.028)
3-months urine (μg/ml)	0.699 (0.152)	0.628 (0.118)	0.655 (0.121)
6-months urine (μg/ml)	0.507 (0.114)	0.467 (0.031)	0.480 (0.053)
	Sham-FR	**Furosemide-FR**	**Hydrochlorothiazide-FR**
PND 8 plasma (μg/ml)	0.155 (0.030)	0.152 (0.035)	0.140 (0.020)
3-months urine (μg/ml)	0.633 (0.116)	0.652 (0.139)	0.648 (0.124)
6-months urine (μg/ml)	0.492 (0.100)	0.502 (0.059)	0.481 (0.129)

Data are presented as mean (standard deviation) and statistical analysis was performed with univariate analysis with Tukey post-hoc. No statistically significant findings were noted. n=6-8 at PND 8; n= 4-6 at 3- and 6-months.

Figure 4 Blood pressure measurements in rats over the study period showing diastolic blood pressure (**A**), systolic blood pressure (**B**), mean blood pressure (**C**) and pulse pressure (**D**)



Means ± SD of normal food (NF) animals are plotted with dots and food restricted (FR) with triangles. Sham-dosed groups are indicated with a solid line, furosemide groups with a striped line and hydrochlorothiazide groups with a dotted line. n=4-6 /group, with exception of 9-month time point where n=2-4/group. Univariate analysis with Tukey post-hoc test was performed for all individual time points. * NF vs. FR $p \leq 0.05$). A multivariate analysis for repeated measurements was performed to investigate the effect of study time and showed a decrease of diastolic and mean blood pressure within nest type over course of the study period ($p=0.018$).

Kidney weight at the end of the experiment (table 2) was significantly lower in FR animals compared with NF animals (mean 2.80 (SD.0.27) g vs. 3.08 (SD.0.12) g; f=11.440, p=0.002). After correction for body weight, no difference could be noted. Neither drug-related effects nor abnormal findings were observed on tubular and glomerular morphology in the kidney after microscopic evaluation.

Discussion

The goal of this study was to determine whether the clinical neonatal dose of furosemide and hydrochlorothiazide affected kidney development and function on a short and/or long-term basis.

Overall, we concluded that these drugs do not impact kidney development. Furosemide was able to induce slight changes in *Fgf7*, *Casr* and *Gdnf* gene expression levels. However, these changes were less than two-fold and therefore the biological relevance of these changes with respect to a disturbed organ development is questionable. Indeed, no alterations in any of the structural renal parameters were noted (table 2). In addition, no abnormalities in any of the renal functions tested were observed. A renal concentration effect, as was noted after prenatal dosing of furosemide (10), could not be found within our experimental conditions. It may therefore be concluded that the renal phenotype after prenatal and postnatal furosemide exposure is different. However, more plausibly we did not find this effect because we administered more clinically relevant dose levels which are significantly lower compared with the dose levels used in that study (i.e. 75 mg/kg).

Similar to furosemide, treatment with hydrochlorothiazide did not change any parameters of kidney development. No alteration in *Ncc* expression level was noted, which was contrary to our expectations because hydrochlorothiazide blocks this thiazide-sensitive $Na^{+}Cl^{-}$ cotransporter and it has been shown to affect gene expression (15). The lack of effect could be due to either the slightly lower dose levels used in this study, the different route of administration or the time between the last dose administration and sampling. Similarly, we did not see any response on *Nkcc2* after furosemide treatment.

Our second hypothesis stated that a multi-hit on the kidney may modulate developmental pathways and that extrauterine growth retardation would modify drug toxicity. Based on our results, such a modulating effect of the combination of growth restriction with diuretic treatment could not be demonstrated. The interactions noted between treatment and nest type were limited to hydrochlorothiazide and FR leading to a slightly higher kidney weight at day 35. No interactions were detected between furosemide and extrauterine growth retardation.

Our model was successful in obtaining growth retardation evidenced by the changes in body weight observed throughout the study. Additionally, we confirmed that extrauterine growth retardation has an impact on glomerular numbers (12), which were decreased by 11% after food restriction. Of note was the absence of any difference between the male sham-FR and sham-NF animals. This may indicate that the decrease noted in the FR group was more drug driven, but this was not confirmed in our analysis. In other studies, the effect between NF and FR was more pronounced also substantiated with the higher average total count of glomerular numbers, which

was relatively low in this study (16)(Bueters et al. unpublished). Therefore, we believe that the observation of low glomerular numbers in the sham-NF group was probably related to chance. However a more drug driven effect could be explained as well. Although the changes that were observed were small and we question the biological relevance, furosemide did down-regulate the gene expression of *Gdnf* and *Fgf7*, which both are considered important for ureteric bud development and nephron formation (17-19). This observation in combination with the added stressor of food restriction could lead to a state where nephrogenesis is disturbed. Such a link is less clear for hydrochlorothiazide. Main effect on the kidney described for hydrochlorothiazide is activation of the renin angiotensis system (RAS), but no changes in our mRNA targets of the RAS after either food restriction or hydrochlorothiazide treatment were seen. Woods et al showed that maternal protein restriction in rats resulted in decreased glomerular numbers but also in a suppression of the RAS (20). Other studies have been performed investigating the relations between reduced nephron endowment and the RAS in sheep, but are not in agreement whether there is a real role for the RAS system or not (21,22). At the moment the relation between the RAS and nephron endowment is not clear.

The changes in glomerular number did not correlate with the number of glomerular generations, which were slightly reduced in male FR vs. NF animals. Again changes in mRNA expression of two targets, *Fgf7* and Grf*a1* with a role in kidney development were detected (17,18,23). The observed changes were very small and the impact on organogenesis was questionable. However, it may be that the effects of small reductions in several pathways cumulate into a significant reduction in the final common pathway, i.e. nephron formation.

Brenner et al. described in the hyperfiltration hypothesis that glomerular damage leads to hyperfiltration, and that the first sign of kidney damage is mostly noted by an increase in creatinine clearance caused by hyperfiltration (24) instead of the classical creatinine clearance decrease which is more evident in more serious kidney damage (25). In this study this was evidenced by a minimally higher creatinine clearance in FR at 3 months and the lower creatinine plasma levels at 9 months. Although these findings are in line with our hypothesis, the observed changes are very small and without any confirmed histopathological kidney abnormalities at the end of the experiment it is difficult to determine whether these were related to treatment.

In the same hypothesis, Brenner described that hyperfiltration would lead eventually to a systemic increase in blood pressure (24). Indeed, it has been described that intrauterine growth retardation leads to a reduced glomerular number (26) and an increase in blood pressure (27). However there are also studies performed in which this phenomenon was not observed (28). We could not detect any differences in blood pressure variables between our groups. This might be because the effects noted in the study of Schreuder et al. were quite small in absolute values and a larger variation

was noted with our experimental set-up (27). Although we detected an increase in the MBP of the NF groups at the 9-month time point, we believe this may be caused by a bias in the measurements because we were not able to record signals of approximately half of the animals on that time point. This was probably related to the age of the animals, which resulted in a suboptimal tail blood flow.

There are some limitations to the experimental study design. Although this experiment tried to simulate a multi-hit of both drug use and extrauterine growth retardation, these can actually only approach the real situation in the neonatal care units. By example there is no full control on the nutrients that every rat food restricted pup received. Although the mothers are well fed with a balanced diet, it is unknown whether the reduced food intake of the pups might have led to an imbalance of nutrient intake and whether this is similar to the clinical practice. Additionally, our rat pups were healthy pups in contrary to the patients, which are likely suffering from chronic lung disease and are therefore fluid restricted. This physiological condition could potentially modify the adverse effects on nephrogenesis as well and should be investigated separately in fluid restriction models. Because of the multi-factorial situation in the clinic, our results are most likely an underestimation of the true effect.

Another limitation was the use of a non-invasive blood pressure technique (tail cuff) instead of a golden standard technique such as telemetry. Another option to measure blood pressure is the transducer method, which is considered a terminal experiment. For a long term follow-up study this would have resulted in adding many animals to the study. All methods have their advantages and disadvantages (29). Although telemetry has a very sensitive read-out it does require the rats to undergo surgery and requires an expensive set-up. Due to extensive drop-out in a previous telemetry study, we decided to use the tail cuff.

In conclusion, furosemide and hydrochlorothiazide did not influence the development or function of the kidneys when administered in a clinically relevant dose to young rat pups at a stage of ongoing nephrogenesis. Growth retardation based on the increase of litter size led to a reduction in glomerular numbers but had no modifying effect on furosemide or hydrochlorothiazide treatment. In the future, experiments should be performed in which the neonatal environment is even more precisely simulated preferably in a disease model to investigate other potential synergies. In regard of neonatal safety, care should be taken to avoid growth retardation as best as possible.

References

1. Allegaert K, van de Velde M, van den Anker J. Neonatal clinical pharmacology. *Paediatr Anaesth* 2014;24(1):30-38.
2. Hughson M, Farris AB, 3rd, Douglas-Denton R, Hoy WE, Bertram JF. Glomerular number and size in autopsy kidneys: The relationship to birth weight. *Kidney Int* 2003;63(6):2113-2122.
3. Lackland DT, Bendall HE, Osmond C, Egan BM, Barker DJ. Low birth weights contribute to high rates of early-onset chronic renal failure in the southeastern united states. *Arch Intern Med* 2000;160(10):1472-1476.
4. Schreuder MF. Safety in glomerular numbers. *Pediatr Nephrol* 2012;27(10):1881-1887.
5. Quaggin SE, Kreidberg J. Embryology of the kidney; in Brenner BM (ed): Brenner and rector's the kidney. Philadelphia, Saunders, Elsevier, 2008, pp 3-24.
6. Bertram JF, Douglas-Denton RN, Diouf B, Hughson MD, Hoy WE. Human nephron number: Implications for health and disease. *Pediatr Nephrol* 2011;26(9):1529-1533.
7. Hartman HA, Lai HL, Patterson LT. Cessation of renal morphogenesis in mice. *Dev Biol* 2007;310(2):379-387.
8. Stewart A, Brion LP, Ambrosio-Perez I. Diuretics acting on the distal renal tubule for preterm infants with (or developing) chronic lung disease. *Cochrane Database Syst Rev* 2011;(9):CD001817.
9. Gimpel C, Krause A, Franck P, Krueger M, von Schnakenburg C. Exposure to furosemide as the strongest risk factor for nephrocalcinosis in preterm infants. *Pediatr Int* 2010;52(1):51-56.
10. Mallie JP, Boudzoumou P. Functional renal maturation in rat neonates after prenatal exposure to furosemide. *Pediatr Nephrol* 1996;10(4):458-460.
11. Mallie JP, Gerard A, Gerard H. Does the in-utero exposure to furosemide delay the renal maturation? *Pediatr Pharmacol (New York)* 1985;5(2):131-138.
12. Bueters RR, Klaasen A, van den Heuvel LP, Schreuder MF. Effect of nsaids and diuretics on nephrogenesis in an ex vivo embryogenic kidney model. *Birth Defects Res B Dev Reprod Toxicol* 2013;98(6):486-492.
13. Reckelhoff JF, Zhang H, Srivastava K, Granger JP. Gender differences in hypertension in spontaneously hypertensive rats: Role of androgens and androgen receptor. *Hypertension* 1999;34(4 Pt 2):920-923.
14. Hinchliffe SA, Sargent PH, Chan YF, van Velzen D, Howard CV, Hutton JL, Rushton DI. "Medullary ray glomerular counting" as a method of assessment of human nephrogenesis. *Pathol Res Pract* 1992;188(6):775-782.
15. Nijenhuis T, Hoenderop JG, Loffing J, van der Kemp AW, van Os CH, Bindels RJ. Thiazide-induced hypocalciuria is accompanied by a decreased expression of ca2+ transport proteins in kidney. *Kidney Int* 2003;64(2):555-564.
16. Schreuder MF, Nyengaard JR, Remmers F, van Wijk JA, Delemarre-van de Waal HA. Postnatal food restriction in the rat as a model for a low nephron endowment. *Am J Physiol Renal Physiol* 2006;291(5):F1104-F1107.
17. Towers PR, Woolf AS, Hardman P. Glial cell line-derived neurotrophic factor stimulates ureteric bud outgrowth and enhances survival of ureteric buc cells in vitro. *Exp Nephrol* 1998;6(4):337-351
18. Qiao J, Uzzo R, Obara-Ishihara T, Degenstein L, Fuchs E, Herzlinger D. Fgf-7 modulates ureteric bud growth and nephron number in the developing kidney. *Development* 1999;126(3):547-554.
19. Bush KT, Sakurai H, Steer DL, Leonard MO, Sapogna RV, Meyer TN, Schwesinger C, Qiao J, Nigam SK. TGF-beta superfamily members modulate growth, branching, shaping, and patterning of the ureteric bud. *Dev Biol* 2004; 266(2): 285–298
20. Woods LL, Ingelfinger JR, Nyengaard JR, Rasch R. Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. *Pediatr Res* 2001;49(4): 460–467.
21. Zhang DY, Lumbers ER, Simonetta G, Wu JJ, Owens JA, Robinson JS, McMillen IC. Effects of placental insufficiency on the ovine fetal renin-angiotensin system. *Exp Physiol* 2000;85(1):79–84.
22. Zohdi V, Moritz KM, Bubb KJ, Cock ML, Wreford N, Harding R, Black MJ. Nephrogenesis and the renin-angiotensin system in fetal sheep: effects of intrauterine growth restriction during late gestation. *Am J Physiol Regul Integr Comp Physiol* 2007;293(3):1267-1273
23. Keefe Davis T, Hoshi M, Jain S. Stage specific requirement of gfralpha1 in the ureteric epithelium during kidney development. *Mech Dev* 2013;130(9-10):506-518.
24. Brenner BM, Lawler EV, Mackenzie HS. The hyperfiltration theory: A paradigm shift in nephrology. *Kidney Int* 1996;49(6):1774-1777.

25. Mogensen CE. Prediction of clinical diabetic nephropathy in iddm patients. Alternatives to microalbuminuria? *Diabetes* 1990;39(7):761-767.
26. Hinchliffe SA, Lynch MR, Sargent PH, Howard CV, Van Velzen D. The effect of intrauterine growth retardation on the development of renal nephrons. *Br J Obstet Gynaecol* 1992;99(4):296-301.
27. Schreuder MF, Fodor M, van Wijk JA, Delemarre-van de Waal HA. Association of birth weight with cardiovascular parameters in adult rats during baseline and stressed conditions. *Pediatr Res* 2006;59(1):126-130.
28. Zimanyi MA, Bertram JF, Black MJ. Does a nephron deficit in rats predispose to salt-sensitive hypertension? *Kidney Blood Press Res* 2004;27(4):239-247.
29. Kurtz TW, Griffin KA, Bidani AK, Davisson RL, Hall JE; Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research. Recommendations for blood pressure measurement in humans and experimental animals. part 2: blood pressure measurement in experimental animals: a statement for professionals from the Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research. *Arterioscler Thromb Vasc Biol*. 2005 Mar;25(3):e22-e33.

9

Discussion and future perspectives

A. Methodological considerations

B. General discussion

C. Suggestions for future studies

A Methodological considerations

To be able to answer the questions stated in **chapter 1** we assessed different endpoints in kidney development. These endpoints needed to relate to either short- or long term effects on kidney morphology or function. Additionally a method to model for extra-uterine growth restriction was included. A more thorough rationale for our considered methodology is written in the following paragraphs.

A.1 Evaluation of short-term effects on early kidney development

A.1.1 Considerations for the selection of an ex-vivo model

We used embryo's from CD-1 mice to investigate early effects on direct pathways of nephrogenesis in our studies. The rudimental kidneys (metanephroi) were explanted 13 days after gestation and cultured on filters in DMEM supplemented with insulin, transferrin and sodium selenite. In the field of developmental kidney biology most models used are varieties of this particular model. Depending on the research question there are subtle changes in the models. For instance the time point of explantation may be slightly different (1,2). Other changes are different consistencies of culture medium such as adding fecal bovine serum, or the use of different matrices, such as gels to better preserve the 3d structure of the rudimentary organ (3,4). Generally, the models used are quite similar and most are established in the mouse, however there are reports of successfully applying similar methodology in rats (5-7).

Apart from this "classic" *ex vivo* approach, genetic modification of mice is used to improve the read-out of these models by expressing fluorescent proteins in specific morphological structures of the kidney. A good example of such a model is the HoxB7 modified mouse developed in the lab of Frank Costantini (8). Other models rely on fluorescent expression after crossing animals using lox-cre techniques to specifically express a fluorescent protein in a renal specific structure (9,10). Using these mice, whole mount staining procedures for complex structures are no longer necessary and more standardized results may be obtained. We did not have access to these models, therefore we stained the embryo's via a calbindin D28k whole mount staining method, which had been reported to provide good results in immunofluorescent ureteric bud visualisation (11).

A.1.2 Nephrogenesis endpoints in the ex vivo model

The *ex vivo* experiments were performed in the budding phase of development, an early phase of nephrogenesis. In this phase the ureteric bud (part of the ureteric tract) starts to branch due to reciprocal interactions with the metanephrogenic mesenchyme (12). Many of the genes of the molecular pathways involved in this process have been identified in the last decade and have been summarized in a large project called the 'GenitoUrinary Development Molecular Anatomy Project' (GUDMAP, www.gudmap.

org) which analyzed expression patterns at different stages of development (13). Still the function and interaction of all the identified targets has not yet been elucidated. We selected five relatively well-described targets, which were shown to be important in the general pathway of developing nephrons. These targets were *Wt1, Sox9*, *Gdnf, Bmp7* and *Fgf8*. Additionally genes for the prostaglandin synthetase 1 and 2 were added in the NSAIDs experiments as their respective proteins are targets of these drugs. More detailed roles of the investigated genes are described in table 1 below.

Table 1 Selected targets and their role in kidney function/development

Gene symbol	Common name	Role
Actb	Beta actin	Cytoskeleton protein, used as high abundance reference gene.
Hmbs	Hydroxymethylbilane synthase	Heme production enzyme, used as medium abundance reference gene.
Wt1	Wilms Tumor 1	Essential for mesenchyme survival. (14,15)
Sox9	Sex determining region Y-Box-9	Controls epithelial branching by activating Ret. (16,17)
Gdnf	Glial cell line-derived neurotrophic factor	Important player in ureteric bud differentiation processes. Loss results in agenesis. (18-22)
Bmp7	Bone morphogenetic protein 7	Inhibits and induces ureteric bud growth and maintains nephrogenic progenitors. (23-25)
Fgf8	Fibroblast growth factor 8	Maintains nephrogenic progenitors. (26-27)
Ptgs1	Prostaglandine synthetase 1	Enzyme converts arachidonate to prostaglandin H2 and is a target of NSAIDs.
Ptgs2	Prostaglandine synthetase 2	Enzyme converts arachidonate to prostaglandin H2 and is a target of NSAIDs.

In addition to these molecular markers morphology-based endpoints were used. Multiple types of immunostainings have been described in the *ex-vivo* models to identify branching (calbindin D28k, keratin) or ureteric buds (lectin, peanut agglutinin, e-cadherin). Other methods rely on morphometric characteristics such as surface area or tissue circumference. We started out to use metanephron surface area and Calbindin D28k immunohistochemistry as morphology markers for kidney development as had been used by the research groups of Woolf and Davies (2,28). However after the initial experiments, metanephron surface area was considered to be an endpoint of low value regarding the prediction of kidney development as is demonstrated in **chapter 4** of this thesis. It can be used however to partially standardize for slightly different individual stages of growth and development at the time of explantation as demonstrated in the same chapter. Therefore we used the combination

of ureteric bud count (using surface area at explantation for correction of developmental stage) and mRNA target expression to identify drug effects on early kidney development. Protein expression analysis was considered, but abandoned due to the large amount of embryo's required to obtain a sufficient sample size for analysis.

A.1.3 Rationale for dose selection

Doses selected for the *ex vivo* studies were calculated based on the clinical pediatric dose and were corrected for the drug plasma binding and volume of distribution as determined from the literature (29-50) to determine an appropriate tissue target dose. In addition, concentrations at a factor of 10 above and below were studied to account for calculation uncertainties and to investigate a potential dose response relationship.

A.2 Evaluation of short and long term effects in an *in vivo* model including extrauterine growth retardation

A.2.1 Selection of an extrauterine growth restriction model

Premature infants are prone to suffer from extra uterine growth restriction (EUGR). Literature reports frequencies up to 67% for infants born ≤ 32 weeks (51,52). A supply of nutrients in the right amount and consistency should be delivered to the newborn infant to mimic normal development occurring during the last trimester *in utero*. Although there are charts and literature available for clinicians to monitor a neonate's growth and identify the onset of EUGR, providing the optimal balance of nutrients remains a challenge (53-56). Therefore, despite careful monitoring of growth velocity, median weight of neonates at hospital discharge is often lower compared with the birth weight of term neonates of the same postmenstrual age (57).

The rodent species was chosen because kidney development, in contrary to human kidney development, is not yet completed at birth and continues up to 8-10 days after birth. This provides a window to dose the pups in a stage of ongoing nephrogenesis. Wistar rats were chosen because they are a strain commonly used in general toxicology work (which meant the ample availability of reference data) and by using an outbred strain, we may avoid characterizing a specific genotype only, by accident. More importantly, most EUGR models are established in the rat.

The method of inducing extrauterine growth retardation was selected out of multiple established models for 'malnourishment' of pups. Although all models lead to a similar phenotype, they are based on different mechanisms. Most models rely on low protein/low caloric feeding regime's for the maternal animals before or during lactation (58-60). Other models rely on adaptation of lactation to litter size. In this model a decrease of litter size with the right timing causes a reduction of mother milk production, which eventually leads to food restriction of the animals (61). We chose a model in which litter size enlargement leads to food restriction of all pups and induces

9

a general lower consumption of total energy. This approach avoids to trigger only specific pathways by creating an imbalance in the diet consistency. One may argue that survival of the fittest may occur in such a situation. The variation on the average litter body weight between normal food and food restricted animals was similar, however, and no increased mortality was observed in our studies performed in **chapters 5, 7 and 8**. Additionally this specific extrauterine growth retardation model specifically was known to reduce nephron numbers (59,62), which enabled this model for identifying additive or synergistic interactions between drug treatment and EUGR.

A.2.2 Short term endpoints of in vivo kidney development

A.2.2.1 Important mRNA targets

A panel of mRNA targets was selected to investigate direct effects of early postnatal treatment on different parts of the kidney (table 2). It included targets in the developmental pathway, transporters in different tubular sections and major players of the renin-angiotensin system. Although some targets may play a larger role in early kidney development we believe that the most important targets were captured in this list.

A.2.2.2 General parameters of kidney growth & development

To get a general idea of kidney development we examined kidney weight relative to body weight, although we have demonstrated in **Chapter 3** that (at least in adult humans) kidney size is a poor predictor for nephron count. Nevertheless, differences in size observed shortly after treatment can be indicative of a disturbed general growth process.

Besides morphometric parameters such as kidney weight, we investigated the balance between proliferating and apoptotic cells as organogenesis can be generally described in cycles of controlled proliferation and apoptosis. Stainings for Ki67 or caspase 3 were used to investigate these respective processes. Ki67 is a very general proliferating marker and stains all cells that are not in G0 phase (91). Caspase 3 stains all cells that are apoptotic, but does not indicate all intermediate phases of apoptosis. An alternative staining for the caspase 3 could have been a TUNEL staining, which detects DNA fragmentation. Both staining determines the same phase, however, and the overall outcome should therefore be similar. (92,93).

A.2.2.3 Glomerular numbers and generations

To identify glomerular numbers we used the current gold standard methodology being stereology with the physical fractionator/disector principle. This technique replaced the old acid maceration technique and has proven to be an invaluable tool in kidney research (94-97). Paraffin was used to embed the kidneys and glomerular count was determined. Morphometric parameters such as volume and density of

Table 2 Selected mRNA targets for *in vivo* studies and their role in kidney function/development

Gene symbol	Common name	role
Reference genes		
Actb	Beta actin	Cytoskeleton protein, used as high abundant reference transcript.
Hmbs	Hydroxymethylbilane synthase	Heme production enzyme, used as medium abundant reference transcript.
Developmental pathway genes		
Bmp7	Bone morphogenetic protein 7	Inhibits and induces ureteric bud growth and maintains nephrogenic progenitors (23-25).
Fgf7	Fibroblast growth factor 7	Promotes ureteric bud growth and branching formation (63).
Gdnf	Glial cell line-derived neurotrophic factor	Important player in ureteric bud differentiation processes. Loss results in agenesis (18-22).
Grf1a	Gdnf receptor alpha 1	Receptor for Ggnf in Ret signaling Loss results in agenesis (64).
Lim	Lim homeobox 1	Involved in multiple phases of kidney development from the intermediate mesoderm up to the mature glomerulus (65).
Hgf	Hepatocyte growth factor	Involved in branching morphogenesis and metanephric growth (66).
Ret	Ret proto oncogene	Receptor for Gdnf-family ligands. Loss results in agenesis (67,68).
Sox9	Sex determining region Y-Box-9	Controls epithelial branching by activating Ret (16,17).
Wnt4	Wingless-type MMTV integration site family, member 4	Regulates nephron progression to S-shaped body state (69,70).
Wt1	Wilms Tumor 1	Essential for mesenchyme survival (14,15).
Nphs1	Nephrin	Important in development and function of the glomerular filtration barrier (71,72).
Nphs2	Podocin	Important for correct slit diaphragm formation. knockout results proteinuria during the antenatal period and renal failure shortly after birth (73).
Transporters and water channels		
ENaC	Epithelial sodium channel	Located in the cortical- and outer medullary collecting duct and plays a major role in Na^{+} K^{+} homeostasis (74).
Nhe3	Sodium hydrogen exchanger 3	Localized to the proximal tubule, thin and thick loop of Henle and maintains sodium balance. Detected from late stages of S-shaped body (75).

Table 2 Continued

Gene symbol	Common name	role
Transporters and water channels		
Nhe8	Sodium hydrogen exchanger 8	Expressed in the proximal tubule where it contibutes to apical membrane transport to maintain sodium balance 76.
Ncc	Sodium-chloride symporter	Reabsorbs sodium and cloride from the tubular lumen of the distal convoluted tubule and important in maintaining blood pressure (77,78). Also target of hydrochlorothiazide.
Nkcc2	Na^{+}-K^{+}-Cl^{-} cotransporter	Reabsorbs sodium and potassium in the thick ascending limb and is important in maintaining blood pressure (78). Also target of furosemide.
Oat1	Organic anion transporter 1	Basolateral organic anion uptake transporter in the proximal tubule which is inhibited by most of our tested drugs (79-81).
Aqp1	Aquaporin 1	Present in both apical and basolateral membranes and most prominent in the proximal tubule (82) and functions as a water reabsorption channel (83).
Aqp4	Aquaporin 4	Basolateral waterchannel localized in the inner medullary collecting duct (84) and has a role in urine concentration.
Regulatory proteins		
Renin	Renin	Important protein for renal vasculature formation and regulation of fluid homeostasis (85).
Agtr1a	Angiotensin receptor type 1	Receptor for angiotensin 1 involved in postnatal vasculature development (86,87).
Agtr2	Angiotensin receptor 2	Receptor for angiotensin 2: Plays a counterregulatory role to the function of angiotensin 1 in renal development (88,89).
Casr	Calcium sensing receptor	Present throughout the nephron either luminal or basolateral and plays roles in Calcium/inorganic phosphate homeostasis, cation transport, urinary acidification and concentration, and renin release (90).

glomeruli were not obtained based on the observed shrinkage of the tissue. Paraffin embedding is known to cause deformation effects, however the precise nature of the deformation determines if the Cavalieri principle is still valid and unbiased observations may be made (98). Additionally we investigated the glomerular generations to determine whether a reduction in glomerular number was caused by a total arrest of

glomerular growth or whether the effect was diffuse and individual glomeruli are affected. The direct method of medullary ray counting as described by Hinchlife was used (99).

A.2.3 long-term endpoints of in vivo kidney development

A.2.3.1 Clinical biomarkers

A basic panel of established clinical markers for kidney damage or deteriorated kidney function (blood: sodium, potassium, magnesium, phosphate, urea and creatinine concentrations; urine: urea and creatinine concentrations, volume and osmolality; calculated creatinine and urea clearances) was included in our studies. Additionally we included NGAL to serve as a biomarker for kidney damage: Higher levels of NGAL in the urine could indicate proximal tubular damage (100), damage to the thick ascending limb and/or the collecting ducts (101). Additionally NGAL was found higher in patients with chronic kidney disease (102). Therefore it can be used as a marker for both acute as well as chronic kidney damage.

A.2.3.2 Blood pressure measurements

We selected radio- telemetry in free moving animals because this is the most accurate and animal friendly way of obtaining blood pressure as recommended by the Professional and Public Education of the American Heart Association Council (103). Its greatest advantage is that animals are in an awake state, the influence of circadian rhythms can be detected, animals can be followed-up for a long period of time and overall there will be a very low variation due to the large amount of data points collected. The biggest disadvantage is its high operational cost and relatively low throughput. We encountered difficulties maintaining stable and physiological meaningful signal intensities in our studies and switched to a non-invasive tail vein volume pressure measurement system to perform the study described in **chapter 8**. This method has some disadvantages compared with the radio telemetry method because it required the rats to be restrained and warmed for a certain period of time introducing stress as another variable and more variation was introduced as a result. Nonetheless the method is considered to be appropriate for identifying larger signal differences between groups (103).

A.2.3.3 Histopathological examination

At the end of the study period microscopic examination was performed to investigate the kidney for any persistent tubular or glomerular damage that may have occurred as a result of the interventions. Special attention was given to focal glomerular sclerosis, a condition one may expect based upon the hyperfiltration hypothesis by Brenner (104). To be able to detect this glomerular damage, Periodic Acid Schiffer staining was performed in addition to the classical heamatoxyline eosine staining. All slides were

evaluated by an experienced renal pathologist, who was blinded to all treatment groups except the non-food restricted control.

A.2.4. Rationale for dose selection
The dose levels and the treatment interval were chosen to be similar to clinical practice. Because the drugs are administered parentally in the clinic, we selected the intraperitoneal route to circumvent first-pass metabolism and approach the clinical route of administration. From the range of dose levels administered in neonates we always selected the highest dose level without correction for allometric scaling. We made this choice to enable easier comparison with other studies in the field. If this correction is applied we have a slight overestimation of the human neonatal dose. For gentamicin we studied the actual plasma levels in our rat pups and concluded that these were more or less within the same range as human neonates **(Chapter 5)**. This may however not be the case for all the drugs we have tested. Gentamicin does not have major metabolites and is excreted mostly unchanged as is hydrochlorothiazide. On the other hand NSAIDs and furosemide are metabolized which could result in a larger species difference. However it has been shown that the metabolic pathways are rather similar between rats and humans for these compounds (105-107). More importantly it is known that neonates of both species have a different and slower metabolism compared with the respective adults in whom kinetic data was generated (108-110). Therefore without having measured exposure levels in all our studies, this is an uncertainty and limitation of our research.

B General discussion

In the introduction of this thesis we formulated some specific questions we aimed to answer by the work performed in this thesis. In the paragraphs below I will elaborate on our findings to each of these questions.

B.1 Are currently used kidney morphometric parameters such as size or surface area good predictors for the state of kidney development?

In **chapter 3 and 4** we investigated kidney morphometric parameters such as size or surface area and their prediction for the state of kidney development in humans and mice respectively. Both studies indicated that these kind of measurements are generally poor predictors for the actual state of kidney development as determined by glomerular counting or ureteric bud branching. Nonetheless metanephron surface area at the time of kidney explantation can partially predict the amount of bud formation after 24 hours, as we discussed in **chapter 4** and can therefore be used as a marker for the state of development at the time of explantation.

The field of imaging is advancing rapidly and intravital imaging of the kidney by means of multiphoton microscopy is slowly becoming a standard (111). Although these are large improvements for researchers, this does not directly translate to the clinic. There, the advances in magnetic resonance imaging resolution are more closely followed. Bennet et al. were able to visualize glomeruli within a 10% range from stereological assessment in the rat kidney in a magnetic field of 11.7 Tesla (112). It is definitely possible that in the future such methods may find an application in the clinic. However until that moment no easy way of identifying structural kidney developmental damage in human individuals exists.

B.2 What is the impact of drug treatment on early- and late kidney development? How does such an impact affect the function of the kidney in later life? Are there alternatives for drugs used in the current first-line treatment?

One of the main aims of this thesis was to study clinical dose levels of frequently used drugs and identify whether these cause subtle kidney toxicity that would not have been noted in regulatory (general) toxicity studies, but may result in long term sequelae. Drugs from three different classes (antibiotics, NSAIDs and diuretics) were studied and the results and implications are described in the following paragraphs.

B.2.1 Impact of antibiotics

We showed that the aminoglycoside gentamicin did not affect the rudimentary kidney development in the mouse **(Chapter 4)**. This was an unexpected finding

because Gilbert et al showed effects of gentamicin in the past, be it at a higher dose level (5,6). The subsequent in vivo study we performed at a later stage of development in **chapter 5** did also not identify any effect of gentamicin on kidney development, but was in line with the findings of other researchers in the field (113, 114). Overall no impact of gentamicin on kidney development was noted at clinically relevant dose levels.

The cephalosporin ceftazidime showed more potential for disturbing normal kidney development. Most cephalosporins are less associated with kidney toxicity compared with the aminoglycoside drug class, but can have effects on the proximal tubule (115). However the cephalosporin ceftriaxone was reported to reduce the number of glomeruli in an ex vivo model of early kidney development (116). Our experiments described in **chapter 4** showed down-regulation in expression of *Fgf8* and *Gdnf,* two important genes in nephrogenesis, and reduced ureteric bud formation in metanephroi. *Fgf8* is important in cell survival of renal progenitor cells, which are an important source of Gdnf production (26,27). Gdnf is considered the major protein involved in ureteric outbud growth and loss results in agenesis. However even a decrease was reported to impact glomerulogenesis (21). It may be questioned whether a general apoptosis process was ongoing, but unchanged expression levels in *Bmp7, Caspase 3 and Caspase 9* mRNA targets did not indicate general progenitor cell loss or large apoptotic insults (24,117). Although these results were obtained in a developmental phase of less importance for neonatal treatment, it is important to consider when prescribing this drug to pregnant women. Ceftazidime can readily pass the placenta barrier and the dose levels administered to the metanephroi cultures are comparable with adult human dose levels (118). These findings alone do not indicate that the pregnant population is at risk. For once there can be species dependency as we have used mouse embryos. Secondly our *ex vivo* model is a static model and does not account for drug elimination. Therefore the actual intracellular concentration in our model may be much higher than would be the case in an *in vivo* situation, where the equilibrium might be completely different. This reasoning is supported by the data provided in the FDA label for this compound. A 40-fold of the human dose was dosed to pregnant rats without observed abnormalities in the pups. However no pregnant women were included in the clinical trials.

The ceftazidime findings of these *ex vivo* studies were not confirmed in our *in vivo* study **(Chapter 5)** where *Gdnf* expression was not affected and no direct effect was noted on glomerulogenesis in the kidney. The only effect of ceftazidime noted was a slight down-regulation of renin expression, however it is doubted whether this has implications for development.

We selected ceftazidime as a possible alternative treatment for gentamicin, however the results obtained in **chapter 4 and 5** showed that the potential of disrupting nephrogenesis and kidney development is higher for ceftazidime compared with gentamicin. Another broad spectrum alternative meropenem was only tested in our *ex vivo* model and did not show any effects on early nephrogenesis. The effect of meropenem on kidney development *in vivo* has yet to be determined. Putting all these results into perspective it shows that the acute toxicity profile of gentamicin is of more concern in the clinic, compared with the concern of disturbing organ development and based on our data there is no direct reason to change existing treatment protocols.

B.2.2 Impact of non-steroidal anti-inflammatory drugs

At present, there is an ongoing debate whether indomethacin or ibuprofen is safer to use as treatment for patent ductus arteriosus. There are reports of toxicity for both compounds and multiple studies investigated or compared the safety profiles resulting in either a similar profile or a slightly less favorable profile for indomethacin (119-126). All these papers studied acute endpoints (spontaneous intestinal perforation, necrotizing enterocolitis, renal blood flow changes or dysfunction, death), but less is known on subtle changes which may influence kidney programming. In the *ex-vivo* studies described in **chapter 6** we showed that both compounds do not seem to affect the ureteric budding process and did not find a potential for impairing nephrogenesis. We anticipated effects because the cyclo oxygenase systems, especially COX-2, are regarded important in kidney development (127-131). However the time point of our experiment may have been too early to evaluate a full effect of both NSAID compounds as the most prominent role of the cyclo oxygenase system may be found at the vascular developmental phase, which starts around embryonic day 16 (130).

In the *in vivo* study, described in **chapter 7**, ibuprofen, but not indomethacin, showed some slight potential to reduce glomerular numbers. These findings were different compared with the earlier work performed by Kent et al, who did not observe differences in glomerular numbers (114), but detected glomerular alterations in the form of extensive foot process effacement for both NSAIDs after a 3 or 5 day neonatal treatment period (132). A more recent publication of this group confirmed the absense of findings on glomerular numbers 30 days after dosing, but did show a decrease in glomerular numbers 6 months after a 3-5 day postnatal indomethacin, but not ibuprofen treatment (133).

Although our study showed a slight decrease on glomerular numbers after ibuprofen treatment, no delayed effects were noted in other measured parameters, but for a slight tendency to an increased NGAL concentration 9 months after treatment.

The current data on effects of ibuprofen and indomethacin regarding to kidney development remain sparse and are not in full agreement. There are not very large differences between treatment protocols of different study groups, however all changes together may tip the balance from coping to toxicity for either compound. More research is therefore required to provide a definite answer whether these two drugs have an effect on kidney development at low clinical dose levels.

B.2.3 Impact of diuretics

Both our performed *ex vivo* **(Chapter 6)** and *in vivo* **(Chapter 8)** studies did not demonstrate an effect of furosemide or hydrochlorothiazide dosed in a clinical range on either early or late stage kidney development. These results were somewhat expected because the described effects regarding furosemide toxicity in human neonates are few (134). Malie et al, however identified furosemide to affect kidney development after prenatal exposure, but the dose levels used in their experiments were much higher compared with the clinically used dose levels (135,136). Concerning hydrochlorothiazide, there could have been an effect on the renin angiotensin system which is debated (137-139), but our results do not support such a link.

B.3 Does an additional stressor in the form of extrauterine growth retardation indeed modulate drug toxicity according to the two-hit hypothesis?

We confirmed the presence of extra-uterine growth retardation in our *in vivo* studies, described in **chapters 5, 7, and 8** based on the significantly decreased body weight of pups in the enlarged litters. Additionally we showed that the intervention caused a 7.5 to 20% reduction of glomeruli in a single right kidney. This was lower than expected based on the earlier publication of Schreuder et al., which showed a 25% reduction (62). The explanation for this different observation may be related to the kidney sampled. In our studies the right kidney was examined contrary to the left kidney in the study of Schreuder et al. There are indications that the left kidney is more prone to abnormalities compared with the right kidney (140,141). Therefore glomerular numbers may be affected differently between the kidneys as well.

Interestingly we did not see any clear long term sequelae of these reductions in glomeruli in the food restricted group. This could have remained undetected because our follow-up time was too short. According to Sengupta et al. at the time of necropsy our rats were still relatively young compared with human years (approximately 25 years old) (142). At a comparable age in humans, effects on human blood pressure after intrauterine growth restriction were substantial from a clinically point of view, but small in the absolute sense (143). Therefore our used measuring methods may not have had enough discriminative power to detect changes at this stage.

A second explanation may be that the glomerular reduction was too small to lead to significant hyper filtration or still has overcapacity for its normal function. This means there is a threshold to be exceeded before showing negative effects. This supports the multi-hit toxicity theory in chronic kidney disease, where the adult kidney needs multiple, prolonged, or toxic stressors in combination with a congenital defect before the function of the organ is reduced to a state as noted in renal disease. Significant effects in renal function may therefore only be apparent when challenged in adulthood.

We showed that postnatal food restriction has a direct structural effect on kidney development, and we hypothesized that it may have synergistic effects if combined with other interventions. However we could not find evidence that this occurred. Glomerular numbers were not substantially more affected in any of the groups combining drug treatment and food restriction and therefore the combination did not lead to further developmental impairment.

Although we did not find any direct evidence for additive effects or synergism between food restriction and drug treatment in our studies, the multi-hit hypothesis for congenital defects remains plausible. Initial work of our group identified a 20% decrease after gentamicin treatment in combination with food restriction (not published). These rat pups were suffering from an unintentional additional stressor in the form of heat stress (approximately 30-35 °C) due to an inadequately performing climate control system. However the combination of all these stressors also resulted in more than average mortality among the pups and resulted in a poorly controlled study, hence this was not published. However when we repeated this study, under standard laboratory conditions (without heat stress) in **chapter 5**, the gentamicin effects could not be reproduced. Whether the reason was the absence of heat stress or a better standardized study remains to be determined. A separate study will be needed to confirm this, but it suggests that the interaction of the specific environmental circumstances, the specific stressor type and the total amount of stressors may be important for the final congenital effect on glomerulogenesis.

B.4 Translation of the research outcomes to the clinic

The findings of ceftazidime in our *ex vivo* studies raises cause for concern for the use of ceftazidime in pregnant women. Currently no warning label is present for pregnancy as these findings were not identified in the regulatory toxicity studies. Nonetheless the current strategy for ceftazidime usage in pregnant women is to avoid these drugs unless necessary, which seems a sufficient precaution considering the data available. We did not observe clear effects of drugs affecting kidney development in neonatal rats with or without food restriction, this does not mean that there is no cause for concern. We only studied one additional stressor, which is a simplification of the actual situation in the neonatal care unit, where the number of stressors is ample.

Each of these stressors may have a different interaction with the other stressors and therefore it is important that these interactions are investigated.
Additionally our experiments were performed in neonatal, but healthy rats. This situation is again quite different from the clinical practice, where the neonate suffers from an actual disease condition for which he requires the specific drug treatment. It is difficult to predict whether a neonate in a disease state will react similarly to the additional stressors compared with a healthy neonate.

Food restriction in rats was identified in three separate experiments to cause a decrease in glomeruli compared with normally fed rats. Although clinicians are already well aware that extra uterine growth retardation should be limited as much as possible, the findings in this thesis show once again the importance of doing so. This is not an easy task because the required drug treatments often have a negative effect on the nutrient status. By example NSAID's may cause gastrointestinal side effects.

There remains no easy way in determining whether kidney development in children is disturbed. Morphometric data such as size or surface area do not predict whether structural loss of nephrons has occurred. But even if we could determine the amount of nephrons, the reported large intra-human variation renders it difficult to evaluate the result of such measurements. Advancements in imaging techniques may overcome this hurdle in the future, by counting glomeruli *in vivo* and establishing a more standardized reference number of the general population. To accomplish this advancement, stronger magnetic fields would have to be used in the clinic and specially developed contrast agents such as the recently demonstrated polymer-based contrast agent by Hlushshuk et al will have to be proven safe in general toxicity studies (144).

B.5 Conclusion

In conclusion, all drugs tested in this manuscript (gentamicin, ceftazidime, meropenem, ibuprofen, indomethacin, furosemide and hydrochlorothiazide), when dosed in a clinically similar dose, did not cause a direct toxic effect on nephrogenesis or glomerulogenesis except for ceftazidime, which was toxic in early kidney development. Postnatal food restriction by litter enlargement results in a significant decrease of glomeruli, but did not enhance or modify the effect of drug treatments nor showed long-term sequelae. Therefore a two-hit interaction between these stressors was not observed. Finally kidney surface area or size are poor markers for renal development and should not be used.

C Future perspectives & recommendations

1. The mechanisms behind decreased glomerular numbers after extrauterine growth retardation should be better understood. Although we could identify that food restriction has a direct impact on many kidney parameters, we did not find a lead to a proposed mechanism. The investigated kidney developmental- and transporter mRNA expression were not affected by food restriction and the findings noted on cytotoxicity were not convincing. A big-data approach including proliferation/apoptosis, developmental and energy metabolism pathways at multiple time points would be a logical next step to identify the disturbed processes. By elucidating the responsible pathways, drugs and environmental stressors may be tested in a more targeted approach.
2. It will be interesting to further evaluate the possibility of a programming effect as there were no clear long-term sequelae of the reduced glomerular numbers in a healthy state. Additional studies may be performed with a longer follow-up time to study the effect at increasing age, where more 'wear and tear' damage is expected. Another possibility is to challenge the kidney function by putting it under great stress. One could combine extrauterine food restriction with subsequent challenges of different drugs that increase the workload of the kidney or are slightly toxic and observe whether the kidney is still able to cope and responds similarly to a normally developed kidney.
3. A follow-up experiment may be performed to study the risk of early pregnancy ceftazidime treatment. Although the regulatory studies showed that a severe effect on nephrogenesis as was noted in our experiments does not occur *in vivo* in rats, a dedicated study in pregnant rats to evaluate the effect on glomerular numbers would be informative to determine ceftazidime's potential of subtle developmental toxicity and programming effects.
4. Morphologically the left- and right kidneys are not completely the same and left-right susceptibility differences are described in the literature. Currently these differences are poorly understood and we need to generate more data to understand better if these susceptibility differences are true and in which situations they become relevant. To gather this data it will be important to always study both kidneys for each end-point in future research projects. In many study designs, including ours, kidneys are examined unilateraly for separate end points or are even used as a control-treatment pair. These kinds of designs should be avoided as much as possible or if unavoidable at least the influence of left-right susceptibility should be described. Additionally the time point at which the kidneys start to differentiate in terms of susceptibility should be determined, which can be done with time-related experiments.

5. In the recent years many researchers were involved in investigating nephrogenesis and possible programming effects. However all studies were performed in animals or tissues because the tools to evaluate these processes in the clinic are currently lacking. Although recent advances in imaging techniques and contrasting agents are very promising, more effort and research is required before these can actually be adapted to the clinic. Continuing this field of research and finding the technical solutions will be paramount for fully understanding the process of nephrogenesis in humans. Stem cell research may also greatly contribute to this understanding, but may also be used to better evaluate the translatability of all animal research.
6. In this thesis we studied a two-hit scenario of different drugs and extra-uterine growth retardation, which is a simplification of the real clinical environment for neonates. To fully study the impact on nephrogenesis more complex studies are needed where multiple stressors are introduced at the same time and interactions can be described both in healthy and in disease models. This will allow ranking of stressors in order of importance for development and will give clinicians tools to build risk mitigation strategies.

Reference list

1. Cullen-McEwen LA, Fricout G, Harper IS, Jeulin D, Bertram JF. Quantitation of 3D ureteric branching morphogenesis in cultured embryonic mouse kidney. *Int J Dev Biol* 2002;46(8): 1049-1055.
2. Chan SK, Riley PR, Price KL, McElduff F, Winyard PJ, Welham SJ, Woolf AS, Long DA. Corticosteroid-induced kidney dysmorphogenesis is associated with deregulated expression of known cystogenic molecules, as well as Indian hedgehog. Am J Physiol Renal Physiol 2010;298(2): F346-356.
3. Rosines E, Sampogna RV, Johkura K, Vaughn DA, Choi Y, Sakurai H, Shah MM, Nigam SK. Staged in vitro reconstitution and implantation of engineered rat kidney tissue. *Proc Natl Acad Sci USA* 2007;104(52): 20938-20943.
4. Rosines E, Johkura K, Zhang X, Schmidt HJ, Decambre M, Bush KT, Nigam SK. Constructing kidney-like tissues from cells based on programs for organ development: toward a method of in vitro tissue engineering of the kidney. *Tissue Eng Part A* 2010;16(8): 2441-2455.
5. Gilbert T, Gaonach S, Moreau E, Merlet-Benichou C. Defect of nephrogenesis induced by gentamicin in rat metanephric organ culture. *Lab Invest* 1994;70(5): 656-666.
6. Gilbert T, Cibert C, Moreau E, Geraud G, Merlet-Benichou C. Early defect in branching morphogenesis of the ureteric bud in induced nephron deficit. *Kidney Int* 1996;50(3): 783-795.
7. Singh RR, Moritz KM, Bertram JF, Cullen-McEwen LA. Effects of dexamethasone exposure on rat metanephric development: in vitro and in vivo studies. *Am J Physiol Renal Physiol* 2007;293(2): F548-554.
8. Srinivas S, Goldberg MR, Watanabe T, D'Agati V, al-Awqati Q, Costantini F. Expression of green fluorescent protein in the ureteric bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis. *Dev Genet* 1999;24(3-4): 241-251.
9. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999;21(1): 70-71.
10. Hohenstein P, Slight J, Ozdemir DD, Burn SF, Berry R, Hastie ND. High-efficiency Rosa26 knock-in vector construction for Cre-regulated overexpression and RNAi. *Pathogenetics* 2008; 1(1): 3.
11. Bullock SL, Johnson TM, Bao Q, Hughes RC, Winyard PJ, Woolf AS. Galectin-3 modulates ureteric bud branching in organ culture of the developing mouse kidney. *J Am Soc Nephrol* 2001;12(3): 515-523.
12. Saxen L, Sariola H. Early organogenesis of the kidney. *Pediatr Nephrol* 1987;1(3): 385-392.
13. RichardsonL, Venkataraman S, Stevenson P, Yang Y, Burton N, Rao J, Fisher M, Baldock RA, Davidson DR, Christiansen JH. EMAGE mouse embryo spatial gene expression database: 2010 update. *Nucleic Acids Res* 2010;38(Database issue): D703-709.
14. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R. WT-1 is required for early kidney development. *Cell* 1993;74(4): 679-691.
15. Dehbi M, Ghahremani M, Lechner M, Dressler G, Pelletier J. The paired-box transcription factor, PAX2, positively modulates expression of the Wilms' tumor suppressor gene (WT1). *Oncogene* 1996;13(3): 447-453.
16. Airik R, Trowe MO, Foik A, Farin HF, Petry M, Schuster-Gossler K, Schweizer M, Scherer G, Kist R, Kispert A. Hydroureternephrosis due to loss of Sox9-regulated smooth muscle cell differentiation of the ureteric mesenchyme. *Hum Mol Genet* 2010;19(24): 4918-4929.
17. Reginensi A, Clarkson M, Neirijnck Y, Lu B, Ohyama T, Groves AK, Sock E, Wegner M, Costantini F, Chaboissier MC, Schedl A. SOX9 controls epithelial branching by activating RET effector genes during kidney development. *Hum Mol Genet* 2011;20(6): 1143-1153.
18. Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A. Renal and neuronal abnormalities in mice lacking GDNF. *Nature* 1996;382(6586): 76-79.
19. Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* 1996;382(6586): 73-76.
20. Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA, Barbacid M. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* 1996;382(6586): 70-73.
21. Cullen-McEwen LA, Kett MM, Dowling J, Anderson WP, Bertram JF. Nephron number, renal function, and arterial pressure in aged GDNF heterozygous mice. *Hypertension* 2003;41(2): 335-340.

22. Costantini, F. GDNF/Ret signaling and renal branching morphogenesis: From mesenchymal signals to epithelial cell behaviors. *Organogenesis* 2010;6(4): 252-262.
23. Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G. BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev* 1995;9(22): 2808-2820.
24. Dudley AT, Godin RE, Robertson EJ. Interaction between FGF and BMP signaling pathways regulates development of metanephric mesenchyme. *Genes Dev* 1999;13(12): 1601-1613.
25. Goncalves A, Zeller R. Genetic analysis reveals an unexpected role of BMP7 in initiation of ureteric bud outgrowth in mouse embryos. *PLoS One* 2011;6(4): e19370.
26. Grieshammer U, Cebrian C, Ilagan R, Meyers E, Herzlinger D, Martin GR. FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. *Development* 2005;132(17): 3847-3857.
27. Perantoni AO, Timofeeva O, Naillat F, Richman C, Pajni-Underwood S, Wilson C, Vainio S, Dove LF, Lewandoski M. Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. *Development* 2005;132(17): 3859-3871.
28. Sebinger DD, Unbekandt M, Ganeva VV, Ofenbauer A, Werner C, Davies JA. A novel, low-volume method for organ culture of embryonic kidneys that allows development of cortico-medullary anatomical organization. *PLoS One* 2010; 5(5): e10550.
29. Jensen KM. Determination of indomethacin in serum by an extractive alkylation technique and gas-liquid chromatography. *J Chromatogr* 1978;153(1): 195-202.
30. Peterson RG, Simmons MA, Rumack BH, Levine RL and Brooks JG. Pharmacology of furosemide in the premature newborn infant. *J Pediatr* 1980;97(1): 139-143.
31. Lam YW, Duroux MH, Gambertoglio JG, Barriere SL, Guglielmo BJ. Effect of protein binding on serum bactericidal activities of ceftazidime and cefoperazone in healthy volunteers. *Antimicrob Agents Chemother* 1988;32(3): 298-302.
32. Weninger M, Pollak A, Salzer-Muhar U, Vergesslich KA, Salzer HR. Pharmacokinetics of intra-arterial indomethacin treatment for patent ductus arteriosus. *Eur J Pediatr* 1989;149(2): 138-140.
33. van den Anker JN, Schoemaker RC, Hop WC, van der Heijden BJ, Weber A, Sauer PJ, Neijens HJ, de Groot R. Ceftazidime pharmacokinetics in preterm infants: effects of renal function and gestational age. *Clin Pharmacol Ther* 1995;58(6): 650-659.
34. Aranda JV, Varvarigou A, Beharry K, Bansal R, Bardin C, Modanlou H, Papageorgiou A, Chemtob S. Pharmacokinetics and protein binding of intravenous ibuprofen in the premature newborn infant. *Acta Paediatr* 1997;86(3): 289-293.
35. Craig WA. The pharmacology of meropenem, a new carbapenem antibiotic. *Clin Infect Dis* 1997;24 Suppl 2: S266-275.
36. Bailey DN, Briggs JR. Gentamicin and tobramycin binding to human serum in vitro. *J Anal Toxicol* 2004;28(3): 187-189.
37. Smyth JM, Collier PS, Darwish M, Millership JS, Halliday HL, Petersen S, McElnay JC. Intravenous indometacin in preterm infants with symptomatic patent ductus arteriosus. A population pharmacokinetic study. *Br J Clin Pharmacol* 2004;58(3): 249-258.
38. Hirt D, Van Overmeire B, Treluyer JM, Langhendries JP, Marguglio A, Eisinger MJ, Schepens P, Urien S. An optimized ibuprofen dosing scheme for preterm neonates with patent ductus arteriosus, based on a population pharmacokinetic and pharmacodynamic study. *Br J Clin Pharmacol* 2008;65(5): 629-636.
39. Basow, D. ed. (2013a). Furosemide: Drug information. UpToDate. Waltham, MA. www.uptodate.com
40. Basow, D. ed. (2013b). Hydrochlorothiazide:drug information. UpToDate. Waltham,MA. www.uptodate.com
41. Basow, D. ed. (2013c). Ibuprofen: Drug information. UpToDate.Waltham, MA. www.uptodate.com
42. Basow, D. ed. (2013d). Indomethacin: Drug information. UpToDate. Waltham, MA. www.uptodate.com
43. Basow, D. ed. (2013e). Meropenem: pediatric drug information. UpToDate. Waltham, MA. www.uptodate.com
44. Dutch Healt Institute (2013a). [Ceftazidime]. www.fk.cvz.nl/preparaatteksten/c/ceftazidim.asp.
45. Dutch Health Institute (2013b).[Furosemide.]. www.fk.cvz.nl/preparaatteksten/f/furosemide.asp)
46. Dutch Health Institute (2013c). [Gentamicin]. (www.fk.cvz.nl/preparaatteksten/g/gentamicine%20(systemisch).asp.)

47. Dutch Health Institute (2013d).[Hycrochloorthiazide.]. www.fk.cvz.nl/preparaatteksten/h/hydrochloorthiazide.asp)
48. Dutch Health Institute (2013e).[Ibuprofen.]. www.fk.cvz.nl/preparaatteksten/i/ibuprofen.asp)
49. Dutch Health Institute (2013f) .[Indomethacine.]. www.fk.cvz.nl/preparaatteksten/i/indomethacine.asp)
50. Dutch Health Institute (2013g).[Meropenem.]. www.fk.cvz.nl/preparaatteksten/m/meropenem.asp
51. Sakurai M, Itabashi K, Sato Y, Hibino S, Mizuno K. Extrauterine growth restriction in preterm infants of gestational age < or =32 weeks. *Pediatr Int* 2008;50(1): 70-75.
52. Lima PA, Carvalho M, Costa AC, Moreira ME. Variables associated with extra uterine growth restriction in very low birth weight infants. *J Pediatr (Rio J)* 2014;90(1): 22-27.
53. Wright K Dawson JP, Fallis D, Vogt E, Lorch V. New postnatal growth grids for very low birth weight infants. *Pediatrics* 1993;91(5): 922-926.
54. Clark RH, Wagner CL, Merritt RJ, Bloom BT, Neu J, Young TE, Clark DA. Nutrition in the neonatal intensive care unit: how do we reduce the incidence of extrauterine growth restriction? *J Perinatol* 2003;23(4): 337-344.
55. Niklasson A, Albertsson-Wikland K. Continuous growth reference from 24th week of gestation to 24 months by gender. *BMC Pediatr* 2008;8: 8.
56. Horemuzova E, Soder O, Hagenas L. Growth charts for monitoring postnatal growth at NICU of extreme preterm-born infants. *Acta Paediatr* 2012;101(3): 292-299.
57. Ehrenkranz RA, Younes N, Lemons JA, Fanaroff AA, Donovan EF, Wright LL, Katsikiotis V, Tyson JE, Oh W, Shankaran S, Bauer CR, Korones SB, Stoll BJ, Stevenson DK, Papile LA. Longitudinal growth of hospitalized very low birth weight infants. *Pediatrics* 1999;104(2 Pt 1): 280-289.
58. Langley-Evans SC, Welham SJ, Jackson AA. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sci* 1999;64(11): 965-974.
59. Huizinga CT, Engelbregt MJ, Rekers-Mombarg LT, Vaessen SF, Delemarre-van de Waal HA, Fodor M. Ligation of the uterine artery and early postnatal food restriction - animal models for growth retardation. *Horm Res* 2004;62(5): 233-240.
60. de Oliveira JC, Grassiolli S, Gravena C, de Mathias PC. Early postnatal low-protein nutrition, metabolic programming and the autonomic nervous system in adult life. *Nutr Metab (Lond)* 2012;9(1): 80.
61. O'Dowd R, Kent JC, Moseley JM, Wlodek ME. Effects of uteroplacental insufficiency and reducing litter size on maternal mammary function and postnatal offspring growth. *Am J Physiol Regul Integr Comp Physiol* 2008;294(2): R539-548.
62. Schreuder MF, Nyengaard JR, Remmers F, van Wijk JA, Delemarre-van de Waal HA. Postnatal food restriction in the rat as a model for a low nephron endowment. *Am J Physiol Renal Physiol* 2006;291(5): F1104-1107.
63. Qiao J, Uzzo R, Obara-Ishihara T, Degenstein L, Fuchs E, Herzlinger D. FGF-7 modulates ureteric bud growth and nephron number in the developing kidney. *Development* 1999;126(3): 547-554.
64. Cacalano G, Farinas I, Wang LC, Hagler K, Forgie A, Moore M, Armanini M, Phillips H, Ryan AM, Reichardt LF, Hynes M, Davies A, Rosenthal A. GFRalpha1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* 1998;21(1): 53-62.
65. Kobayashi A, Kwan KM, Carroll TJ, McMahon AP, Mendelsohn CL, Behringer RR. Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. *Development* 2005;132(12): 2809-2823.
66. Santos OF, Barros EJ, Yang XM, Matsumoto K, Nakamura T, Park M, Nigam SK. Involvement of hepatocyte growth factor in kidney development. *Dev Biol* 1994;163(2): 525-529.
67. Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* 1994;367(6461): 380-383.
68. Schuchardt A, D'Agati V, Pachnis V, Costantini F. Renal agenesis and hypodysplasia in ret-k- mutant mice result from defects in ureteric bud development. *Development* 1996;122(6): 1919-1929.
69. Stark K, Vainio S, Vassileva G, McMahon AP. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* 1994;372(6507): 679-683.
70. Kispert A, Vainio S, McMahon AP. Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. *Development* 1998;125(21): 4225-4234.

71. Holzman LB, St John PL, Kovari IA, Verma R, Holthofer H, Abrahamson DR. Nephrin localizes to the slit pore of the glomerular epithelial cell. *Kidney Int* 1999;56(4): 1481-1491.
72. Ruotsalainen V, Patrakka J, Tissari P, Reponen P, Hess M, Kestila M, Holmberg C, Salonen R, Heikinheimo M, Wartiovaara J, Tryggvason K, Jalanko H. Role of nephrin in cell junction formation in human nephrogenesis. *Am J Pathol* 2000;157(6): 1905-1916.
73. Roselli S, Heidet L, Sich M, Henger A, Kretzler M, Gubler MC, Antignac C. Early glomerular filtration defect and severe renal disease in podocin-deficient mice. *Mol Cell Biol* 2004;24(2): 550-560.
74. Butterworth MB. Regulation of the epithelial sodium channel (ENaC) by membrane trafficking. *Biochim Biophys Acta* 2010;1802(12): 1166-1177.
75. Biemesderfer D, Rutherford PA, Nagy T, Pizzonia JH, Abu-Alfa AK, Aronson PS. Monoclonal antibodies for high-resolution localization of NHE3 in adult and neonatal rat kidney. *Am J Physiol* 1997;273(2 Pt 2): F289-299.
76. Goyal S, Vanden Heuvel G, Aronson PS. Renal expression of novel Na+/H+ exchanger isoform NHE8. *Am J Physiol Renal Physiol* 2003;284(3): F467-473.
77. Mastroianni N, De Fusco M, Zollo M, Arrigo G, Zuffardi O, Bettinelli A, Ballabio A, Casari G. Molecular cloning, expression pattern, and chromosomal localization of the human Na-Cl thiazide-sensitive cotransporter (SLC12A3). *Genomics* 1996;35(3): 486-493.
78. Ji W, Foo JN, O'Roak BJ, Zhao H, Larson MG, Simon DB, Newton-Cheh C, State MW, Levy D, Lifton RP. Rare independent mutations in renal salt handling genes contribute to blood pressure variation. *Nat Genet* 2008;40(5): 592-599.
79. Hosoyamada M, Sekine T, Kanai Y, Endou H. Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney. *Am J Physiol* 1999;276(1 Pt 2): F122-128.
80. Ljubojevic M, Balen D, Breljak D, Kusan M, Anzai N, Bahn A, Burckhardt G, Sabolic I. Renal expression of organic anion transporter OAT2 in rats and mice is regulated by sex hormones. *Am J Physiol Renal Physiol* 2007;292(1): F361-372.
81. VanWert AL, Gionfriddo MR, Sweet DH. Organic anion transporters: discovery, pharmacology, regulation and roles in pathophysiology. *Biopharm Drug Dispos* 2010;31(1): 1-71.
82. Nielsen S, Smith BL, Christensen EI, Knepper MA, Agre P. CHIP28 water channels are localized in constitutively water-permeable segments of the nephron. *J Cell Biol* 1993;120(2): 371-383.
83. Ma T, Yang B, Gillespie A, Carlson EJ, Epstein CJ, Verkman AS. Severely impaired urinary concentrating ability in transgenic mice lacking aquaporin-1 water channels. *J Biol Chem* 1998;273(8): 4296-4299.
84. Terris J, Ecelbarger CA, Marples D, Knepper MA, Nielsen S. Distribution of aquaporin-4 water channel expression within rat kidney. *Am J Physiol* 1995;269(6 Pt 2): F775-785.
85. Sequeira-Lopez ML, Nagalakshmi VK, Li M, Sigmund CD, Gomez RA. Vascular versus tubular renin: role in kidney development. *Am J Physiol Regul Integr Comp Physiol* 2015;309(6): R650-657.
86. Tufro-McReddie A, Romano LM, Harris JM, Ferder L, Gomez RA. Angiotensin II regulates nephrogenesis and renal vascular development. *Am J Physiol* 1995;269(1 Pt 2): F110-115.
87. Madsen K, Marcussen N, Pedersen M, Kjaersgaard G, Facemire C, Coffman TM, Jensen BL. Angiotensin II promotes development of the renal microcirculation through AT1 receptors. *J Am Soc Nephrol* 2010;21(3): 448-459.
88. Meffert S, Stoll M, Steckelings UM, Bottari SP, Unger T. The angiotensin II AT2 receptor inhibits proliferation and promotes differentiation in PC12W cells. *Mol Cell Endocrinol* 1996;122(1): 59-67.
89. Maric C, Aldred GP, Harris PJ, Alcorn D. Angiotensin II inhibits growth of cultured embryonic renomedullary interstitial cells through the AT2 receptor. *Kidney Int* 1998;53(1): 92-99.
90. Riccardi D, Brown EM. Physiology and pathophysiology of the calcium-sensing receptor in the kidney. *Am J Physiol Renal Physiol* 2010;298(3): F485-499.
91. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984;133(4): 1710-1715.
92. Negoescu A, Guillermet C, Lorimier P, Brambilla E, Labat-Moleur F. Importance of DNA fragmentation in apoptosis with regard to TUNEL specificity. *Biomed Pharmacother* 1998;52(6): 252-258.
93. Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, Brambilla C, Brambilla E. In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. *J Histochem Cytochem* 1996;44(9): 959-968.

94. Nyengaard JR. Stereologic methods and their application in kidney research. *J Am Soc Nephrol* 1999;10(5): 1100-1123.
95. Lodrup AB, Karstoft K, Dissing TH, Pedersen M, Nyengaard JR. Kidney biopsies can be used for estimations of glomerular number and volume: a pig study. *Virchows Arch* 2008;452(4): 393-403.
96. Cullen-McEwen LA, Armitage JA, Nyengaard JR, Bertram JF. Estimating nephron number in the developing kidney using the physical disector/fractionator combination. *Methods Mol Biol* 2012;886: 109-119.
97. Bertram JF, Cullen-McEwen LA, Egan GF, Gretz N, Baldelomar E, Beeman SC, Bennett KM. Why and how we determine nephron number. *Pediatr Nephrol* 2014;29(4): 575-580.
98. Dorph-Petersen KA, Nyengaard JR, Gundersen HJ. Tissue shrinkage and unbiased stereological estimation of particle number and size. *J Microsc* 2001;204(Pt 3): 232-246.
99. Hinchliffe SA, Sargent PH, Chan YF, van Velzen D, Howard CV, Hutton JL, Rushton DI. "Medullary ray glomerular counting" as a method of assessment of human nephrogenesis. *Pathol Res Pract* 1992;188(6): 775-782.
100. Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, Yang J, Barasch J, Devarajan P. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 2003;14(10): 2534-2543.
101. Paragas N, Qiu A, Zhang Q, Samstein B, Deng SX, Schmidt-Ott KM, Viltard M, Yu W, Forster CS, Gong G, Liu Y, Kulkarni R, Mori K, Kalandadze A, Ratner AJ, Devarajan P, Landry DW, D'Agati V, Lin CS, Barasch J. The Ngal reporter mouse detects the response of the kidney to injury in real time. *Nat Med* 2011;17(2): 216-222.
102. Bolignano D, Lacquaniti A, Coppolino G, Donato V, Campo S, Fazio MR, Nicocia G, Buemi M. Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. *Clin J Am Soc Nephrol* 2009;4(2): 337-344.
103. Kurtz TW, Griffin KA, Bidani AK, Davisson RL, Hall JE, Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure. Recommendations for blood pressure measurement in humans and experimental animals: part 2: blood pressure measurement in experimental animals: a statement for professionals from the Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research. *Arterioscler Thromb Vasc Biol* 2005;25(3): e22-33.
104. Brenner BM, Lawler EV, Mackenzie HS. The hyperfiltration theory: a paradigm shift in nephrology. *Kidney Int* 1996;49(6): 1774-1777.
105. Rowe JS, Carless JE. Investigations on the in vitro dose-related metabolism of indomethacin in four laboratory species. *Eur J Drug Metab Pharmacokinet* 1982;7(3): 159-163.
106. Williams DP, Antoine DJ, Butler PJ, Jones R, Randle L, Payne A, Howard M, Gardner I, Blagg J, Park BK. The metabolism and toxicity of furosemide in the Wistar rat and CD-1 mouse: a chemical and biochemical definition of the toxicophore. *J Pharmacol Exp Ther* 2007;322(3): 1208-1220.
107. Sanoh S, Horiguchi A, Sugihara K, Kotake Y, Tayama Y, Uramaru N, Ohshita H, Tateno C, Horie T, Kitamura S, Ohta S. Predictability of metabolism of ibuprofen and naproxen using chimeric mice with human hepatocytes. *Drug Metab Dispos* 2012;40(12): 2267-2272.
108. Milner RD, Milner GR, Lancaster D. Tissue gentamicin concentrations in the newborn and adult rat. *Pediatr Res* 1979;13(3): 161-166.
109. Marre R, Tarara N, Louton T, Sack K. Age-dependent nephrotoxicity and the pharmacokinetics of gentamicin in rats. *Eur J Pediatr* 1980;133(1): 25-29.
110. Nassberger L, DePierre JW. Uptake of gentamicin in foetal and newborn rats after a single intraperitoneal injection. *Pharmacol Toxicol* 1989;64(1): 126-131.
111. Peti-Peterdi J, Kidokoro K, Riquier-Brison A. Novel in vivo techniques to visualize kidney anatomy and function. *Kidney Int* 2015;88(1): 44-51.
112. Bennett KM, Bertram JF, Beeman SC, Gretz N. The emerging role of MRI in quantitative renal glomerular morphology. *Am J Physiol Renal Physiol* 2013;304(10): F1252-1257.
113. Gilbert T, Lelievre-Pegorier M, Malienou R, Meulemans A, Merlet-Benichou C. Effects of prenatal and postnatal exposure to gentamicin on renal differentiation in the rat. *Toxicology* 1987;43(3): 301-313.
114. Kent AL, Douglas-Denton R, Shadbolt B, Dahlstrom JE, Maxwell LE, Koina ME, Falk MC, Willenborg D, Bertram JF. Indomethacin, ibuprofen and gentamicin administered during late stages of glomerulogenesis do not reduce glomerular number at 14 days of age in the neonatal rat. *Pediatr Nephrol* 2009;24(6): 1143-1149.

115. Tune BM. Nephrotoxicity of beta-lactam antibiotics: mechanisms and strategies for prevention. *Pediatr Nephrol* 1997;11(6): 768-772.
116. Nathanson S, Moreau E, Merlet-Benichou C, Gilbert T. In utero and in vitro exposure to beta-lactams impair kidney development in the rat. *J Am Soc Nephrol* 2000;11(5): 874-884.
117. Araki T, Hayashi M, Nakanishi K, Morishima N, Saruta T. Caspase-9 takes part in programmed cell death in developing mouse kidney. *Nephron Exp Nephrol* 2003;93(3): e117-124.
118. Pacifici GM, Nottoli R. Placental transfer of drugs administered to the mother. *Clin Pharmacokinet* 1995;28(3): 235-269.
119. Varvarigou A, Bardin CL, Beharry K, Chemtob S, Papageorgiou A, Aranda JC. Early ibuprofen administration to prevent patent ductus arteriosus in premature newborn infants. *JAMA* 1996;275(7): 539-544.
120. Pezzati M, Vangi V, Biagiotti R, Bertini G, Cianciulli D, Rubaltelli FF. Effects of indomethacin and ibuprofen on mesenteric and renal blood flow in preterm infants with patent ductus arteriosus. *J Pediatr* 1999;135(6): 733-738.
121. Guthrie SO, Gordon PV, Thomas V, Thorp JA, Peabody J, Clark RH. Necrotizing enterocolitis among neonates in the United States. *J Perinatol* 2003;23(4): 278-285.
122. Thomas RL, Parker GC, Van Overmeire B, Aranda JV. A meta-analysis of ibuprofen versus indomethacin for closure of patent ductus arteriosus. *Eur J Pediatr* 2005;164(3): 135-140.
123. Attridge JT, Clark R, Walker MW, Gordon PV. New insights into spontaneous intestinal perforation using a national data set: (1) SIP is associated with early indomethacin exposure. *J Perinatol* 2006;26(2): 93-99.
124. Peitz GJ, Hoie EB, Hoy S, Anderson-Berry A. Repeated bowel perforations with Ibuprofen lysine: a case report. *J Pediatr Pharmacol Ther* 2008;13(3): 166-169.
125. Katakam LI, Cotten CM, Goldberg RN, Dang CN, Smith PB. Safety and effectiveness of indomethacin versus ibuprofen for treatment of patent ductus arteriosus. *Am J Perinatol* 2010;27(5): 425-429.
126. Rao R, Bryowsky K, Mao J, Bunton D, McPherson C, Mathur A. Gastrointestinal complications associated with ibuprofen therapy for patent ductus arteriosus. *J Perinatol* 2011;31(7): 465-470.
127. Avner ED, Sweeney Jr. WE, Piesco NP, Ellis D. Growth factor requirements of organogenesis in serum-free metanephric organ culture. *In Vitro Cell Dev Biol* 1985;21(5): 297-304.
128. Komhoff M, Grone HJ, Klein T, Seyberth HW, Nusing RM. Localization of cyclooxygenase-1 and -2 in adult and fetal human kidney: implication for renal function. *Am J Physiol* 1997;272(4 Pt 2): F460-468.
129. Zhang MZ, Wang JL, Cheng HF, Harris RC, McKanna JA. Cyclooxygenase-2 in rat nephron development. *Am J Physiol* 1997;273(6 Pt 2): F994-1002.
130. Saez F, Reverte V, Salazar F, Castells MT, Llinas MT, Salazar FJ. Hypertension and sex differences in the age-related renal changes when cyclooxygenase-2 activity is reduced during nephrogenesis. *Hypertension* 2009;53(2): 331-337.
131. Yamamoto E, Izawa T, Kuwamura M, Yamate J. Immunohistochemical Expressions of Main PGE(2) Biosynthesis-related Enzymes and PGE(2) Receptor in Rat Nephrogenesis. *J Toxicol Pathol* 2011;24(4): 257-261.
132. Kent AL, Maxwell LE, Koina ME, Falk MC, Willenborg D, Dahlstrom JE. Renal glomeruli and tubular injury following indomethacin, ibuprofen, and gentamicin exposure in a neonatal rat model. *Pediatr Res* 2007;62(3): 307-312.
133. Kent AL, Koina ME, Gubhaju L, Cullen-McEwen LA, Bertram JF, Lynnhtun J, Shadbolt B, Falk MC, Dahlstrom JE. Indomethacin administered early in the postnatal period results in reduced glomerular number in the adult rat. *Am J Physiol Renal Physiol* 2014;307(10): F1105-F1110.
134. Gimpel C, Krause A, Franck P, Krueger M, von Schnakenburg C. Exposure to furosemide as the strongest risk factor for nephrocalcinosis in preterm infants. *Pediatr Int* 2010;52(1): 51-56.
135. Mallie JP, Boudzoumou P. Functional renal maturation in rat neonates after prenatal exposure to furosemide. *Pediatr Nephrol* 1996;10(4): 458-460.
136. Mallie JP, Gerard A, Gerard H. Does the in-utero exposure to furosemide delay the renal maturation? *Pediatr Pharmacol (New York)* 1985;5(2): 131-138.
137. Zhang DY, Lumbers ER, Simonetta G, Wu JJ, Owens JA, Robinson JS, McMillen IC. Effects of placental insufficiency on the ovine fetal renin-angiotensin system. *Exp Physiol* 2000;85(1): 79-84.
138. Woods LL, Ingelfinger JR, Nyengaard JR, Rasch R. Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. *Pediatr Res* 2001;49(4): 460-467.

139. Zohdi V, Moritz KM, Bubb KJ, Cock ML, Wreford N, Harding R, Black MJ. Nephrogenesis and the renal renin-angiotensin system in fetal sheep: effects of intrauterine growth restriction during late gestation. *Am J Physiol Regul Integr Comp Physiol* 2007;293(3): R1267-1273.
140. Schreuder MF. Unilateral anomalies of kidney development: why is left not right? *Kidney Int* 2011;80(7): 740-745.
141. Simonetti GD, Mohaupt MG. Smaller left kidney in low birth weight children. *Kidney Int* 2012;81(4): 418; author reply 418-419.
142. Sengupta P. The Laboratory Rat: Relating Its Age With Human's. *Int J Prev Med* 2013;4(6): 624-630.
143. Keijzer-Veen MG, Finken MJ, Nauta J, Dekker FW, Hille ET, Frolich M, Wit JM, van der Heijden AJ, Dutch POPS-19 Collaborative Study Group. Is blood pressure increased 19 years after intrauterine growth restriction and preterm birth? A prospective follow-up study in The Netherlands. *Pediatrics* 2005;116(3): 725-731.
144. Hlushchuk R, Barre S, Correa Shokiche C, Djonov V. Angio-**μ**CT: New Polymer-Based Contrast Agent Makes Kidney Morphometry Look Attractive. Poster session presented at: Kidney week 2015. 2015 Nov 3-8; San Diego, Ca.

10

Summary
Samenvatting

Summary

Currently preterm neonates have a relatively good survival rate from 24-25 weeks of gestation onwards and this will likely improve even more in the future due to the continuing expansion of knowledge of neonatal physiology, drugs and the better integration of this knowledge with engineering and computational models. However there is a high level of uncertainty on the impact that preterm birth, with concurrent treatments, has on (late) gestational organ development and organ function. There is growing evidence that events around birth program the physiology of our organs for direct optimal survival, but has implications for later life with an increased susceptibility to disease. With regards to the kidney there is currently limited data available, however these suggest that a reduced fetal growth could result in less glomeruli being formed. This paradigm is strongly related with the hyperfiltration hypothesis in kidney disease. The hypothesis states that a low glomerular number, after progressing through several phases, ultimately results in renal disease.

The total number of glomeruli is normally completed just before term birth, with little to no regeneration capacity in later life. Possible risk factors for a lower glomerular number have been previously identified. In this thesis we studied the impact of two factors, being drug treatment and extrauterine growth restriction, and their interaction.

We selected to study (1) antibiotics, (2) NSAIDs and (3) diuretics, which are used to treat three different neonatal indications: (1) sepsis/infection, (2) closure of a patent ductus arteriosis and (3) fluid retention. The use of these drugs is quite frequent as for the three drug classes 62%, 16% and 19% of neonates born before 32 weeks in the Netherlands receive treatment, respectively. In addition to neonatal treatment, the drugs may also be taken by pregnant women (although contraindicated) for similar or other indications (infections, water retention/high blood pressure, pain killer or as tocolycant), resulting in intrauterine exposure.

Extrauterine growth restriction was included was as a second stressor. Due to the immaturity of the intestines and all the necessary treatments (and accompanied side effects) in preterm born children, extrauterine growth restriction is very difficult to prevent in the neonatal care unit. Furthermore, research up till now has mainly focused on evaluating only a single stressor at a time, simplifying the actual situation in the neonatal intensive care unit. We believe it is crucial to study additive or synergic interactions between stressors to make a better risk prediction for preterm born children.

In **chapter 2** we started out by providing a more thorough background overview on the known effects of congenital abnormalities and drug treatment with regard to the kidney**.** One of the early questions that rose was how kidney development could be monitored in the clinic. Currently estimations are made based on renal weight and/

or renal size. We questioned the value of these morphometric predictions and investigated the validity of renal size in **chapter 3** by performing a meta-analysis on the available data in the literature. Papers were included when both renal sizes were reported for individual subjects as well as unbiased results for glomerular numbers using stereology. Our analysis of the available data showed that renal size is a poor predictor for the number of glomeruli and should therefore not be used to evaluate kidney development.

Morphometric markers for kidney development were further examined in **chapter 4,** where we assessed the validity of metanephron (early fetal kidney) surface area measurements for ureteric bud formation, a well-studied kidney development endpoint. The fetal kidneys were explanted from mice embryo's and cultured for 24 hours. Our study showed that there was no correlation between the surface area and the ureteric bud formation in untreated specimens. Therefore we concluded that the use of the surface area of fetal kidneys as a study endpoint is also not wise. We did show that metanephron surface area at the time of kidney explantation is partially predictive for the bud formation after 24 hours and can therefore be used to gauge and correct for small differences in the state of kidney development at the time of explantation.

In the same **chapter 4** we investigated the impact of the antibiotic drugs gentamicin, ceftazidime and meropenem in an approximated clinical dose range on early kidney development. With these experiments we showed that ceftazidime has a detrimental effect on ureteric bud formation *ex vivo* and down-regulated the *Gdnf* and *Fgf8* genes, which are important in this process. No effect of gentamicine nor meropenem was demonstrated. The observed findings for ceftazidime in this developmental stage are more relevant to pregnant women than neonates, especially because ceftazidime can easily pass the placenta.

Effect of antibiotics on kidney development were further investigated in **chapter 5,** where we studied the effect of gentamicine and ceftazidime on glomerular numbers when administered in a clinical neonatal dose range, which we measured and confirmed for gentamicin by pharmacokinetic analysis. This experiment was performed in rats just after birth, which is a comparable developmental stage to that of a human preterm neonate with regards to the kidney. Additionally, we introduced a 'second hit' in this chapter by food restricting a subgroup of the rats by using litter enlargement to model for extrauterine growth retardation. This allowed us to study both stressors and their interaction. The effects on development were evaluated by determining glomerular numbers, proliferation/apoptosis and expression of important genes in renal development or renal function. Methods used for the determination were stereology, immunohistochemistry and quantitative polymerase chain reaction. Our results indicated that food restriction caused a decrease in glomerular number, but drug treatment did not. There was no added or synergistic effect by combining drug

treatment with extrauterine growth restriction and the multi-hit hypothesis was therefore not confirmed in this study.

The effects of the NSAID's ibuprofen and indomethacin, and the diuretics furosemide and hydrochlorothiazide were studied in our *ex vivo* model in **chapter 6**. These drug classes are not only frequently administered to premature neonates, but also to pregnant women. Similar endpoints for toxicity evaluation were used as in chapter 3 and no effects were noted on either ureteric bud formation or important developmental pathways. We concluded that furosemide, hydrochlorothiazide, ibuprofen and indomethacin did not have an intrinsic effect on early kidney development in a clinically relevant dose range.

These drugs were further studied in **chapters 7 and 8** for their effects on late kidney development in rats. In **chapter 7**, the impact of ibuprofen and indometacin was studied in a similar study setup as in chapter 5. Additional endpoints were added, however, to study long term sequelae. Kidney function was monitored by determining electrolytes and creatinine clearance up to 9 months-of-age and radio telemetry blood pressure measurement devices were used to follow-up on blood pressure. We identified a slight decrease in glomerular numbers after ibuprofen treatment, but no delayed effects were noted in the long-term treatment parameters, except for a slight tendency to an increased NGAL concentration 9 months after treatment. Unfortunately, blood pressure measurements were not reliable to analyze. Additionally, our study outcome contrasted some of the results of other researchers in the field. Therefore more studies should be performed to provide a definite answer whether there is a real effect of these drugs. The effect of food restriction on glomerular numbers was small and no interaction between drug treatment and food restriction was observed.

In our final experimental chapter **(chapter 8**) we studied the effects of clinical dose levels of furosemide and hydrochlorothiazide treatment with a similar protocol as described in chapters 5 and 7. Due to the unsuccessful telemetry, we used tail cuff blood pressure measurements in this study. In this study we noted a decrease in glomerular number by food restriction, but drug treatment did not have an effect and no interaction between food restriction and drug treatment was noted. Nor did we see any effect on long term endpoints.

All considerations for our choice of techniques and the overall implications of the work combined in this thesis are discussed in **chapter 9.** Based on this we conclude that all drugs tested (gentamicin, ceftazidime, meropenem, ibuprofen, indomethacin, furosemide and hydrochlorothiazide), when dosed in a clinical relevant dose, did not cause a direct toxic effect on nephrogenesis or glomerulogenesis except for ceftazidime, which was toxic in early kidney development. Postnatal food restriction by litter enlargement results in a significant decrease of glomeruli, but did not enhance or modify the effect of drug treatments nor showed long-term sequelae. Therefore a multiple-hit interaction between these stressors was not observed and adding age had no effect. Finally kidney surface area or size are poor markers for renal development and should better not be used.

Samenvatting

Hedendaags hebben te vroeg geboren kinderen een redelijk goede overlevingskans vanaf week 24-25 en in de toekomst zal dit waarschijnlijk verder toenemen vanwege de groeiende kennis op gebied van neonatale fysiologie, medicatie en de betere integratie van techniek en computersimulaties. Desondanks is er een grote onzekerheid over de invloed die vroeggeboorte, met de bijbehorende behandelingen, heeft op de (late) ontwikkeling van organen en hun functioneren. Er is groeiend bewijs dat gebeurtenissen rondom de geboorte de orgaanfunctie programmeren en optimaliseren voor directe overleving, maar wat gevolgen heeft voor de vatbaarheid voor aandoeningen op latere leeftijd. Voor dit effect op de nier zijn er op dit moment beperkte onderzoeksgegevens beschikbaar, maar deze suggereren dat een gereduceerde foetale groei mogelijk een vermindering in aanleg van het aantal glomeruli ten gevolg kan hebben. Dit paradigma is sterk verwant met de hyperfiltratie theorie in het veld van nierziekten. Deze theorie beweert dat een laag aantal glomeruli, na progressie via verscheidene stadia's , uiteindelijk kan leiden tot nierziekte.

Het totaal aantal glomeruli is normaal gesproken volledig aangelegd vlak voor de geboorte en het lichaam heeft weinig tot geen herstelmogelijkheid indien deze glomeruli verloren gaan. Mogelijke risicofactoren voor een lager aantal glomeruli zijn voorheen geïdentificeerd. In dit proefschrift is de impact van twee factoren: medicijngebruik en extra uterine groeivertraging, en de interactie daartussen bestudeerd. We hebben gekozen om (1) antibiotica, (2) NSAIDs en (3) diuretica te bestuderen, welke gegeven worden ter behandeling van 3 verschillende neonatale indicaties: (1) sepsis/infectie, (2) sluiting van een persisterende ductus arteriosis en (3) vocht retentie. Het gebruik van deze medicijnen is frequent aangezien voor de 3 medicijnklassen respectievelijk 62%, 16% en 19%, van de neonaten geboren in Nederland vóór 32 weken hiermee worden behandeld. Naast neonatale behandeling, kunnen deze medicijnen ook gebruikt worden door zwangere vrouwen (ook al contrageïndiceerd) voor vergelijkbare indicaties (infecties, vocht retentie/hoge bloeddruk) of als pijnstiller of weeënremmer, resulterend in intra uterine blootstelling. Een tweede prikkel was geïntroduceerd in de vorm van extra uterine groeivertraging. Vanwege de onvolgroeidheid van de darmen en alle noodzakelijke behandelingen (en bijbehorende neveneffecten) bij te vroeg geboren kinderen is het zeer moeilijk om dit te vermijden in een neonatale care unit. Wetenschappelijk onderzoek heeft zich tot op heden voornamelijk gefocussed om één enkele prikkel tegelijkertijd te bekijken, waardoor de daadwerkelijke situatie in de neonatale intensive care unit sterk vereenvoudigd wordt. Wij geloven dat het cruciaal is om additieve en synergetische interacties tussen prikkels te bestuderen om zo een betere risico evaluatie te kunnen maken voor te vroeg geboren kinderen.

In **hoofdstuk 2** geven we een achtergrond overzicht over bekende effecten van aangeboren afwijkingen en medicijngebruik op de nier. Een van de eerste vragen die opkwam was hoe nierontwikkeling opgevolgd zou kunnen worden in de kliniek. Dit wordt momenteel gedaan door het bepalen van nier gewicht en/of nier grootte. Wij betwijfelden de waarde van deze morphometrische voorspellingen en hebben daarom de validiteit onderzocht in **hoofdstuk 3** door een meta-analyse uit te voeren op beschikbare data in de literatuur. Onderzoeks artikelen werden geïncludeerd wanneer zowel niergrootte alswel objectieve metingen het behulp van stereologie voor het aantal glomeruli in individuele proefpersonen werden gerapporteerd. Onze analyse van de beschikbare data toont aan dat niergrootte geen goede voorspeller is voor het aantal glomeruli en zou daarom niet gebruikt dienen te worden om een voorspelling voor nierontwikkeling te geven.

Morphometrische merktekens voor nierontwikkeling zijn verder bestudeerd in **hoofdstuk 4** waarin we de validiteit van metanefron (vroege foetale nier) oppervlakte voor ureterknop formatie, een goed bestudeerd eindpunt van nierontwikkeling, hebben bepaald. De foetale nieren werden geëxplanteerd vanuit muizen embryo's en 24h in kweekcultuur gebracht. Onze studie toonde aan dat er geen correlatie was tussen de oppervlakte en ureterknop formatie bij onbehandelde specimens. Daaruit concludeerden we dat het gebruik van oppervlakte van de foetale nier als een studie eindpunt ook niet verstandig is. Bijkomend toonden we aan dat de oppervlakte van de foetale nier op het moment van explanteren gedeeltelijk voorspellend is voor de knop formatie na 24 uur. De oppervlakte meting kan daarom wel gebruikt worden om het stadium van nierontwikkeling te schatten op het moment van de explantatie en voor kleine verschillen daarin te corrigeren.

In hetzelfde **hoofdstuk 4** hebben we het effect van de antibiotica gentamicine, ceftazidime en meropenem in een benaderde klinische doserings range, op vroege nier ontwikkeling onderzocht. Met deze experimenten toonden we aan dat ceftazidime een negatief effect op ureter knop formatie heeft *ex vivo* en de expressie van twee belangrijke genen in dit proces, namelijk *Gdnf* en *Fgf8*, verminderd. Behandeling met gentamicine of meropenem liet geen effect zien. In dit ontwikkelingsstadium zijn de bevindingen bij ceftazidime behandeling meer relevant voor zwangere vrouwen dan neonaten, voornamelijk omdat ceftazidime ook zonder moeite de placenta kan passeren.

De effecten van gentamicine en ceftazidime op nier ontwikkeling zijn verder onderzocht in **hoofdstuk 5**, waarin we het effect op het aantal glomeruli bestudeerd hebben na een klinische relevante dosering. In het geval van gentamicine hebben we deze ook gemeten en bevestigd door middel van farmacokinetische analyse. Dit experiment is uitgevoerd in ratten, welke kort na de geboorte gedoseerd werden. Op dat moment is het stadium van nierontwikkeling vergelijkbaar met dat van een te vroeg geboren kind. Tegelijkertijd introduceerden we de tweede prikkel in dit hoofdstuk door voerrestrictie toe te passen (d.m.v. nestvergroting) op een subpopulatie van de

ratten om extra uterine groeivertraging te modelleren. Daarmee konden de 2 prikkels en hun interactie bestudeerd worden.

De effecten op ontwikkeling zijn geëvalueerd door het aantal glomeruli te bepalen, proliferatie/apoptose te bekijken en door expressie van belangrijke genen in nier ontwikkeling of nier functie te meten. De methoden die gebruikt werden voor de experimentele bepalingen waren stereologie, immunohistochemie en kwantitatieve polymerase ketting reactie. Onze resultaten lieten zien dat voerrestrictie een verlaging van het aantal glomeruli veroorzaakt, maar medicijn behandeling niet. Er was geen additief of synergetisch effect aanwezig in de kombinatie van medicijn behandeling en extra uterine groei restrictie en de meervoudige 'treffer' hypothese was daarom niet bevestigd in deze studie.

De effecten van de NSAIDs ibuprofen en indometacine, en de diuretica furosemide en hydrochloorthiazide hebben we bestudeerd in ons *ex vivo* model in **hoofdstuk 6**. Deze medicijnklassen worden niet alleen frequent gegeven aan te vroeg geboren kinderen, maar ook aan zwangere vrouwen. Voor de evaluatie van toxiciteit zijn vergelijkbare eindpunten als in hoofdstuk 3 gebruikt. We vonden geen effecten op zowel ureterknop formatie als belangrijke genen in ontwikkeling. We concludeerden daaruit dat furosemide, hydrochloorthiazide, ibuprofen en indomethacine geen intinsiek effect op de nierontwikkeling hebben in een klinische relevante doseer range.

Deze medicijnen zijn verder onderzocht in **hoofdstuk 7 & 8** voor effecten op late nier ontwikkeling bij ratten. In **hoofdstuk 7** is de impact van ibuprofen en indometacine bestudeerd volgens een vergelijkbaar protocol als in hoofstuk 5. Extra eindpunten zijn echter toegevoegd echter om mogelijke lange termijn gevolgen op te volgen. De nierfunctie werd gemonitord door electrolieten concentraties en creatinine klaring te bekijken tot een leeftijd van 9 maanden, en radio telemetrie bloeddruk meters zijn ingezet om bloeddruk op te volgen. Na ibuprofen behandeling zagen we een lichte afname van de hoeveelheid glomeruli, maar verdere effecten werden nier gevonden in de lange termijn eindpunten. De uitzondering hierop was een lichte stijgende trend in NGAL concentratie 9 maanden na behandeling.

Helaas bleken de verkregen bloeddruk resultaten niet betrouwbaar genoeg om te analyseren. Daarnaast waren de bevindingen uit onze studie tegenstrijdig met de resultaten van andere wetenschappers in het werkveld. Daarom zullen er meer studies nodig zijn om een definitief antwoord te vinden of er daadwerkelijk een effect is van deze medicijnen. Het effect van voerrestrictie op nier ontwikkeling was klein en er is geen interactie waargenomen tussen medicijn behandeling en voerrestrictie.

In het laatste experimentele hoofdstuk **(hoofstuk 8**) hebben we de effecten van een klinisch relevante dosering furosemide en hydrochloorthiazide bestudeerd volgens een vergelijkend protocol zoals beschreven in hoofdstukken 5 en 7. Vanwege de mislukte telemetrie experimenten, zijn er dit maal bloeddrukken gemeten met een staartcuff. In deze studie zagen we wederom een afname van glomeruli in de

voerrestrictie groep, maar medicijn behandeling had geen invloed en ook werd er geen interactie waargenomen. Tevens zagen we ook hier geen effecten in de lange termijn eindpunten.

Alle overwegingen met betrekking tot de toegepaste technieken en de algehele bevindingen van het gecombineerde werk in deze theses zijn bediscussiëerd in **hoofdstuk 9.** Hierin concluderen we dat all geteste medicijnen (gentamicine, ceftazidime, meropenem, ibuprofen, indomethacine, furosemide en hydrochlorothiazide), wanneer gedoseerd in een klinisch relevante dosering niet een direct toxisch effect op nefrogenese of glomerulogenese hebben, met uitzondering van ceftazidime, welke schadelijk was in vroege nier ontwikkeling. Postnatale voerrestrictie door middel van nestvergroting leidde tot een significante afname van glomeruli, maar beïnvloede niet het effect van medicijn behandeling, noch liet het een lange termijn consequentie zien. Daardoor is een meervoudige treffer interactie tussen deze prikkels niet aangetoond en had gevorderde leeftijd geen effect. Ter afsluiting, nier oppervlakte of grootte zijn ongeschikte markers voor nier ontwikkeling en kunnen beter niet gebruikt worden.

11

Dankwoord
List of abbreviations
Curriculum Vitae
List of publications

Dankwoord

Veel personen hebben in de afgelopen jaren op één of andere manier een bijdrage geleverd aan het tot stand komen van dit proefschrift. Waaronder alle medewerkers van **Laboratorium geneeskunde, kindernefrologie, pathologie en het centraal dieren laboratorium** van het Radboud UMC/ Radboud universiteit**.** Naast hulp bij experimenten en veel wetenschappelijke conversaties zijn er ook vele leuke activiteiten georganiseerd zoals o.a. een luxe overnachting op vakantiepark de Berckt met een (bijna) privé zwembad, culinair eten en drinken bij Café de Beugel, pubquizzen en de whiskey proeverij bij Versailles (zeker géén Velpon **Roel**!). Buiten de bijzondere uitjes zoals deze waren de regelmatige bijeenkomsten in de Aesculaaf of Maxim ook zeer memorabel (**Maurice**, ik ben je nog steeds een wiel schuldig....). Naast mijn oud-collega's in Nijmegen wil ik ook mijn huidige collega's bij **Janssen** bedanken voor alle steun. Zonder al deze mensen tekort te doen (maar vooral om deze sectie kort te houden) wil ik een aantal mensen nog speciaal bedanken.

Bert en Michiel, allereerst wil ik jullie graag bedanken voor de mogelijkheid om de experimenten die beschreven staan in dit proefschrift beschreven staan uit te voeren. Tevens was jullie inzicht en soms andere kijk op data van grote waarde voor mijn wetenschappelijke ontwikkeling. Michiel, ik wil je uitzonderlijk bedanken voor je feilloze instinct om me bij te sturen op de momenten waar ik weer (te) kritisch was over de resultaten van mijn experimenten. Ondanks dat ik nog steeds zeer kritisch blijf heb ik geleerd dit beter te relativeren en te kunnen plaatsen in een groter geheel.

Annelies, ik wil je graag bedanken voor al het werk wat je verricht hebt voor de experimenten beschreven in dit proefschrift. Menig dag zul je naar huis gegaan zijn met een lamme arm van het snijden van coupes voor het stereologie werk. Alhoewel de uiteindelijke analyses niet de vooropgestelde hypotheses bevestigden hebben we enkele leuke publicaties kunnen schrijven. Ik wens je veel succes in je carrière en veel geluk en gezondheid samen met je gezin.

Mijn andere directe research nephro collega's**: Dineke, Carolien, Elena, Thea en Nicole** dank voor al jullie wetenschappelijke input op mijn experimenten en vooral voor alle flauwekul daaromheen. Bij de voortdurende stroom aan hypothese ontkrachtende data is het fijn om dit te kunnen delen met zulke fijne collega's. Niet te vergeten wil ik ook mijn stagiair **Lisanne** bedanken. Naast al het werk wat je gedaan hebt, heb ik ook door jou leren coachen.

Buiten mijn collega's wil ik ook enkele familieleden niet onbesproken laten.

Pap, Mam, ik wil jullie bedanken voor alle liefde, steun en kansen die jullie mij gegeven hebben. Jullie opvoeding heeft mede geholpen om mij te vormen tot wie ik nu ben. Het verplicht moeten aanhoren van klassieke muziek op de zondagochtend (na een hele week van rock en metal) heeft zijn nut gehad. Ik heb ondervonden dat het

uiterst concentratieverhogend werkt bij het uitvoeren van stereologie analyses. Verder wil ik jullie bedanken voor alle keren dat ik even lekker heb kunnen klagen over alles en niets aan de eettafel om het hoofd weer leeg te krijgen.

Carin,Oscar (Damian, Jessica): wanneer je zoveel tijd en energie investeert in één project raak je er soms in verstrikt. Ik heb jullie te danken om de inhoud van het directe werk te relativeren en om in te zien dat er ook nog andere belangrijke zaken zijn, zoals bijvoorbeeld barbecueën met familie! **Niklas**, waar ik uiteindelijk niet verder gekomen ben dan een paar setjes in Dollars tijdens de 4daagse, bespeel jij nu hele festival menigtes. Jouw toewijding aan de muziek is voor mij een grote inspiratiebron geweest om te blijven doorzetten en dit proefschrift te voltooien. **Tjerk, Sandra (Lisa, Tycho),** jullie eerdere ervaringen vanuit het promoveer traject en als mede vakgenoten hebben mij geholpen om het einddoel voorogen te blijven houden en te begrijpen wat ik daadwerkelijk geleerd heb tijdens dit traject. Tjerk, laten we onze PubMed competitie in stand houden als voorlopig de enige twee 'Buetersen' ter wereld die in de auteurslijst voorkomen. Je hebt misschien een "lichte" voorsprong wat betreft het aantal artikelen, maar bij het schrijven van dit dankwoord staat er een van mij bovenaan!

Mijn schoonfamilie: **Riekie, Gerard, Bart & Krista (Eef), Paul**: Dank voor al jullie steun en interesse in mijn werk. Jullie warmte heeft er mede voor gezord dat ik gemotiveerd bleef om dit werk te voltooien naast mijn functie bij Janssen. Gerard, Ik vind het zeer spijtig dat je de voltooiing van dit manuscript niet meer mag meemaken, want ik weet zeker dat je van de verdediging zou genieten. Toch ga ik nog zorgen dat er een boekje bij jou terecht komt.

Verder wil ik al mijn **vrienden, buren en kennissen** bedanken voor al hun steun, gezelligheid, maar voornamelijk begrip. Ik heb menigeen verjaardagfeestje en activiteit overgeslagen vanwege de vervaardiging van dit proefschrift en dat was zeker niet makkelijk. Met het afronden van dit manuscript gaan we daar verandering in brengen. Ten laatste, maar niet ten minste wil ik mijn vriendin **Anke** toespreken.

We hebben samen wat pittige jaren gehad, waarin ik een grote belasting op jouw schouders heb gelegd. Door mijn grote tijdsinvestering, kwam een groot deel van de huishoudelijke taken bij jou terecht, naast jouw eigen voltijdse baan. Ik voelde me dan ook vaak schuldig als ik na een weekend werken uiteindelijk niet veel op papier gekregen had, maar zoals je weet de raderen in het hoofd draaien altijd (en soms iets te veel). Met het voltooien van dit proefschrift komt er weer ruimte om samen meer leuke dingen te doen en hoef je niet meer alleen naar verjaardagen. Eén ding is zeker, zonder jouw onvolwaardelijke steun was dit proefschrift niet tot stand gekomen. Laten we daar nu samen van gaan genieten.

En nu heb ik geen goesting meer om te schrijven, cheers!

Ruud

List of abbreviations

ACEIs: Angiotensin converting enzyme inhibitors
Actb: Beta actin
Agtr1a: Angiotensin receptor type 1
Agtr2: Angiotensin receptor 2
ANCOVA: Analysis of covariance
ANOVA: Analysis of variance
Aqp1: Aquaporin 1
Aqp4: Aquaporin 4
ARBs: angiotensin receptor blockers
ASF: Area Sample Fraction
AUC: Area Under the Curve
BAX: B-cell lymphoma 2-like protein 4
BCL-2: B-cell lymphoma 2
BMI: Body Mass Index
Bmp7: Bone morphogenetic protein 7
BSA: Bovine Serum Albumine
BSA: Body Surface Area
CAKUT: Congenital Abnormalities of the Kidney and Urinary Tract
Casp3: Caspase 3
Casp9: Caspase 9
Casr: Calcium sensing receptor
C_{max}: Maximum concentration
COX: Cyclooxygenase
C_T: Cycle time
DBP: Diatolic Blood Pressure
DMEM: Dulbecco's Modified Eagle Medium
DNA: Deoxyribonucleic Acid
DOHaD: The developmental origins of health and disease
ELISA: Enzyme-Linked Immuno Sorbent Assay
EM: Electron Microscopy
ENaC: Epithelial sodium channel
ESRD: End Stage Renal Disease
EUGR: Extrauterine Growth Retardation
FDA: Food and Drug Administration
Fgf7: Fibroblast growth factor 7
Fgf8: Fibroblast growth factor 8
FR: Food restricted (regime)
Gdnf : Glial cell line-derived neurotrophic factor
GRf1a: Glial cell line-derived neurotrophic factor receptor alpha 1
GUDMAP: Genitourinary development molecular anatomy project
HCTZ: Hydrochlorothiazide
Hgf: Hepatocyte growth factor
HIV: Human immunodeficiency virus
Hmbs: Hydroxymethylbilane synthase
HRP: Horse radish peroxidase
Lim: Lim homeobox 1
MBP: mean blood pressure
MCDK: multicystic dysplastic kidney
M-MLV: Moloney Murine Leukemia Virus
MRI: magnetic resonance imaging

mRNA: messenger Ribonucleic Acid
Ncc: Sodium-chloride symporter
NF: Normal Food (regime)
NGAL: Neutrophil Gelatinase-Associated Lipocallin
N(glom): Number of glomeruli
Nhe3: Sodium hydrogen exchanger 3
Nhe8: Sodium hydrogen exchanger 8
Nkcc2: Na^+-K^+-Cl^- cotransporter
*Nphs1:*Nephrin
Nphs2: Podocin
NSAIDs: Non Steroidal Anti Inflammatory Drugs
Oat1: Organic anion transporter 1
PBS: Phosphate Buffered Saline
PBS-T: Phosphate Buffered Saline - Tween80
PDGF-B: Platelet Derived Growth Factor subunit B
Pf: Number of counting frame corners that hit evaluated kidney tissue
PND: Post Natal Day
PP: Pulse Pressure
PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses
Ps: Number of counting frame corners that hit kidney tissue
Ptgs1: Prostaglandin synthetase 1
Ptgs2: Prostaglandin synthetase 2
qPCR: quantitative Polymerase Chain Raction
RAS: Renin-angiotensin system
Ret: Ret proto oncogene
RNA: Ribonucleic Acid
SBP: Systolic Blood Pressure
SD: Standard Deviation
Sox9: Sex determining region Y-Box-9
SSF: Section Sample Fraction
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
Wnt4: Wingless-type MMTV integration site family, member 4
Wt1: Wilms Tumor 1

Curriculum Vitae

Ruud Bueters werd op 9 mei 1983 te Eindhoven geboren. In Juni 2001 haalde hij zijn gymnasium diploma aan scholengemeenschap het Udens college te Uden, waarna hij in hetzelfde jaar begon aan de studie Biomedische Wetenschappen aan de Radboud Universiteit in Nijmegen. Het propedeutisch examen van deze studie behaalde hij in 2002 en de bachelor fase werd afgerond in 2004 na een kortdurende stage o.l.v. Dr. B van Balkom in de vakgroep farmacologie/toxicologie van de Radboud universiteit betreffende de functionele karakterisatie van de Oatp5 transporter in een MDCK2 cellijn. Daarna heeft hij de masterfase van zijn opleiding afgerond in 2006 met een specialisatie in toxicologie en een bijvak in geneesmiddelen onderzoek. In het kader van het bijvak is een stage uitgevoerd o.l.v. Dr. Miriam Huls, tevens bij de afdeling farmacologie/toxicologie in Nijmegen met als titel "Phenotyping of the bcrp 1 knockout mice & Investigation of the role of the bcrp 1 transporter in the repair of ischemic renal damage". Vervolgens heeft hij de specialisatie toxicologie afgerond met onderzoek op de afdeling van cognitive neurosciences Radboud universiteit Nijmegen met het onderzoek getiteld: "Phenotyping of the Pmch mutant rat: Study on locomotor, anxiety and feeding behavior", waarbij de MCH knockout rat gefenotypeerd werd door middel van challenges met neuropharmaceutische middelen. Na het afronden van de studie Biomedische wetenscappen is hij van januari 2007-october 2009 werkzaam geweest bij NOTOX B.V. (Huidige Wil Research, a Charles River Company) als studieleider generale toxicologie. Van November 2009 tot November 2013 was hij werkzaam of de aldeling kindernefrologie in het UMC St. Radboud. Onder leiding van Dr. M. Schreuder en Prof. Dr. L. van den Heuvel voerde hij door de Nederlandse nierstichting gesubsidieerd onderzoek uit na de effecten van medicijnen op nierontwikkeling, zoals in dit proefschrift beschreven staat. Op het symposium van de Nederlandse Vereniging van Toxicologie in 2014 presenteerde hij een gedeelte van het werk uit dit proefschrift en ontving daarvoor een prijs. Sinds December 2013 is hij werkzaam bij de afdeling 'Preclinical Development & Safety' van Janssen in de rol van toxicoloog.

List of publications

1: **Bueters RR**, Jeronimus-Klaasen A, Maicas N, Florquin S, van den Heuvel LP, Schreuder MF. The Effects of Early Postnatal Diuretics Treatment on Kidney Development and Long-Term Kidney Function in Wistar Rats. Nephron. 2016; 132(2):110-8.

2: **Bueters RR**, Klaasen A, Maicas N, Florquin S, van den Heuvel LP, Schreuder MF. Impact of Early Postnatal NSAID Treatment on Nephrogenesis in Wistar Rats. Birth Defects Res B Dev Reprod Toxicol. 2015 Oct;104(5):218-26.

3: **Bueters RR**, Kusters LJ, Klaasen A, van den Heuvel LP, Schreuder MF. Antibiotics and renal branching morphogenesis: comparison of toxicities. Pediatr Res. 2014 Dec;76(6):508-14.

4: **Bueters RR,** Klaasen A, van den Heuvel LP, Schreuder MF. Effect of NSAIDs and diuretics on nephrogenesis in an ex vivo embryogenic kidney model. Birth Defects Res B Dev Reprod Toxicol. 2013 Dec;98(6):486-92.

5: **Bueters RR,** van de Kar NC, Schreuder MF. Adult renal size is not a suitable marker for nephron numbers: an individual patient data meta-analysis. Kidney Blood Press Res. 2013;37(6):540-6.

6: Schreuder MF, **Bueters RR**, Allegaert K. The interplay between drugs and the kidney in premature neonates. Pediatr Nephrol. 2014 Nov;29(11):2083-91.

7: Schreuder MF, **Bueters RR**, Huigen MC, Russel FG, Masereeuw R, van den Heuvel LP. Effect of drugs on renal development. Clin J Am Soc Nephrol. 2011 Jan;6(1):212-7.