AN ABSTRACT OF THE DISSERTATION OF

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Title: Programming of Furosemide Pharmacokinetics by Intrauterine Growth Restriction and High Fat Diet in the Adult Rat.

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Ganesh Cherala

Perinatal growth restriction programs higher risk for chronic disease during adulthood via morphological and physiological changes in organ systems. Perinatal growth restriction is highly correlated with a decreased nephron number, altered renal function and subsequent hypertension. I hypothesize that such renal maladaptations result in altered pharmacologic patterns for life. Pregnant and lactating rats were fed either a purified control diet (18% protein) or low protein diet (9% protein) to produce perinatal growth restriction, also known as intrauterine growth restriction (IUGR) in the offspring. The diuretic response of furosemide (2mg/kg single i.p dose) in adult IUGR rats was investigated. Diuresis, natriuresis and renal excretion of furosemide were significantly reduced relative to controls, indicative of decreased efficacy. In the follow-up study, offspring were weaned onto either lab chow (11% fat) or high fat diet (45% fat). Adult offspring were dosed with furosemide (10mg/kg i.p. dose), and serum

and urine collected. The pharmacokinetic parameters were significantly altered by both IUGR and the high-fat diet; these effects appeared to be additive. The overall exposure profile in IUGR males was significantly reduced due to a ~35% increases in both clearance and volume of distribution. Females appeared resistant to the IUGR phenotype. The effects of the high fat diet trended in the opposite direction to that of IUGR, with increased drug exposure due to decreases in both clearance (31% males, 46% females) and volume of distribution (24% males, 44% females), with a 10% longer half-life in both genders. The alterations in furosemide pharmacokinetics and pharmacodynamics were explained by changes in the expression of renal organic anion transporters-1 and -3, and NKCC2. In summary, this study suggests that IUGR and diet interact to produce sub-populations with similar body weights but dissimilar pharmacokinetic profiles; this underlines the limitation of one-size-fits-all dosing which does not account for physiological differences in body composition resulting from IUGR and diet.

Programming of Furosemide Pharmacokinetics by Intrauterine Growth Restriction and High Fat Diet in the Adult Rat

by

Barent Nathan DuBois

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Barent Nathan DuBois, Author

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CONTRIBUTION OF AUTHORS

Mr. Jacob Pearson and Mr. Tahir Mahmood assisted with the design, data collection, and data interpretation for Chapters 2 and 3. Dr. Duc Nyguyen assisted with data collection in Chapter 2. Dr. Kent Thornburg assisted with data interpretation and writing of Chapters 2 and 3. Dr. Ganesh Cherala assisted with design, data interpretation, and writing for Chapters 2 and 3.

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INTRODUCTION

For the clinician prescribing drugs, the patient is a "black box" of diverse physiological processes which ultimately determine a drug's fate. Pharmacokinetics is the branch of pharmaceutical science which studies the body's effect on drugs; how a patient's physiology creates a terrain across which drugs traverse. Four processes known as 'ADME' or absorption, distribution, metabolism, and elimination are described by pharmacokinetic parameters. These parameters are typically estimated from the *in vivo* blood concentration-time profiles for a given drug, but data from other biological fluids (such as urine or saliva) and *in vitro* assays inform these parameter estimates. Parameters of interest include the area-under-the-curve of concentration time profile, a measure of overall drug exposure; clearance, which describes the elimination of drug; volume of distribution, or the extent to which drug permeates into tissues; and half-life, an amalgam of distribution and elimination which illustrates how long the drug persists in the body.

Many adverse drug events are the result of our poor understanding of inter-individual variability in pharmacokinetics. The pharmacokinetic parameters described above are a consequence of an array of molecular mechanisms; examples include a drug's affinity for protein in the blood, metabolizing enzymes, transporter enzymes, and tissue permeability. Given this complexity of human biology, weight-based or one-size-fits-all dosing may not be sufficient to optimize therapy in the general population. Therefore identifying demographic factors that explain pharmacokinetic variability is crucial. Such factors exert their influence on drug distribution and

clearance because of the underlying mechanistic differences in physiology between men and women, and the old and the young. The purpose of the work presented in this dissertation is to examine if a new factor – birth weight – has similar expository potential.

The importance of birth weight as a predictor of health throughout the lifespan was first observed by Dr. David J.P. Barker, a British clinician and epidemiologist examining geographic disease rates in the UK [1]. Regional data of obstetric records were linked to death certificates and survivor health records were obtained for 5654 men. These data showed a startling association where men who had the lowest weights at birth and 1 year of age had the highest mortality rates of ischemic heart disease. A follow-up study of this cohort found that among 468 men aged 64 years those with impaired glucose tolerance had smaller mean weights at birth and 1 year of age; they also observed an inverse relationship between birth weight and adult blood pressure [2]. Subsequent studies in India, China, continental Europe, and the USA have shown similar associations between risk for heart disease, diabetes, and obesity with early life growth rates, especially those born small with a following period of rapid catch-up growth in later childhood and adolescence [3-6]. These studies began a paradigm shift in biology and medicine, described as “fetal programming” or more recently “the developmental origins of health and disease” hypothesis [7].

What has been learned from subsequent studies in humans and animal models is that an adverse intrauterine environment – one where nutrient flow to the developing fetus is suppressed – produces suboptimal growth and development of fetal organ systems, now called “intrauterine growth restriction” (IUGR), increasing the risk for

metabolic syndrome [3, 7]. The IUGR phenotype in human and animals is consistently associated with decreased (30%) numbers of nephrons and cardio myocytes, leading to elevated risks for renal and cardiac dysfunction. These deficits also contribute to an increased risk for hypertension and many forms of cardiovascular disease [8-13]. Thus the etiology of chronic disease in IUGR adults is not simply the current environment or lifestyle, but in the adaptations which occurred *in utero*. Mechanistic studies in animals have been done in a variety of animal species which include guinea pigs, rats, sheep, pigs, and non-human primates; and a variety of growing strategies to produce the IUGR phenotype include intrauterine ligation, maternal low protein diet, calorie restriction, glucocorticoid exposure, hypoxia, and iron restriction [7, 14]. We chose the maternal low protein diet in the rat, where pregnant rats are fed a low protein diet during gestation and lactation, to produce the IUGR phenotype for our studies. It is a well characterized, less invasive model for IUGR where the resultant offspring have low birth weight, stunted growth, and altered renal function [13, 15-18]. The particular disease pathologies can vary depending on the type of stress and the developmental period during which the insult is experienced, such as the window during the perinatal period when the protein restriction occurs (mid vs. late gestation vs. lactation, or all three), the gender of the offspring, and the extent of the protein dietary deficit [13, 17-20]. But overall, these diseases fall under the umbrella of cardiovascular disease and the metabolic syndrome.

The predisposition for chronic disease increases the likelihood that IUGR individuals will need to be treated with pharmaceutical agents over their lifetime. However, it is not clear whether one-size-fits-all dosing will be sufficient to optimize

pharmacotherapy in this population. Given these baseline differences in physiology, especially in organs such as the kidneys which are so critical for the clearance and elimination of drugs from the body, we would not assume that drug distribution and clearance would behave similarly to "normal" birth weighted subjects. Previous reports have associated IUGR with altered drug responses; these include blood pressure and diuresis with antihypertensive agents, hexobarbital-induced sleep time, and indomethacin anti-inflammatory response [16, 21, 22]. These associations between IUGR and altered drug response, and the resultant physiological maladaptations observed in IUGR models in animals and humans led us to the studies presented in this dissertation.

The primary objective of the two studies presented here was to examine whether pharmacokinetic factors are altered by the IUGR phenotype. The associations between IUGR and renal dysfunction led us to test this hypothesis using a drug which undergoes extensive renal clearance. The loop diuretic furosemide was our proof of concept drug of choice for these studies for this reason and because its disposition and clearance are dependent on several different mechanisms. It is a low extraction, highly protein bound drug where differences in free-fraction contribute to variability in action [23, 24]. Furosemide is cleared primarily by the renal transport proteins, Oat-1 and -3, but non-renal clearance (an amalgam of biliary excretion and hepatic biotransformation) is a significant confounder [25-28].

The principle focus of the 1st study (chapter 2) was to demonstrate if the renal excretion (estimated from urine data) and diuretic effect of furosemide was altered by the IUGR phenotype. A decrease in both furosemide's renal excretion and the extent

of diuresis in adult IUGR offspring was observed, and mechanistic experiments were performed to determine if protein binding, metabolizing enzyme activity, and transporter expression explained this observation. The limitation of this 1st study was the lack of *in vivo* serum concentration-time data from which we could estimate *in vivo* pharmacokinetic parameters. The 2nd study (chapter 3) connects the 1st by including *in vivo* serial blood sampling and along with urine data and *in vitro* assays. It is well known that the compromised IUGR physiology can be further stressed in later life by the "mismatch" between the poor nutritional milieu *in utero* followed by diets high in fat and sugar after birth [6, 11, 29, 30]. Thus the 2nd study also included a high fat post-weaning diet to test the hypothesis that environmental mismatch exacerbates compromised pharmacokinetics beyond that found by IUGR alone.

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PERINATAL GROWTH RESTRICTION DECREASES DIURETIC ACTION OF FUROSEMIDE IN ADULT RATS

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Abstract

Perinatal growth restriction programs higher risk for chronic disease during adulthood via morphological and physiological changes in organ systems. Perinatal growth restriction is highly correlated with a decreased nephron number, altered renal function and subsequent hypertension. We hypothesize that such renal maladaptations result in altered pharmacologic patterns for life. Maternal protein restriction during gestation and lactation was used to induce perinatal growth restriction in the current study. The diuretic response of furosemide (2mg/kg single i.p dose) in perinatally growth restricted rats during adulthood was investigated. Diuresis, natriuresis and renal excretion of furosemide were significantly reduced relative to controls, indicative of decreased efficacy. While a modest 12% decrease in diuresis was observed in males, females experienced 26% reduction. It is important to note that the baseline urine output and natriuresis was similar between treatment groups. The *in vitro* renal and hepatic metabolism of furosemide, the *in vivo* urinary excretion of the metabolite, and the expression of renal drug transporters was unaltered. Creatinine clearance was significantly reduced by 15% and 19% in perinatally growth restricted male and female rats, respectively. Further evidence of renal insufficiency was suggested by decreased uric acid clearance. Renal protein expression of sodium-potassium-chloride cotransporter, a pharmacodynamic target, was unaltered. In summary, perinatal growth restriction could permanently imprint pharmacokinetic processes affecting drug response.

1. Introduction

Poor nutrition during perinatal development leads to growth restriction and low birth weight, the consequences of which are both pernicious and permanent. The relationship between early life growth restriction and the predisposition for adult-onset type-2 diabetes, hypertension, and obesity has been consistently reported in human epidemiological studies and animal models [1-3].

The higher risk for chronic disease in adults who were growth restricted at birth and postnatally stems from maladaptations, both morphological and physiological, that occur during perinatal development [4, 5]. Restricted nutrient flow from mother to fetus, due to poor nutrition or placental insufficiency, prioritizes development such that organs like the brain are preserved at the expense of other organ systems [6]. Of the sacrificial organs, the kidney has received the most attention. Perinatal growth restriction in humans is strongly correlated with fewer nephrons/glomeruli and reduced renal volume, a consequence of reduced nutrient delivery and reduced blood flow to kidneys during fetal development [7-9].

Given their predisposition to chronic diseases, perinatally growth restricted adults are targets for pharmacological therapies; however, very little is known about the comparative effectiveness of pharmacological interventions in this population. The association between perinatal growth restriction and renal dysfunction led us to test the hypothesis that perinatal growth restriction-induced maladaptations in renal physiology could result in altered pharmacology for drugs which undergo extensive renal clearance. The loop diuretic furosemide was our drug of choice for this study because the kidney is its site of action, as well as its major route of elimination [10].

These characteristics make urinary furosemide excretion a robust predictor of diuretic efficacy as well as an estimate of renal clearance [10-13].

In the current study, we used a well-characterized rat model of perinatal growth restriction to examine the pharmacology of furosemide in adult rats that had low birth weight due to maternal protein restriction during pregnancy and lactation. We monitored the extent of diuresis, and urinary excretion of sodium, furosemide and its metabolite, furosemide-glucuronide. Furosemide is extensively plasma/serum protein bound, undergoes vectorial transport into the proximal tubule, and is renally metabolized [14-17]. To characterize these potential mechanisms for variability in renal clearance, we measured *in vitro* expression of drug transporters and metabolism in hepatic and renal microsomes. We also measured creatinine and uric acid clearance as surrogate markers for altered renal physiology.

2. Materials and methods

2.1 Diets

Modified versions of the AIN76A purified diet (control, 19% protein) and the corresponding isocaloric low-protein diet (LPD, 8% protein) formulations were obtained in pellet form from Purina Test Diets (Richmond, IN). Detailed compositions of both diets are available in the literature [18].

2.2 Experiments with dams

The study was approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University (OHSU), Portland, OR. Sprague-Dawley rats (Charles River Laboratories, Inc., Hollister, CA) were mated by housing one male

rat with two virgin female rats. Day 1 of pregnancy was assigned upon observation of sperm in the daily morning vaginal smears, at which time rats were randomly assigned to one of the two diet groups. Each diet group consisted of 5-6 pregnant rats, and these rats received the assigned diet *ad libitum* throughout pregnancy and lactation.

2.3 Experiments with offspring

Upon birth, litter size and sex of pups were recorded. All litters were randomly culled to 12 pups (6 male and 6 female) on the day of birth and further randomly culled to 8 pups (4 males and 4 females) on day 4 after birth. The step-wise culling was practiced to adjust for any loss of pups during the first 72 h following birth [18]. Offspring from both groups were weaned on day 28 and were housed in isosexual groups according to perinatal diet treatment. Different dietary treatments were administered only during gestation and lactation, and all pups were weaned onto a rodent laboratory chow with access to *ad libitum* food and water.

At day 120 and 150, two male and two female offspring from each litter were housed in separate metabolic cages with *ad libitum* food and water. At day 120, urine was collected over a 24-hour period. Blood was collected at the end of 24-hour period (9:00am) the following morning, and spun at 3,000 x g to collect serum within 30min of blood collection. At day 150, between 8 AM and noon, a 4-h baseline urine was collected upon intraperitoneal dosing with 1ml/kg of vehicle (isotonic saline). The following day, a 2 mg/kg intraperitoneal dose of furosemide was administered at 8 AM, followed by a 4-h urine collection. Urine outputs were measured during the same time of the day on both days in order to minimize diurnal variations [19]. Drug and vehicle administration was carried outside of the metabolic cage. Handling of rats prior to

placing on metabolic cage led to voiding of the bladder resulting in less confounding of urine output data. Similarly, at the end of urine collection window, rats were manipulated within the cage to result in the emptying of the bladder. The collection tube was protected from light to minimize photo-degradation of furosemide. The urine volumes were recorded, and an aliquot was stored at -80°C.

About 4-5 days after furosemide administration, animals were euthanized. Prior to organ collection, blood was collected via cardiac puncture; serum was isolated within 30 min of blood collection and stored at -80°C. Liver and kidneys were collected and weighed, snap-frozen in liquid nitrogen, and stored at -80°C.

2.4 Preparation of kidney and liver samples

Crude plasma membranes were generated as previously described [20]. Briefly, half of whole frozen rat kidney or ~2 grams of liver tissue was removed and homogenized in 12 ml of sucrose buffer (0.25 M sucrose, 0.10 M Tris, 0.1 mM phenylmethylsulfonyl fluoride) using a dounce homogenizer. The homogenate was centrifuged at 2,500 × **g** for 15 min at 4°C. The supernatant was then removed and centrifuged at 24,000 × **g** for 20 min at 4°C. The beige fluffy upper layer of the resulting pellet (crude plasma membranes) was resuspended in 1 ml of supernatant and stored at -80°C. The remaining supernatant was further centrifuged at 105,000 × **g** for 60 min at 4°C. The resultant microsomal pellet was resuspended in sucrose buffer and stored at -80°C. The protein concentration of the crude plasma membranes and microsomes were measured using the Bradford method [21].

2.5 Western Blotting

Crude renal plasma membrane samples were subjected to Western blotting. Briefly, samples were reduced with Laemmli sample buffer (Bio-Rad, Hercules, CA) and denatured in a 100°C water bath for 3 min. Samples were diluted to equal protein concentrations and 30 µg was loaded onto a 10× Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, Hercules, CA) and subjected to electrophoresis at 250 mV for 20 min. Samples were then transferred onto a low fluorescence PVDF membrane (Bio-Rad, Hercules, CA), blocked with Odyssey Blocking Buffer (LiCor Biosciences, Lincoln, NE), and incubated with primary antibody (Rabbit anti-rat antibodies; Alpha Diagnostic Inc, San Antonio, Tx). The membrane was then incubated with 1:15000 concentration IRDye® 800RD secondary antibody (LiCor Biosciences, Lincoln, NE) before being imaged on Odyssey Imaging System (Licor Biosciences, Lincoln, NE).

To ensure proper quantification, an absorption test with a manufacturer blocking peptide (Alpha Diagnostic Inc, San Antonio, Tx) was performed. Bands absorbed by the blocking peptide were quantified, using NIH ImageJ software. Recent findings from our work indicated alterations in the expression, albeit at mRNA level, of commonly used endogenous controls in the same samples [22]. Hence, the design of Western blotting experiments did not include an endogenous loading control. Equal loading of samples was ensured through rigorous determination of protein concentrations (in replicates) and loading of equal volumes of the same concentration, further confirmed by Ponceau S staining. Relative densitometric signal of each sample was obtained upon normalization to an assay control sample.

2.6 Determination of in vitro *glucuronidation of furosemide*

The *in vitro* formation of furosemide glucuronide was measured in renal and hepatic microsomes, using a method adapted from Kerdpin *et al.* [23]. Briefly, microsomes were preincubated with alamethicin (12 µg/mg protein) on ice for 30 min. 10-100 µg of activated microsomes were then incubated with 300 µM of furosemide, 10 mM uridine 5'-diphosphoglucuronic acid trisodium salt in 100 µl of 7.4 pH 100 mM potassium phosphate buffer with 1.0 mM EDTA and 5 mM $MgCl_2$ at 37^0C. After 90-120 min of incubation, the reaction was terminated by adding 100 µl of ice cold methanol and 3% acetic acid, vortexed, and spun at 9000 × **g** for 5 min. The supernatant was analyzed using high performance liquid chromatography (HPLC), as described below. The enzyme activity was reported as the amount of furosemide-glucuronide formed/min/mg of microsomal protein. Time-course experiments ensured linearity of reaction-rate and incubation with beta-glucuronidase from *Helix promatia* identified the furosemide-glucuronide peak.

2.7 Determination of in vitro *serum protein binding of furosemide*

To determine whether there are differences in furosemide binding to serum proteins, we performed an *in vitro* binding study. Briefly, rat serum from each group was pooled and spiked with furosemide to obtain a 30 µM final concentration. The above concentration was chosen to mimic serum concentrations of furosemide in adult rats upon dosing with 2mg/kg dose (assuming 100% bioavailability). Serum was incubated at 37^oC in a water bath for 10 min. An aliquot was removed to determine total furosemide. For estimation of unbound concentrations of furosemide, 0.75 ml of serum was filtered through a 30,000 molecular weight cut-off Millipore Centrifree YM-30 (Millipore Corporation, Billerica, MA) cellulose filter by centrifugation at

2,000 × **g** for 60 min at room temperature. Ultra filtrate obtained after centrifugation was processed and analyzed for furosemide, using HPLC method described below.

The free fraction (f_u) was determined as a ratio of unbound concentration to total concentration of furosemide. The percent increase in free fraction in the LPD group ($f_{u, LPD}$), compared to control group ($f_{u, control}$) was computed as ($f_{u, LPD}$ - $f_{u, control}$) × 100/ $f_{u, control}$.

2.8 Quantitative analysis of urinary furosemide and furosemide-glucuronide

Urine samples (100 µl) were diluted with 100 µl methanol and 3% acetic acid, vortexed, and spun at 9000 × **g** for 5 min. The supernatant was analyzed using a HPLC method adapted from Smith *et al.* [24], with a Shimadzu LC-20AD Prominence liquid chromatography on a Phenomenex 2.6 µ C18 reversed-phase Kinetex column (100 cm × 4.60 mm), and quantitated with a Shimadzu RF-10AXL fluorescence spectrophotometer at excitation/emission wavelengths of 345/405 nm. At a flow rate of 0.9 ml/min in a solvent system of 28% acetonitrile and 30 mM H_3PO_4, furosemide and furosemide-glucuronide had retention times of 6.50 and 4.25 min, respectively. The *in vitro* incubation samples as well as serum samples were run at 35% acetonitrile and 15 mM H_3PO_4, with furosemide and furosemide-glucuronide retention times of 3.5 and 1.9 min, respectively. A standard curve of furosemide was constructed in the linear range of 10–100 µg/ml. Inter- and intra-day variability in slopes was <10%. Equality of extinction coefficients was assumed between furosemide and the glucuronide metabolite because the glucuronic-acid cofactor did not fluoresce at 345/405 nm wavelength.

2.9 Quantitative analysis of urinary sodium

Sodium concentrations were measured using flame photometry (Cole-Parmer Instrument Company, Model 2655-10, Vernon Hills, IL).

2.10 Quantitative analysis of serum and urine creatinine and uric acid

Serum and urinary concentrations of creatinine and uric acid were determined by the simultaneous method by Zhiri *et al [25]*. Briefly, 10 µL of urine/plasma was diluted with 495 µL of acetonitrile, vortexed, and spun 9000 x **g** for 10 min. Ten µL of supernatant was injected onto a 100 x 3 mm, 2.6 µ Kinetex XB-C18 column (Phenomenex Inc, Torrance, CA), and quantitated with a Shimadzu SPD-20A UV detector at 235nm. At a flow rate of 0.5 ml/min in solvent system of 30 mM ammonium acetate and 156 mM methanol pH 7.0, uric acid and creatinine had retention times of 3.33 and 4.00 min, respectively. A standard curve for creatinine was constructed in the linear range of 12.5-400 µg/ml, and 3.125-100 µg/ml for uric acid. Inter- and intra-day variability in slopes was <10%.

2.11 Quantitation of albumin

Serum concentrations of albumin were determined as described by Hayashi T *et al.* [26]. Briefly, 2 µl of unprocessed rat serum was injected onto Asahipak ES-502N column (100× 7.5 mm; Phenomenex Inc, Torrance, CA) maintained at 37^{o}C. The mobile phase consisted of buffer (0.05 M sodium acetate 0.40 M sodium sulfate (pH4.85)) and ethanol pumped at 1 ml/min. The gradient program includes ethanol concentration from 0 to 10% v/v from 0 to 50 min, followed by a return to baseline by 55 min, and 10 min of column equilibration. The eluent was monitored using a

fluorescence detector at 280/340 nm excitation and emission wavelengths, respectively.

2.12 Data analysis

Data were tested for normality and equal variance, and expressed as mean ± S.D., unless otherwise specified. Two-way ANOVA was conducted using factors [Diet (control vs. LPD) × gender (male vs. female)] followed by Student-Newman-Keuls post-hoc test. For non-parametric data, two-way ANOVA on ranks was performed. All statistical tests were conducted at $p<0.05$ (SigmaPlot v11.0, San Jose, CA).

All experiments include a sample size of 5-6 litters in each diet group, except for *in vitro* protein binding study where serum from all litters in a diet group were pooled to perform the experiment. Whenever data from two or more littermates were available, an average value was calculated, and considered as a single data point. The arithmetic mean ± SD of the litters (5-6) was reported. This is the recommended method of calculating a mean for multiparous species to avoid artificial inflation of the power of statistical tests [27, 28].

3. Results

3.1 Confirmation of in utero growth restriction (IUGR)

In the current study, both male and female offspring in the LPD group had 16% reduction in birth weight, confirming IUGR (male: 6.61 ± 0.71 g (mean ± S.D., control) vs. 5.53 ± 0.42 g (LPD), $P<0.05$; female: 6.15 ± 0.74 g vs. 5.17 ± 0.34 g; $P<0.05$; Table 1). Birth weight was also significantly lower in females, compared to males, in both diet groups ($P<0.01$). Body weight at day 150 was significantly lower

for both genders in the LPD group (male: 676 ± 48 g vs. 521 ± 54, $p<0.001$; female: 330 ± 29 g vs. 264 ± 17 g, $P<0.05$). Body weight at day 150 was significantly lower in females, compared to males, in both diet groups ($P<0.01$). Absolute kidney weight was significantly lower in LPD diet group (male: 2.48 ± 0.19 g vs. 1.92 ± 0.22; $p<0.001$; female: 1.28 ± 0.11 g vs. 0.98 ± 0.04; $P<0.001$). However, kidney weight normalized to body weight was similar in both diet groups. Gender differences in absolute kidney weight ($P<0.001$) were also ameliorated upon normalization to body weight. Absolute liver weight was also significantly less in LPD diet group (male: 22.22 ± 3.66 g vs. 16.76 ± 2.09; $P<0.001$; female: 11.05 ± 1.19 g vs. 8.32 ± 0.35; $P<0.001$). Similar to kidney, gender differences were present ($P<0.001$), but no differences were observed in liver weight normalized to body weight in either treatment or gender groups.

3.2 Perinatal growth restriction decreases furosemide diuretic response during adulthood

In 150-day old male offspring, pre-dose (baseline) urine output was similar between diet groups (male: 5.56 ± 2.07 ml/kg of body weight, control vs. 5.74 ± 0.94, LPD; female: 6.38 ± 3.88 ml/kg vs. 3.93 ± 1.64; Fig 1A). The extent of diuresis in LPD group animals, however, was decreased compared to the control group (male: 19.21 ± 2.64 ml/kg vs. 16.72 ± 1.90, $P<0.05$; female: 15.74 ± 3.77 ml/kg vs. 11.69 ± 1.54; $P<0.05$; Fig 1B). The altered diuretic response was corroborated by urinary excretion rate of sodium. Significantly lower natriuresis was observed in LPD group offspring upon dosing with furosemide (males: 259.8 ± 61.5 μmoles/hr vs. 184.9 ± 37.6, $p<0.05$; females: 110.8 ± 38.2 μmoles/hr vs. 74.6 ± 15.7, $P<0.05$; Fig 1D). There

was no difference in pre-dose sodium excretion (males: 95.94 ± 54.80 μmoles/hr vs. 97.70 ± 42.88; females: 57.38 ± 14.76 μmoles/hr vs. 46.65 ± 27.66; Fig 1C), Compared to males, females excreted significantly smaller amounts of sodium in both diet groups during pre-dose and dosed periods ($P<0.001$).

As expected, furosemide treatment has resulted in increased urine output in male and female offspring of both diet groups ($P<0.01$). Similarly, increased natriuresis was observed in male and female offspring of control diet groups and male offspring of LPD group ($p<0.05$), but not in female offspring of LPD group.

3.3 Expression of pharmacodynamic receptor is unaltered by perinatal growth restriction

To determine whether the reduced diuretic response could be explained by changes in the furosemide pharmacodynamic receptor Nkcc2, protein expression was quantified using Western blotting was (Fig 2A-B). Nkcc2 expression in LPD rats was unaltered in males (0.96 ± 0.12, control vs. 0.85 ± 0.35, LPD) and in females (0.76 ± 0.18 vs. 0.71 ± 0.29).

3.4 Urinary excretion of furosemide is decreased in perinatally growth restricted rats

We measured urinary excretion rate of furosemide as an overall marker of renal clearance processes (Fig 2C). The percentage of the dose excreted was decreased in the LPD group (male: 61.11 ± 4.53 %dose, control vs. 51.64 ± 5.88, LPD; $P<0.01$; female: 47.78 ± 8.45%dose vs. 40.58 ± 7.56; $P<0.01$). Additionally, females in both diet groups excreted a significantly smaller fraction of the dose, compared to their male counterparts ($P<0.001$).

3.5 Expression of renal drug transporters is unaltered by perinatal growth restriction

To determine whether the reduction in urinary furosemide excretion is caused by altered renal transport of furosemide, we measured the protein expression of Oat1 and Oat3 drug transporters. Molecular weights quantified for Oat1, and Oat3 were 70, and 110 kDa, respectively (Fig 3A-B). There was no perinatal diet effect on the expression of transporter proteins in either male or female rats (Fig 3C-D). The Oat1 and Oat3 expression in males, compared to females, was higher in both diet groups ($P<0.05$).

3.6 Renal and hepatic metabolism is not increased by perinatal growth restriction

The decreased urinary excretion of furosemide could be a result of increased furosemide metabolism. The LPD did not alter the *in vitro* rate of formation of furosemide-glucuronide in renal microsomes (male: 172 ± 27 pmoles/min/mg of microsomal protein, control, vs. 179 ± 16, LPD; female: 86 ± 23 pmoles/min/mg vs. 69 ± 18, Fig 4A). Significantly slower renal *in vitro* metabolism was observed in females of both diet groups compared to males ($P<0.001$). The hepatic *in vitro* rate of formation of furosemide-glucuronide was significantly decreased in LPD males but unchanged in LPD females (male: 119 ± 15 pmoles/min/mg, control vs. 87 ± 13, LPD, $P<0.05$; female: 83 ± 32 pmoles/min/mg vs. 102 ± 9; Fig 4B). Both control- and LPD-group animals excreted similar amounts of metabolite in urine, normalized to the total furosemide dose, in each gender (male: 0.78 ± 0.35% vs. 1.19 ± 0.66%; female: 1.11 ± 0.38% vs. 0.75 ± 0.23%; Fig 4C). There were no gender differences in either group.

3.7 Serum protein binding of furosemide is increased by perinatal growth restriction

The serum protein binding of furosemide was estimated in 150-day old rats in a pooled serum sample (data not shown). Enough serum was available to perform

measurement in singlicate in isosexual animals of each diet group. The percent increase in free fraction, compared to control rats, was 18.6% and 7.1% in male and female rats of the LPD group, respectively. The male rats in each diet group exhibited 20% higher free fraction than their female counterparts. Free-fraction estimates of furosemide in the serum of male rats were 0.0041 (control) and 0.0049 (LPD), and in female rats were 0.0033 (control) and 0.0035 (LPD). The changes in free fraction were noted in the absence of alterations in serum albumin concentrations (data not shown).

3.8 Creatinine and uric acid clearance are decreased in perinatally growth restricted rats

We estimated creatinine and uric acid clearance as surrogate markers of renal function (Fig 5A-B). The estimated creatinine clearance was decreased in LPD group (male: 8.22 ± 1.75 mL/min, control, vs. 6.95 ± 1.51, LPD, $P<0.05$; female: 4.22 ± 0.63 mL/min vs. 3.42 ± 0.25; $P<0.05$). Females in both diet groups had significantly lower creatinine clearance compared to males ($P<0.001$). Similarly, uric acid clearance was decreased in LPD group (male: 0.41 ± 0.11 mL/min, control, vs. 0.24 ± 0.09, LPD, $P<0.001$; female: 0.19 ± 0.02 vs. 0.12 ± 0.04; $P<0.001$). Additionally, females in both diet groups had less uric acid clearance compared to males ($P<0.001$).

4. Discussion

An adverse intrauterine environment leading to growth restriction is robustly associated with a higher risk of chronic diseases during adulthood. Pharmaceutical interventions are a vital mode of treatment and management of these diseases; however, the efficacy of drug treatment in adults who were *in utero* as well as

perinatally growth restricted is poorly understood. In the current study using perinatally growth restricted rats, we found that the diuretic efficacy of furosemide is altered due to programming of molecular pharmacokinetic processes.

In growth restricted rats, furosemide-induced diuresis and natriuresis were decreased despite similar baseline urine outputs. Previous reports indicate that furosemide-induced diuresis and natriuresis are best explained by urinary furosemide excretion, instead of plasma concentrations [10-13]; hence, we compared urinary excretion. The diminished pharmacological response of furosemide was corroborated by reduced urinary excretion of furosemide, suggesting that altered pharmacokinetic processes such as drug transport and metabolism may be at play.

Furosemide is actively secreted across the basolateral membrane by Oat 1 and 3[29]. As reported by others [30], we also observed a male-predominant expression of Oat1 which would correlate with the higher urinary excretion rate of furosemide in males in both diet groups. This is further corroborated by other reports in female rats of decreased renal clearance of furosemide and para aminohippuric acid (a prototypical Oat substrate) compared to males [31]. However, there were no differences in transporter expression in growth restricted rats, indicating that the altered urinary excretion of furosemide may be explained by mechanisms other than renal transport.

The predominant furosemide metabolite in humans and rats is furosemide-glucuronide, mediated by uridine 5'-diphospho-glucuronosyltransferase. It has been observed that approximately two-thirds of the metabolic clearance is attributable to kidney, and the remainder in the liver [32]. Similarly, while the majority of the

systemic clearance (sum of metabolic and non-metabolic clearance) of furosemide occurs in the kidney, the liver does contribute; in the rat, hepatic clearance of furosemide has been estimated to account for 20% of systemic clearance [33], and in rats variability in non-renal metabolism can account for variability in the diuresis [17]. In growth restricted rats, there were no differences in either *in vivo* urinary excretion of furosemide-glucuronide or *in vitro* formation of furosemide-glucuronide in renal microsomes. This would suggest that renal metabolism would not explain the difference in urinary furosemide excretion. Given the relative contribution of liver metabolism, we also measured *in vitro* metabolism of furosemide in hepatic microsomes. Curiously, hepatic microsomes from growth restricted males showed a ~27% decrease in the rate of furosemide-glucuronide formation; in females, there were no differences between growth restricted and control, and no gender difference was observed. These trends would not suggest that the decreased urinary excretion of furosemide is explainable by intrinsic hepatic metabolism.

Lastly, although we did not measure nephron number, multiple reports have indicated up to ~30% reduction in the nephron number in a similar animal model [2]. If the concentration of drug transporters and drug metabolizing enzymes in the kidney are unchanged, what could be rate-limiting for urinary excretion is a decrease in the number of nephrons and/or a decrease in renal sufficiency. Creatinine clearance is a common surrogate marker for renal filtration, and uric acid is a less robust but still relevant marker for renal tubular dysfunction [34-36]. We observed in this study significant decreases in both creatinine clearance and uric acid clearance. While the magnitude of these differences in clearance and serum values would not be interpreted

to suggest renal disease, they are suggestive of an overall decrease in the renal function. In this study, perinatally growth restricted rats may not have diseased kidneys but perhaps less efficient kidneys, and of the mechanisms we studied this could be an explanation for the observed decrease in urinary excretion of furosemide. Additionally, furosemide has been shown to induce vasodilation and reduction of cardiac output [37]. Although untested in the current study, could further potentially explain the renal changes post drug administration.

Other sources for this variability in urinary excretion were not examined in the current study. Significant drug loss as a result of first-pass metabolism is reported with intraperitoneal route of administration [17]. Previous studies and the current study noted 40-60% of drug recovery in urine suggesting ~0.5 bioavailability of furosemide upon dosing intraperitoneally [17]. However, differences in bioavailable fraction between the two groups could possibly explain the diminished furosemide response in perinatally growth restricted rats. While lack of serum data to construct a pharmacokinetic profile is typically a limitation for most drugs, the serum pharmacokinetic profile is less informative in the case of furosemide. On the other hand, serum protein binding data is demonstrated to be highly valuable in predicting furosemide delivery to renal tubules [10-13]. Hence, we have obtained preliminary data on serum protein binding of furosemide *in vitro* that show in perinatally growth restricted males and females an 18% and 7% increase in free-fraction of furosemide compared to their respective controls; serum albumin levels being similar between control and LPD groups (data not shown). Serum protein binding of drugs has been shown to be decreased by hyperglycemia [38] and hyperlipidemia [39], both of which

are often observed in IUGR human subjects. While this data is purely speculative and not subject to statistical interrogation (experiment carried out in a pooled serum sample; n=1), growth restricted rats may have an increase in non-renal clearance given that bound-furosemide is more likely to be secreted into kidneys and escape extra-renal distribution and metabolism [14]. Further studies with urinary and serum concentration data are required to thoroughly understand the influence of serum protein binding alterations on the pharmacokinetics of furosemide, especially the degree to which renal and non-renal (hepatic) clearances are affected.

The above discussed alterations in pharmacokinetic processes of furosemide were observed in the absence of any significant changes in the renal expression of the Nkcc2 receptor protein, the pharmacodynamic receptor of furosemide. The lack of change in Nkcc2 observation in this study is in disagreement with increased expression reported in other studies [40, 41], which could be attributable to differences in duration and extent of protein deprivation, age and strain of the animals, and tissue preparation. In the current study, a 2mg/kg dose was chosen for examining the programming effect. Upon considering 65% bioavailability of furosemide in humans [42], this dose would translates into a human equivalent dose of 80mg which is typically the highest dose prescribed to control edema. We speculate that the diuretic and saluretic responses would be further depressed at lower doses in growth restricted subjects, giving rise to larger inter individual variability in furosemide treatment at lower range of doses. Hence, the authors plan to undertake dose response studies to confirm this speculation as well as understand the potency (parallel shift) and/or activity (changes in maximal response) of furosemide.

In summary, this study implicates perinatal growth restriction in drug efficacy during adult life, and adds to the growing list of drugs, currently hexobarbital [43] and indomethacin [44], with pharmacological responses shown to be programmed *in utero*. Lack of programming of pharmacodynamic processes (Nkcc2 expression) underscores the relevance of pharmacokinetic processes to develop varied strategies to personalize medicine in subjects that are growth restricted in their early life. Additional experimentation is needed to identify and characterize molecular and biochemical mechanisms underlying the fetal programming of pharmacokinetics. The clinical relevance of the current study finding is unclear, as the reduction in diuretic response was small. However, mechanistic insights into inter-individual variability of drug response are very valuable and the current study addresses sources of such variability. Furthermore, it is important to consider that birth weight, a marker of IUGR, is an easily available and highly economical (no cost) patient specific information that could contribute significantly towards optimization of pharmacotherapies.

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Table 1 – Birth, body, and organ weights of day 150 old male and female offspring of dams fed either control or LPD during gestation and lactation.

	Males		Females	
	Control	LPD	Control	LPD
Birth Weight (g)	6.61±0.71	5.53±0.42[a]	6.15±0.74[b]	5.17±0.34[a,b]
Body Weight (g)	676±48	521±54[a]	330±29[b]	264±17[a,b]
Kidney Weight (g)	2.48±0.19	1.92±0.22[a]	1.28±0.11[b]	0.98±0.04[a,b]
Kidney Weight (g)/ Body Weight (kg)	3.67±0.22	3.68±0.09	3.89±0.15	3.70±0.25
Liver Weight (g)	22.22 ± 3.66	16.76 ± 2.09[a]	11.05 ± 1.19[b]	8.32 ± 0.35[a,b]
Liver Weight (g)/ Body Weight (kg)	3.28 ± 0.44	3.22 ± 0.28	3.34 ± 0.27	3.16 ± 0.15

[a] denotes significant difference from control diet within the gender; $p<0.05$.

[b] denotes significant difference from males within the diet group; $p<0.05$.

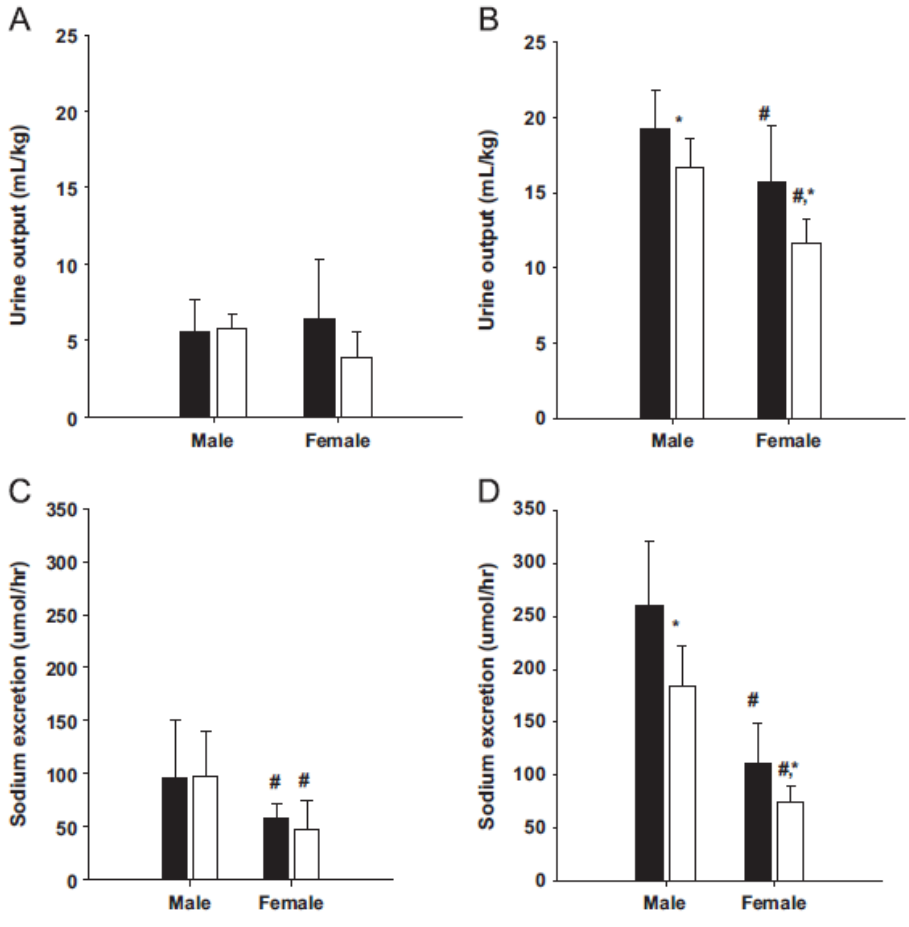

Figure 1. Perinatal programming of diuretic response. Male and female offspring (150 days old) of dams fed either control (closed bars) or low protein (open bars) diets throughout gestation and lactation were administered furosemide (2 mg/kg i.p. dose) at 8 AM. The 4-h urine output was adjusted for body weight (Fig 1A-B). Baseline (pre-dose) urine output was determined the previous day during the same time period (8 AM-noon; Fig 1A). Urinary excretion rate of sodium during pre-dose (Fig 1C) and upon treatment with furosemide (Fig 1D) was also measured. Each bar represents arithmetic mean ± S.D. of 5-6 litters. Whenever data from two or more littermates were available, an average value was calculated and reported as a single data. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) × gender (male vs. female)). Multiple comparisons were tested using Student-Newman-Keuls post-hoc test. * denotes significant differences between control and LPD groups ($P<0.05$). # denotes significant gender differences ($P<0.05$).

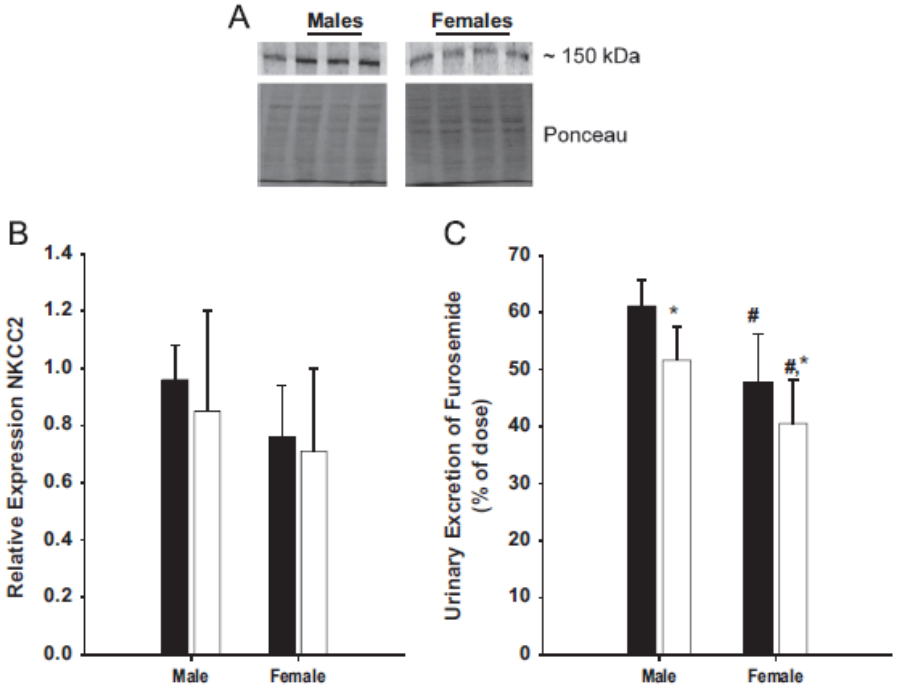

Figure 2. Nkcc2 receptor expression and urinary excretion of furosemide. Representative Western blots of Nkcc2 protein in renal crude plasma membranes of 150-day old rats is shown in panel (A). Alternating control and LPD representative samples are shown for male and female rats, followed by representative section of ponceau stain. Expression of Nkcc2 was detected at 150 kDa, and results were normalized to an assay control (adult renal sample) run simultaneously with samples on all blots to account for blot variability. Relative expression of Nkcc2 (B) and urinary excretion of furosemide (C) in male and female offspring of dams fed either control diet (closed bars) or LPD (open bars) throughout gestation and lactation is shown. Each bar represents arithmetic mean ± S.D. of 5-6 litters. Whenever data from two or more littermates were available, an average value was calculated and reported as a single data. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) × gender (male vs. female)) followed by Student-Newman-Keuls post-hoc test. # denotes significant gender differences ($P<0.05$). * denotes significant differences between control and LPD groups ($P<0.05$).

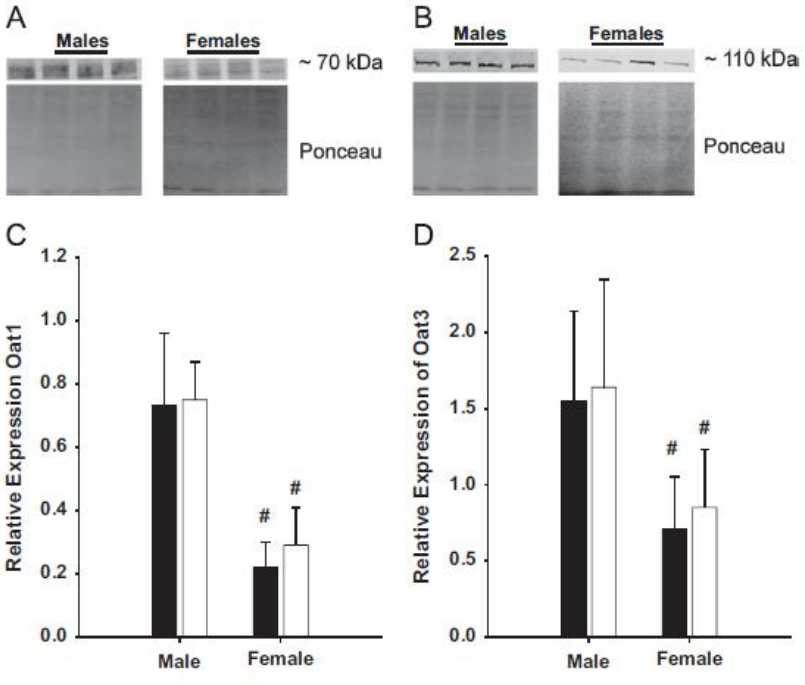

Figure 3. Expression of renal drug transporter proteins in 150-day old rats. Western blots of Oat1, and Oat3 proteins in renal crude plasma membranes is shown in top panels (Oat1 (A); and Oat3 (B)). Alternating control and LPD representative samples are shown for male and female rats, followed by representative section of ponceau stain. Expression results were normalized to an assay control (adult renal sample) run simultaneously with samples on all blots to account for blot variability. Relative expression of Oat1 (C), and Oat3 (D) in male and female offspring of dams fed either control (closed bars) or LPD (open bars) diets throughout gestation and lactation is shown. Each bar represents mean ± S.D. of 5-6 litters. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) × gender (male vs. female)) followed by Student-Newman-Keuls posthoc test. # denotes significant gender differences ($P<0.05$).

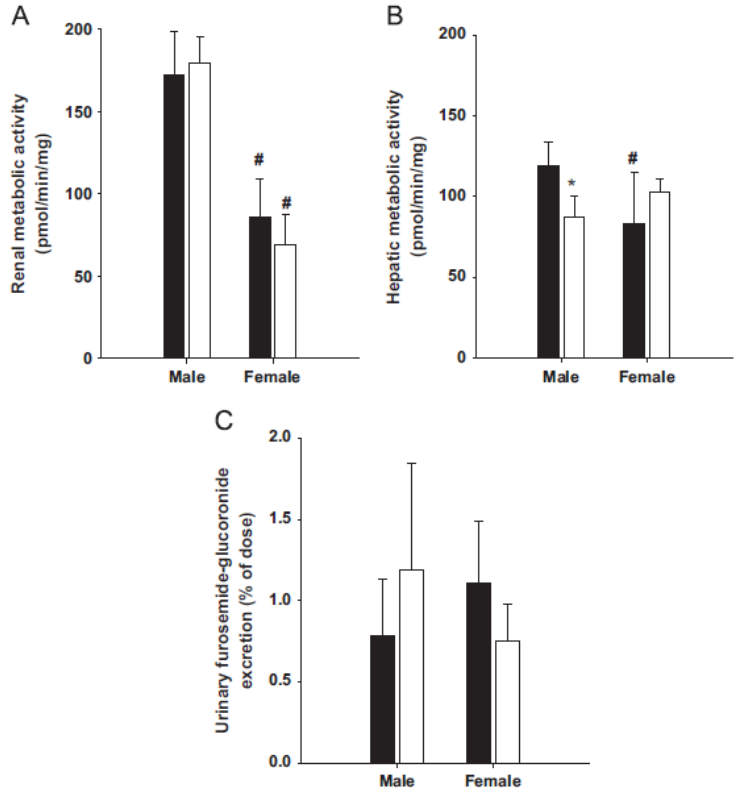

Figure 4. *In vitro* and *in vivo* metabolism of furosemide. The *in vitro* renal (A) and hepatic (B) metabolism of furosemide and *in vivo* renal excretion of furosemide metabolite (C) was determined in 150-day old male and female offspring of dams fed either control (closed bars) or low protein (open bars) diets throughout gestation and lactation. The *in vitro* rate of furosemide metabolism was reported as pmoles of metabolite formed /min /mg of microsomal protein. The urinary excretion of the metabolite was computed as a percent of furosemide dose administered. Each bar represents arithmetic mean ± S.D. of 5-6 litters. Whenever data from two or more littermates were available, an average value was calculated and reported as a single data. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) × gender (male vs. female)) followed by Student-Newman-Keuls post-hoc test. # denotes significant gender differences ($P<0.05$). * denotes significant differences between control and LPD groups ($P<0.05$).

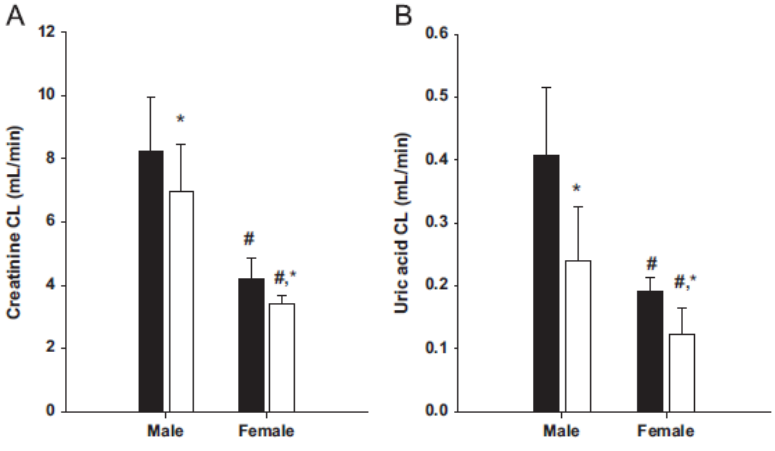

Figure 5. Creatinine and uric acid clearance. Creatinine (A) and uric acid (B) clearance were estimated from serum and urinary concentrations from a 24-hour urine collection in day120 old rats. Each bar represents arithmetic mean ± S.D. of 5-6 litters. Whenever data from two or more littermates were available, an average value was calculated and reported as a single data. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) × gender (male vs. female)) followed by Student-Newman-Keuls post-hoc test. # denotes significant gender differences ($P<0.001$). * denotes significant differences between control and LPD groups ($P<0.05$).

FUROSEMIDE PHARMACOKINETICS IN ADULTS RATS BECOME ABNORMAL WITH AN ADVERSE INTRAUTERINE ENVIRONMENT AND MODULATED BY A POST-WEANING HIGH FAT DIET

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Abstract:

Adult individuals born with intrauterine growth restriction (IUGR) have physiological maladaptations that significantly increase risk for chronic disease. We hypothesized that such abnormalities in organ function would alter pharmacokinetics throughout life, exacerbated by environmental mismatch. Pregnant and lactating rats were fed either a purified control diet (18% protein) or low protein diet (9% protein) to produce IUGR offspring. Offspring were weaned onto either lab chow (11% fat) or high fat diet (45% fat). Adult offspring (5-months old) were dosed with furosemide (10mg/kg i.p. dose), and serum and urine collected. The pharmacokinetic parameters were significantly altered by both IUGR and the high-fat diet; these effects appeared to be additive. The overall exposure profile in IUGR males was significantly reduced due to ~35% increases in both clearance and volume of distribution. Females appeared resistant to the IUGR phenotype. The effects of the high fat diet trended in the opposite direction to that of IUGR, with increased drug exposure due to decreases in both clearance (31% males, 46% females) and volume of distribution (24% males, 44% females), with a 10% longer half-life in both genders. The alterations in furosemide pharmacokinetics and pharmacodynamics were explained by changes in the expression of renal organic anion transporters-1 and -3, and NKCC2. In summary, this study suggests that IUGR and diet interact to produce sub-populations with similar body weights but dissimilar pharmacokinetic profiles; this underlines the limitation of one-size-fits-all dosing which does not account for physiological differences in body composition resulting from IUGR and diet.

1. Introduction

An adverse intrauterine environment – one where nutrient flow to the developing fetus is suppressed – produces suboptimal growth and development of fetal organ systems, now called "intrauterine growth restriction" (IUGR) [1]. Robust evidence in human and animal models demonstrate a link between IUGR and increased risk for cardiovascular disease and the metabolic syndrome [2]; thus, this predisposition for chronic disease increases the likelihood that IUGR individuals will need to be treated with pharmaceutical agents over their lifetime. However, it is not clear whether one-size-fits-all dosing will be sufficient to optimize pharmacotherapy in this population.

The IUGR phenotype in human and animals is consistently associated with decreased (30%) numbers of nephrons and cardiomyocytes, leading to elevated risks for renal and cardiac dysfunction. These deficits also contribute to an increased risk for hypertension and many forms of cardiovascular disease [3-6]. In our model of IUGR, pregnant rats were fed a low protein diet during gestation and lactation resulting in offspring with low birth weight and stunted postnatal growth [7-10]. The IUGR offspring had a reduced urinary excretion of the loop-diuretic, furosemide, compared to rats that had a normal birth weight [11].

The primary objective of the current study was to examine whether pharmacokinetic factors are altered by the IUGR phenotype with furosemide serving as an exemplar. We employed a rigorous protocol with frequent samples of serum and urine to better characterize pharmacokinetic parameters in the low birth weight rats during adulthood. It is well known that the compromised physiology can be further stressed in later life by the "mismatch" between the poor nutritional milieu *in utero*

followed by diets high in fat and sugar after birth [3, 12-14]. Thus this study had as its second objective: to investigate whether mismatched perinatal and post-weaning diets interact to exacerbate alterations in pharmacokinetics. **We tested the hypothesis that the post weaning high fat diet exacerbates compromised pharmacokinetics beyond that found in IUGR alone**.

2. Materials and methods

2.1 Diets

Modified versions of the AIN76A purified diet (control, 19% protein), and the corresponding isocaloric low-protein diet (LPD, 8% protein), and the modified high fat diet 58G8 (high fat, 45% calories from fat) formulations were obtained in pellet form from Purina Test Diets (Richmond, IN). Detailed compositions of all diets are shown in the supplementary table 1.

2.2 Experiments with dams and offspring

The study was approved by the Institutional Animal Care and Use Committee of the Oregon Health & Science University (OHSU), Portland, OR. Sprague-Dawley rats (Charles River Laboratories, Inc., Hollister, CA) were mated by housing one male rat with two virgin female rats. Day 1 of pregnancy was assigned upon observations of sperm in the daily morning vaginal smears, at which time rats were randomly assigned to one of the two diet groups. Each diet group consisted of 6-7 pregnant rats, and these rats received the assigned diet *ad libitum* throughout pregnancy and lactation. At birth, the litter size, birth weights, and sex of pups were recorded. All litters were randomly culled to 12 pups (6 male and 6 females) on the day of birth and further randomly culled to 8 pups (4 males and 4 females) on day 4 after birth. The step-wise culling was practiced to adjust for any loss of pups during the first 72 h following birth [7, 11]. Day 28 old offspring from both groups were weaned onto a rodent laboratory chow (11% energy from fat, 4.05 kcal/g) or the high fat diet (45% energy from fat, 4.74 kcal/g) with access to food and water *ad libitum*, and housed in isosexual groups according to treatment. Restrained animals were measured from the base of the tail to

the nose to estimate length, and body mass index (BMI) was computed as mass normalized to length-squared (kg/m^2). The model produces four distinct treatment groups: normal birth weights fed lab chow (NBW-LC), normal birth weights fed a high fat diet (NBW-HF), low birth weights fed lab chow (IUGR-LC), and low birth weights fed a high fat diet (IUGR-HF).

2.3 Urine and blood sampling scheme for pharmacokinetic study

At day 150, animals were given an intraperitoneal vehicle of isotonic saline at 1ml/kg, and urine collected 6-h and 24-h post dose. The following day, a 10mg/kg intraperitoneal dose of furosemide (injection USP, Hospira Inc, Lake Forest, IL) was administered at 9:00 am, followed by urine collection at 6-h and 24-h post dose. Drug and vehicle administration was carried outside of the metabolic cage. The urine collection tube was protected from light to minimize photo-degradation of furosemide. The urine volumes were recorded, and an aliquot was stored at -80 ^{0}C. After a 24-h wash out period, the animals were dosed a second time intraperitoneally with 10mg/kg furosemide and 0.5ml of blood was collected from the medial saphenous vein at 0, 0.5, 1, 2, 4, 6, 8-h post dose. Samples were allowed to clot on ice for approximately 1-h and spun at 3000 x g to collect serum, which was then stored at -80 ^{0}C.

2.4 Preparation of kidney and liver samples

Animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Kidneys and liver were harvested, patted dry and weighed before snap freezing in liquid nitrogen and storage at -80 ^{0}C. Whole kidney (~2 g) and or minced liver tissue (~2 g) were homogenized in 12 ml of homogenizing buffer (50mM Tries, 0.15M KCl, 7.4 pH). The homogenate was spun at 2500 x g for 15 minutes at 4 ^{0}C. The

supernatant was transferred and spun at 24,000 x g for 20 minutes at 4 ^{0}C. In kidney, the beige fluffy upper layer of the resulting pellet (crude plasma membranes) was collected and resuspended in 1 mL of supernatant and stored at -80 ^{0}C [15]. The remaining supernatant was further centrifuged at 105,000 x g for 1 hr. The resultant microsomal pellet was collected and resuspended in 2 ml of storage buffer (50 mM Tris, 0.25 M sucrose, 7.4 pH).

2.5 Western blotting

Crude renal plasma membrane samples were subjected to Western blotting. Briefly, samples were reduced with laemmli sample buffer (Bio-Rad, Hercules, CA) and denatured in a 100°C water bath for 3 min. For membranes, 20µg of each sample was loaded onto a AnyKD™ Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, Hercules, CA) and subjected to electrophoresis at 250mV for 20 minutes. Samples were then transferred onto a low fluorescence PVDF membrane (Bio-Rad, Hercules, CA), blocked with Odyssey Blocking Buffer (LiCor Biosciences, Lincoln, NE), and incubated with 1:1000 target primary antibody (Rabbit anti-rat antibodies; Alpha Diagnostic Inc, San Antonio, Tx) or 1:100 control (gapdh) primary antibody (Rabbit anti-rat antibodies; Abcam Cambridge, MA). The membrane was then incubated with 1:15000 concentration IRDye® 800CW secondary antibody (LiCor Biosciences, Lincoln, NE) before being imaged on Odyssey Imaging System (Licor Biosciences, Lincoln, NE). Bands were quantified using Licor Image Studio™ Lite software (Licor Biosciences, Lincoln, NE), relative to a gapdh control. Prior to normalization with endogenous control (gapdh), equal loading of samples was ensured through rigorous

determination of protein concentrations (in replicates) and loading of equal volumes of the same concentration, further confirmed by Ponceau S staining.

2.6 Determination of in vitro *glucuronidation of furosemide*

The *in vitro* formation of furosemide glucuronide was measured in hepatic microsomes from previously adapted methods [11, 16, 17]. Briefly, microsomes were preincubated with alamethicin (250 µg/mg protein) on ice for 30 minutes. Activated microsomes (0.1 mg/ml) were then mixed with 600 µM furosemide, 10 mM uridine 5'-diphosphoglucuronic acid trisodium salt in 7.4 pH 100 mM potassium phosphate buffer with 1.0 mM EDTA and 5 mM $MgCl_2$ (total volume 100 µl). After 60 minutes incubation at 37 ^{0}C the reaction was terminated by adding 100 µl of ice cold methanol and 10% acetic acid, vortexed, and spun at 9000 x g for 5 minutes. The supernatant was analyzed using high performance liquid chromatography, as described below.

2.7 Determination of in vivo *furosemide protein binding in serum*

For each animal, serum from several time points after dosing with furosemide was pooled. An aliquot was used to determine total concentrations of furosemide as described below. For estimation of unbound concentrations of furosemide, 0.13 ml of pooled serum was filtered through a 30,000 molecular weight cut-off Millipore Centrifree YM-30 (Millipore Corporation, Billerica, MA) cellulose filter by centrifugation at 2000 x g for 40 min at 4 ^{0}C. Ultrafiltrate obtained after centrifugation was processed and analyzed for furosemide, using the HPLC method described below. The free fraction (f_u) was determined as the ratio of unbound concentration to total concentration of furosemide.

2.8 Quantitative analysis of furosemide and furosemide-glucuronide in urine and serum

Urine, serum, and microsomal samples were diluted 1:10, 1:1.5, and 1:1 respectively, with ice cold methanol and 10% acetic acid, vortexed, and spun at 9000 x g for 5 minutes. The supernatant was analyzed using a HPLC method adapted from previous methods [11, 18], with a Shimadzu LC-20 AD Prominence liquid chromatography on a Phenomenex 2.6 µ C18 reversed-phase Kinetex column (100 cm x 4.60 mm), and quantitated with a Shimadzu RF-10AXL fluorescence spectrophotometer at excitation/emission wavelengths of 345/405 nm. Serum supernatant (5 µl) was injected into a solvent system of 28% acetonitrile and 30 mM H_3PO_4 at a flow rate of 0.9 ml/min, with retention times of 2.8 and 5.7 minutes for furosemide-glucuronide and furosemide, respectively. Urine and microsomal supernatants (5 µl), and for protein binding experiments, total serum supernatant (5 µl) and ultrafiltrate supernatant (30 µl), were injected onto a solvent system of 22% acetonitrile and 30 mM H_3PO_4 at a flow rate of 1.0 ml/min, with retention times of 5.3 and 11 minutes for furosemide-glucuronide and furosemide, respectively.

2.9 Pharmacokinetic analysis

Furosemide serum data were analyzed by noncompartmental methods using WinNonlin v 6.3 (Pharsight, Certara™, St. Louis, MO). Maximum serum furosemide concentrations (C_{max}) were observed values. Area under the curve (AUC) was calculated from time zero to 8 h. Drug half-life ($t_{1/2}$), apparent total clearance (CL/F), and apparent volume of distribution (V_D/F) were generated using standard PK calculations ($t_{1/2}= 0.693/\lambda_z$, where λ_z is the terminal elimination rate constant; CL/F=

dose/AUC; $V_D/F = (CL/F)/\lambda_z$). Apparent renal clearance (CL_{renal}) = (dose excreted in urine/body weight)/serum AUC; apparent non-renal clearance ($CL_{nonrenal}$) = (CL/F) – CL_{renal}.

2.10 Data analysis

Data are expressed as mean ± standard deviation with sample sizes between 5 – 7 litters in each of the four treatment groups. Whenever data from two or more litter mates within the same treatment group were available, an average value was calculated and reported as a single data point. This is the recommended method of calculating a mean for multiparous species to avoid artificial inflation of the power of statistical tests [19, 20].

Data were tested for normality and equal variance, and two-way ANOVA was conducted using factors perinatal diet (maternal low-protein vs. control) and post-weaning diet (high fat vs. lab chow) followed by a Student-Newman-Keuls post-hoc test. Males and females were analyzed separately, except for birth-weight where the two-way ANOVA used the factors of perinatal diet and gender. For non-parametric data, two-way ANOVA on ranks was performed. All statistical tests were conducted at $p < 0.05$ (SigmaPlot v11.0, San Jose, CA). The %-differences are shown in the results when a difference was observed to be significant ($p < 0.05$). For analyses which used two-way ANOVA, the %-difference reflects the average difference within a factor for all levels, unless a significant interaction was observed.

The percentage differences are shown only when a difference was observed to be significant ($p < 0.05$). Unless an interaction was observed, the percentage-effect of IUGR compares the average differences among IUGR-LC and IUGR-HF to NBW-LC

and NBW-HF. The percentage-effect of HF diet compares the average differences among IUGR-LC and NBW-LC to IUGR-HF and NBW-HF.

3. Results

3.1 Morphometrics

A summary of the body measurements taken at birth and in adulthood are shown in Table 1. Birth weight of both male and female offspring of the low protein diet fed dams decreased by 14%. There were no significant differences in litter size or sex ratios between the two diet groups (data not shown).

Adult IUGR males were 25% smaller in body weight, 5% shorter in length, and had a 16% reduction in BMI, compared to NBW males. HF diet males were 12% heavier, 3% longer, and but the BMI was unchanged relative to LC diet males. The liver and kidney weights of IUGR males, relative to NBW males, were 28% and 23% smaller, respectively. However, these differences were not evident upon normalization to body weight. On the other hand, HF diet males had disproportionally smaller livers and kidneys relative to body weight (12% and 15%, respectively).

The adult IUGR females were 19% smaller in body weight, 4% shorter in length, and the BMI was 13% smaller. HF diet females were 18% heavier, similar in length, and BMI was 15% increased compared to LC diet females. The liver and kidney weights of IUGR females were 18% and 27% smaller, respectively, compared to NBW females. The difference persisted after body weight normalization in the kidney, 9% smaller in IUGR females, but the IUGR female liver weight was proportional to

body weight. HF diet females had disproportionally smaller livers and kidneys relative to body weight (14% and 18%).

With respect to morphometrics, there was not a significant interaction between perinatal diet and post-weaning diets; the differences between NBW and IUGR, and between LC and HF diets, were conserved within each group.

3.2 Non-compartmental pharmacokinetics and urinary excretion

The furosemide serum concentration vs. time profiles are shown in Figure 1. A summary of the non-compartmental pharmacokinetic parameters and urinary excretion of furosemide are detailed in Table 2.

In adult IUGR males, C_{max} and AUC were significantly decreased by 27% and 32%. CL_{total} was increased by 34% (CL_{renal} by 38% and $CL_{nonrenal}$ 35%). The V_z was increased by 34%. The $t_{1/2}$ was unchanged among IUGR males. HF diet males had higher C_{max} and AUC, 25% and 32% respectively. The CL_{total} was decreased by 31% (CL_{renal} by 30% and $CL_{nonrenal}$ by 30%). The V_z was decreased by 24%. The $t_{1/2}$ was increased by 10%.

In adult IUGR females, the estimated pharmacokinetic parameters were not changed significantly, except for $CL_{nonrenal}$ which was increased by 15%. Among HF diet females within the NBW and IUGR groups, C_{max} and AUC were increased by 40% and 45%. The CL_{total} decreased by 46% (CL_{renal} by 50% and $CL_{nonrenal}$ 41%). The V_z was decreased by 44%, and the $t_{1/2}$ decreased by 10%.

In males and females, the %-furosemide bound to protein and the renal excretion of furosemide normalized to dose was unchanged by either IUGR or the HF diet. HF

diet males had an increased diuretic effect of 141%, but was unchanged in IUGR males. The diuretic effect was not modulated by either IUGR or HF diet in females. Among the estimated parameters, we did not observe a significant interaction between IUGR and the HF diet: the differences were conserved within each group. As we found for the morphometric features among males, the estimated pharmacokinetic parameters between NBW-LC males were similar to those of IUGR-HF males. However, among females this was not the case; IUGR-HF were similar in size to NBW-LC females but their pharmacokinetics are significantly different.

3.3 Metabolite analysis

The *in vivo* ratios of furosemide-glucuronide (metabolite) to furosemide (parent) in serum and urine and the *in vitro* formation of furosemide-glucuronide by hepatic microsomes are shown in Table 3.

In males, no changes were observed in the ratio of $AUC_{metabolite}$ to AUC_{parent}, between NBW and IUGR or LC and HF diets. In IUGR females, the AUC ratio was decreased by 24%; the HF diet did not have an observable effect in females. In urine, the ratio of metabolite to parent was unchanged by IUGR in males and females. In HF diet males, the ratio was 93% lower, and in females 82% lower relative to LC diet group. Hepatic *in vitro* formation of furosemide-glucuronide was significantly decreased by 25% in HF diet males; it was unchanged by IUGR in males, and was unchanged by IUGR or HF diet in females.

3.4 Expression of Oat 1, Oat 3, and Nkcc2

The expression of Oat-1 and -3 in renal plasma membranes is shown in Table 4. Oat-1 abundance was 48% greater in IUGR males than in NBW-LC males, but

unchanged in the HF diet group. Oat-3 had 187% greater expression in HF fed males but no change with respect to IUGR. The estimated abundance of Oat-1 and -3 in females did not differ significantly by IUGR, but Oat-3 was 29% lower in HF diet group. The expression of Nkcc2 was not modulated by IUGR in males or females. Nkcc2 had 98% decreased expression in HF fed males, and 38% decreased expression in HF fed females.

4. Discussion

In this study, the pharmacokinetics of furosemide was significantly altered by IUGR and further changed by the addition of a high-fat diet. The additivity of the two factors suggests that they operate via different mechanisms, as well as in a sex-dependent fashion. The drug exposure profile in IUGR males was decreased by enhanced clearance and volume of distribution, but this was not so female rats. This finding is in keeping with published observations in humans and rats where females are more resistant than males to other physiological maladaptations of pre-and post-natal protein restriction [5]. The physiological and pharmacokinetic changes associated with the high fat diet were generally opposite to that of IUGR: greater exposure of the drug (higher C_{max} and AUC), a longer half-life, and decreased total clearance and distribution. Increased BMI was observed in females, but not in males.

There appears to be a sex-dependent response between IUGR and HF diet with regard to body composition. IUGR-HF and NBW-LC rats grew to a similar size. While the males in these two groups had similar pharmacokinetic parameters, females in the two groups were different. Although similar in body size, furosemide

pharmacokinetics were significantly different between IUGR-HF and NBW-LC females. These observations underline two important limitations of weight-based dosing: 1) the effects of IUGR and the high fat diet on pharmacokinetics were not allometrically scaled by size, and 2) this model produce subjects who have similar BMI but do not have equivalent pharmacokinetics. Thus, one should be cautious about using BMI alone as a way to normalize data among the groups.

Furosemide was used as a proof of concept test drug in this study because its disposition and clearance are dependent on several different mechanisms. It is a low extraction, highly protein bound drug where differences in free-fraction contribute to variability in action [21, 22]. Furosemide is cleared primarily by the renal transport proteins, Oat-1 and -3, but non-renal clearance (an amalgam of biliary excretion and hepatic biotransformation) is a significant confounder [23-26]. In IUGR males, clearance and volume of distribution were increased; given the similar differences in clearance and distribution such that half-life is unchanged, it is likely that eliminating organs (liver and kidneys) contribute a significant portion of the volume of distribution of furosemide in the rat. Thus, mechanisms that would enhance the volume of distribution (increases in free fraction or transporter expression) would also necessarily increase clearance.

We did not observe changes in percentage of free furosemide in plasma. The percentage of dose excreted in urine was unchanged despite lower serum AUC in IUGR, hence greater renal clearance; this correlates with the increased renal Oat-1 expression observed in IUGR males. Non-renal clearance was also elevated in IUGR males. In hepatic microsomes we did not observe differences in furosemide-

glucuronidation. The ratio of metabolite to parent compound was similar but the amount of metabolite (normalized to parent) secreted in IUGR urine was lower. These data suggest that the increased non-renal clearance is not necessarily enhanced biotransformation to the glucuronide metabolite in IUGR males, but leads to our speculation that enhanced biliary excretion of furosemide occurs in IUGR males. IUGR females also had increased non-renal clearance, but without changes in other pharmacokinetic parameters. IUGR females had disproportionately smaller kidneys for body size coupled with similar renal Oat-1/3 expression. This could result in more excretion by the liver, the weight of which was unchanged. Thus, the resulting increase in non-renal clearance was sufficient to compensate for renal changes such that the overall exposure profile remains unchanged in females.

In our previous study, using 2-mg/kg furosemide IP dose, we found a significant decrease in renal excretion and diuresis [11]. A higher 10 mg/kg IP dose was chosen for this study to better characterize the metabolite kinetics (given their relatively low concentrations), and to observe a larger pharmacodynamic effect. Instead, we observed the opposite; renal excretion and diuresis were similar despite the lower serum AUC in IUGR, hence greater renal clearance. The sampling interval for urine was extended from 4 hours to 6 hours; a longer sampling time provides a better estimate of renal clearance. Even with the increased renal clearance, non-renal clearance and volume of distribution were also increased such that these processes worked against the renal excretion rate so that it was reduced or unchanged. Furosemide-glucuronide was the only metabolite characterized in the present study. The differences in the serum AUC could be explained by changes in other

biotransformation pathways. Phase I metabolism and glutathione conjugation have been observed in rats and mice to contribute to furosemide hepatic clearance [27].

We expected to observe more profound differences in the IUGR phenotype when exposed to the chronic high fat diet, though we now understand the biology in a new way. Others have shown that the perinatal low protein diet animal model produces offspring with low birth weight, but offspring may have less sensitivity for catch-up growth and environmental mismatch [3, 5, 6]. In a rat model where pregnant dams had a low protein diet and offspring were placed on a high fructose diet after weaning, hypertension was due to both the independent effects of the maternal low protein diet as well as the fructose diet after birth [14]. In a separate study that used a similar perinatal low protein diet followed by a "cafeteria style" high fat diet after weaning, the authors found additive effects of small size and diet at one year in causing hypertension [10].

The pharmacokinetic data in the present study suggest that drug distribution and clearance processes are abnormal in HF diet animals. In males, this could be explained by the decreased glucuronidation in HF hepatic microsomes. In addition, furosemide metabolite excretion in urine of HF males was reduced compared to excretion of the parent molecule. However, in HF males we observed a significant increase in Oat-3 expression relative to control LC diet animals. However the interpretation of this finding is not clear because the relationship between Oat expression and *in vivo* renal clearance of furosemide is not known.

HF diet females had a lower percentage of free unbound drug; given furosemide is a low extraction drug, a low free concentration would result in decreased clearance

and volume of distribution. HF females also had a decreased Oat-3 expression, which would predict a lower renal clearance. The increase in diuretic efficacy in HF diet males (but not HF diet females) could be attributable to the dramatic decrease in Nkcc2 expression. Studies in high fat fed obese mice and rats have shown decreased Nkcc2, and increased phosphorylated Nkcc2, decreased basal urine output, and increased diuretic and natriuretic response when dosed with furosemide compared to normal diet non-obese controls [28, 29]. Our results are congruent with these findings, although our data and the current literature has not examined why obese female rats appear resistant to furosemide's enhanced diuretic effect in obese rodents.

Although it is somewhat counter-intuitive, the larger body size and presumably greater adiposity of the HF animals would not necessarily result in a larger volume of distribution of the drug. The reason is that furosemide is an organic anion at physiologic pH, and its distribution into tissues is largely dependent on drug-transporters in liver and kidney. Relative to body weight, the livers and kidneys were smaller in HF animals. Given that the animals are dosed based on total body weight, the livers and kidneys would be presented with a proportionally larger amount of drug relative to normal-sized animals. Additional experiments are needed to better understand this study, including 1) serial sampling from cannulated bladders, 2) multiple dosing experiments to determine if kinetics are linear in these groups, 3) sampling from bile and characterization of multiple furosemide metabolites, and 4) immunohistochemistry to better determine the distribution and quantity of renal Oats transporters.

There is a growing body of literature, in humans and animal models, showing how an individual's early life environment can 'program' a dramatically increased risk for hypertension, cardiovascular disease, diabetes, and obesity later in life. This process is called the 'developmental origins of health and disease' [2]. People who born with low birthweight are more vulnerable to chronic diseases. Thus they are even more likely than most people to need pharmacotherapy as a first line of intervention at some point in their lives. Yet, very little is known about the safety and efficacy of drugs in this sub-population. Previous reports have associated IUGR with altered diminished drug responses; these include blood pressure and diuresis with antihypertensive agents, hexobarbital-induced sleep time, and indomethacin anti-inflammatory response [8, 30, 31]. Our previous study showed a decrease in furosemide-induced diuresis and decreases in urinary excretion of furosemide [11]. The findings of the present study suggest that the changes in drug efficacy associated with IUGR could be explained by powerful changes in pharmacokinetic mechanisms.

The results of this study suggest that maternal diet during perinatal period, post-weaning diet, and gender interact to yield sub-populations of rats with unique pharmacokinetic features. The nature of these interactions produces animals with similar body weights and BMIs but dissimilar pharmacokinetic profiles upon dosing by body-weight. These studies have important clinical implication, the most important being the limitation of weight-based dosing which does not account for physiological differences in body composition resulting from adverse intrauterine stress. While furosemide is a drug with a wide therapeutic window, the mechanisms underlying its kinetics are shared with other drugs such as beta-lactams whose therapeutic windows

are far narrower, and where subtle changes in renal clearance can have a dramatic clinical effect. Future studies are needed to determine if the changes observed with furosemide are conserved in other similar biopharmaceutical classification system (BCS) class III and IV compounds; if so, a strong case could then be made to include birth weight, a surrogate marker for IUGR, as a covariate for pharmacovigilance in clinical trials. Understanding the trajectory of one's growth from the womb to adulthood could explain variability in drug response by pharmacokinetic mechanisms.

5. Acknowledgements

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Table 1 – Morphometrics of d150 old male and female offspring of dams fed either control or LPD during gestation and lactation, and fed a control or high fat diet post-wean (d28 to d150).

Males	**NBW-LC**	**IUGR-LC**	**NBW-HF**	**IUGR-HF**
Birth weight (g)	6.5 ± 0.2*	5.6 ± 0.2	---	---
Body weight (g)	638 ± 31*,†	497 ± 37†	756 ± 127*	545 ± 57
Length (mm)	267 ± 6*,†	257 ± 8†	281 ± 16*	261 ± 7
BMI (kg/m^2)	8.99 ± 0.75*	7.51 ± 0.48	9.53 ± 0.86*	7.96 ± 0.69
Liver weight (g)	22.8 ± 2.0*	16.9 ± 2.4	23.5 ± 4.2*	16.3 ± 1.8
Liver weight (g) / body weight (kg)	35.7 ± 2.6†	33.8 ± 2.7†	31.1 ± 2.0	30.0 ± 1.4
Kidney weight (g)	2.3 ± 0.1*	1.9 ± 0.3	2.4 ± 0.3*	1.7 ± 0.2
Kidney weight (g) / body weight (kg)	3.6 ± 0.2†	3.8 ± 0.3†	3.2 ± 0.2	3.1 ± 0.2
Females				
Birth weight (g)	6.2 ± 0.2*	5.3 ± 0.2	---	---
Body weight (g)	305 ± 21*,†	245 ± 25†	373 ± 50*	301 ± 31
Length (mm)	214 ± 9*	206 ± 11	218 ± 8*	209 ± 7
BMI (kg/m^2)	6.67 ± 0.54*,†	5.78 ± 0.31†	7.81 ± 0.78*	6.87 ± 0.46
Liver weight (g)	11.5 ± 1.9*	8.8 ± 1.2	11.1 ± 0.8*	9.8 ± 0.7
Liver weight (g) / body weight (kg)	37.4 ± 3.9†	36.1 ± 3.7†	30.2 ± 4.3	32.7 ± 2.0
Kidney weight (g)	1.3 ± 0.2*	0.9 ± 0.1	1.3 ± 0.1*	1.0 ± 0.1
Kidney weight (g) / body weight (kg)	4.4 ± 0.3*,†	3.9 ± 0.5†	3.5 ± 0.3*	3.3 ± 0.3

* Significant difference from IUGR within post-weaning diet; $P < 0.05$.
†Significant difference from high fat diet within birth-weight; $P < 0.05$.
Males: NBW-LC (n = 6), IUGR-LC (n = 7), NBW-HF (n = 6), IUGR-HF (n = 7)
Females: NBW-LC (n = 6), IUGR-LC (n = 7), NBW-HF (n = 6), IUGR-HF (n = 7)

Table 2 – Non-compartmental pharmacokinetics of furosemide.

Males	**NBW-LC**	**IUGR-LC**	**NBW-HF**	**IUGR-HF**
Cmax (µg/mL)	28 ± 2*,†	20 ± 5†	36 ± 5*	27 ± 7
AUC (hr* µg/mL)	26 ± 4*,†	18 ± 4†	39 ± 5*	26 ± 8
Total CL/F (mL/hr/kg)	385 ± 57*,†	580 ± 119†	260 ± 33*	404 ± 102
Renal CL/F (mL/hr/kg)	169 ± 26*,†	256 ± 91†	110 ± 15*	190 ± 47
Non-renal CL/F (mL/hr/kg)	198 ± 16*,†	323 ± 75†	149 ± 22*	214 ± 75
Vz/F (mL/kg)	288 ± 18*,†	436 ± 99†	219 ± 25*	330 ± 78
Half-life (hr)	0.52 ± 0.06†	0.52 ± 0.02†	0.59 ± 0.05	0.57 ± 0.05
Protein binding (%-free)	0.50 ± 0.14	0.46 ± 0.13	0.43 ± 0.11	0.43 ± 0.11
Urinary furosemide excretion				
%-excreted of dose (0-6hrs)	46 ± 3	42 ± 4	44 ± 10	43 ± 7
%-excreted of dose (6-24hrs)	7 ± 5	7 ± 5	6 ± 3	5 ± 2
Diuretic effect (dosed/predosed urine volume)	3.3 ± 0.9†	3.8 ± 2.3†	7.4 ± 3.0	9.7 ± 3.9
Females	**NBW-LC**	**IUGR-LC**	**NBW-HF**	**IUGR-HF**
Cmax (µg/mL)	23 ± 3†	21 ± 4†	40 ± 8	34 ± 3
AUC (hr* µg/mL)	25 ± 8†	20 ± 3†	45 ± 14	37 ± 4
Total CL/F (mL/hr/kg)	436 ± 125†	514 ± 81†	237 ± 62	273 ± 33
Renal CL/F (mL/hr/kg)	204 ± 71†	227 ± 35†	106 ± 30	112 ± 40
Non-renal CL/F (mL/hr/kg)	233 ± 93*,†	263 ± 49†	131 ± 36*	162 ± 17
Vz/F (mL/kg)	382 ± 80†	429 ± 96†	211 ± 36	242 ± 33
Half-life (hr)	0.53 ± 0.02†	0.58 ± 0.05†	0.63 ± 0.09	0.61 ± 0.05
Protein binding (%-free)	0.36 ± 0.08†	0.42 ± 0.12†	0.31 ± 0.10	0.31± 0.10
Urinary furosemide excretion				
%-excreted of dose (0-6hrs)	47 ± 10	44 ± 5	46 ± 6	40 ± 11
%-excreted of dose (6-24hrs)	10 ± 8	9 ± 5	7 ± 2	8 ± 3
Diuretic effect (dosed/predosed urine volume)	4.2 ± 1.2	2.3 ± 1.5	4.5 ± 2.1	4.2 ± 3.1

* Significant difference from IUGR within post-weaning diet; $P < 0.05$.
†Significant difference from high fat diet within birth-weight; $P < 0.05$.
Males: NBW-LC (n = 5-6), IUGR-LC (n = 7), NBW-HF (n = 6), IUGR-HF (n = 7).
Females: NBW-LC (n = 6), IUGR-LC (n = 5-7), NBW-HF (n = 6), IUGR-HF (n = 7).

Table 3 – Comparison of *in vivo* and *in vitro* formation of the glucuronide metabolite of furosemide.

Males	**NBW-LC**	**IUGR-LC**	**NBW-HF**	**IUGR-HF**
Serum AUC – Metabolite to Parent ratio (%)	1.05 ± 0.37	1.09 ± 0.30	1.09 ± 0.30	0.91 ± 0.30
Urine – amount excreted (0-24hrs) Metabolite to Parent ratio (%)	1.43 ± 0.48†	1.53 ± 1.20†	0.15 ± 0.08	0.04 ± 0.04
UGT activity (nmoles/min/mg)	265 ± 105†	252 ± 63†	185 ± 73	204 ± 65

Females	**NBW-LC**	**IUGR-LC**	**NBW-HF**	**IUGR-HF**
Serum AUC – Metabolite to Parent ratio (%)	0.93 ± 0.18*	0.62 ± 0.14	0.81 ± 0.18*	0.68 ± 0.08
Urine – amount excreted (0-24hrs) Metabolite to Parent ratio (%)	1.98 ± 0.65†	1.27 ± 0.61†	0.65 ± 0.57	0.91 ± 0.76
UGT activity (nmoles/min/mg)	188 ± 72	253 ± 44	203 ± 46	217 ± 73

* Significant difference from IUGR within post-weaning diet; $P < 0.05$.
†Significant difference from high fat diet within birth-weight; $P < 0.05$.
Males: NBW-LC (n = 5-6), IUGR-LC (n = 7), NBW-HF (n = 6), IUGR-HF (n = 7).
Females: NBW-LC (n = 6), IUGR-LC (n = 5-7), NBW-HF (n = 6), IUGR-HF (n = 7).

Table 4 – Expression of renal Oat-1, Oat -3, and Nkcc2.

Males	**NBW-LC**	**IUGR-LC**	**NBW-HF**	**IUGR-HF**
Oat-1 (%)	100 ± 108*	123 ± 66	75 ± 93*	239 ± 102
Oat-3 (%)	100 ± 34†	111 ± 13†	246 ± 99	339 ± 177
Nkcc2 (%)	100 ± 114†	105 ± 156†	4 ± 1	5 ± 4
Females	**NBW-LC**	**IUGR-LC**	**NBW-HF**	**IUGR-HF**
Oat-1 (%)	100 ± 89	64 ± 54	87 ± 52	84 ± 58
Oat-3 (%)	100 ± 35†	82 ± 24†	64 ± 36	61 ± 28
Nkcc2 (%)	100 ± 35†	78 ± 42†	44 ± 29	58 ± 22

* Significant difference from IUGR within post-weaning diet; $P < 0.05$.
†Significant difference from high fat diet within birth-weight; $P < 0.05$.
Males: NBW-LC (n = 6), IUGR-LC (n = 5-6), NBW-HF (n = 6), IUGR-HF (n = 7).
Females: NBW-LC (n = 6), IUGR-LC (n = 7), NBW-HF (n = 6), IUGR-HF (n = 6-7).

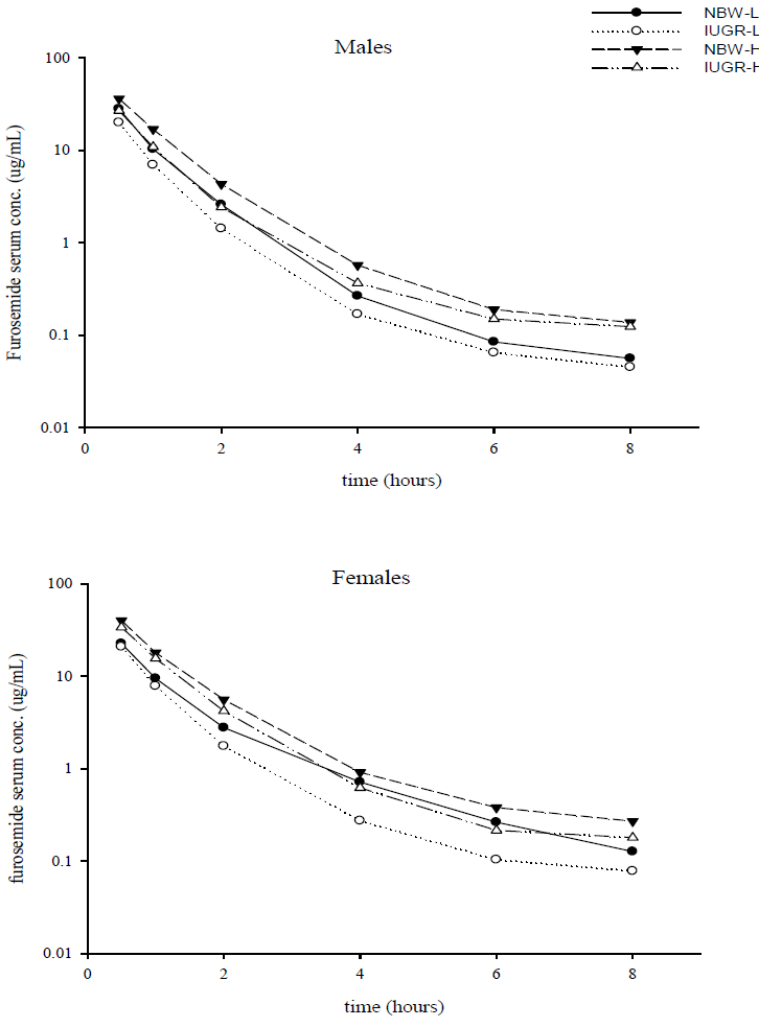

Figure 1. Furosemide serum concentration time profiles. Male (A) and female (B) adult offspring (5-months old) were dosed at 9:00 am with 10mg/kg i.p. furosemide and serum collected over 8 hours. Each concentration time point is an average: NBW-LC (n=6) are closed circles, IUGR-LC (n=6-7) are open circles, NBW-HF (n=6) are closed triangles, and IUGR-HF (n=7) are open triangles. Error bars were omitted for clarity.

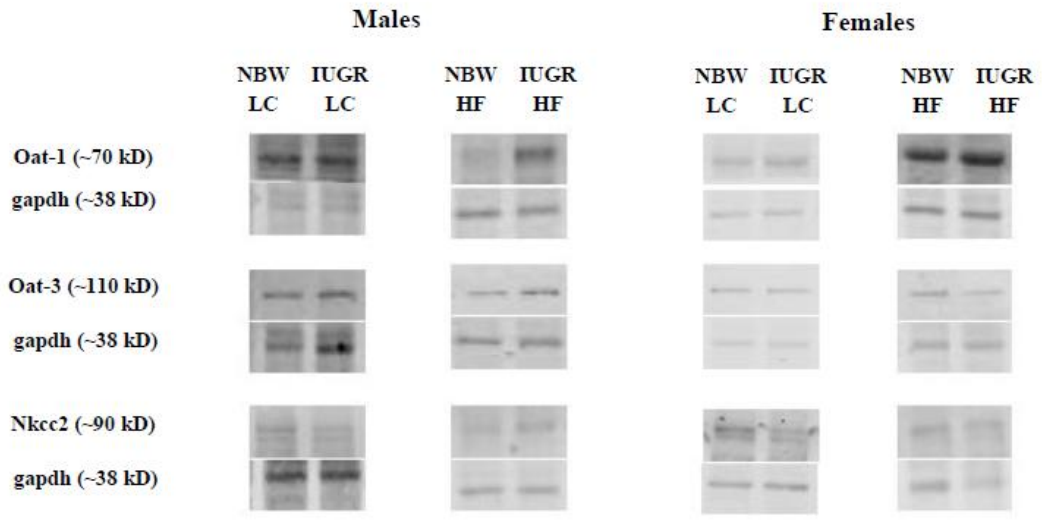

Figure 2. Expression of Oat-1, Oat-3, and Nkcc2 transporter proteins. Western blots of renal crude plasma membrane proteins Oat-1 (~70 kD), Oat-3 (~110 kD), and Nkcc2 (~90kD) are shown in upper bands, lower bands are gapdh (~38kD).

Supplemental table 1 – Composition of the diets

	Control diet (58E7)	Low protein diet (58E6)	High fat diet (58G8)
		Ingredients (%)	
Casein- vitamin tested	21.1000	8.8435	24.1420
Dextrin	43.6000	43.4435	30.1182
Sucrose	15.0000	27.4000	10.3465
Lard	5.0000	5.0000	17.7170
Corn oil	5.0000	5.0000	5.7480
Powdered cellulose	3.0000	3.0000	1.7244
RP vitamin mix #10[1]	2.0000	2.0000	2.2992
RP mineral mix #10[2]	5.0000	5.0000	5.7480
DL-methionine	0.1500	0.0630	0.1724
Choline chloride	0.2000	0.2000	0.2299
Gross energy, kcal/g	4.08	4.13	4.74
Protein, % energy	18.632	7.752	18.1
Fat, % energy	22.047	21.783	44.5
Carbohydrate, % energy	59.321	70.465	37.4

[1] Provided in diet: vitamin A 25.4IU/g, vitamin D-3 2.5IU/g, vitamin E 57.5IU/kg, vitamin K 11.96ppm, thiamin hydrochloride 23.8ppm, riboflavin 23.8ppm, niacin 103ppm, pantothenic acid 65ppm, folic acid 4.8ppm, pyridoxine 19ppm, biotin 0.5ppm, choline chloride 1609ppm, ascorbic acid 0.0ppm .

[2] Provided in diet: calcium 0.70%, phosphorus 0.65%, potassium 0.46%, magnesium 0.08%, sodium 0.27%, chloride 0.32%, fluorine 5.7ppm, iron 76ppm, zinc 31ppm, manganese 75ppm, copper 17.3ppm, cobalt 3.7ppm, iodine 0.66ppm, chromium 3.5ppm, molybdenum 0.94ppm, selenium 0.34ppm.

Non-purified diet (Purina catalog #5012) so called standard lab chow diet contained 22.5% crude protein, 4% crude fat, 52.9% carbohydrate, and 4.6% crude fiber in addition to the minerals and vitamin mixes. It provided 3.38 of kcal per gram and derived 27%, 11%, and 63% of its energy from protein, fat, and carbohydrate respectively.

CONCLUSION

The studies conducted in this dissertation examined the long-term effects of intrauterine growth restriction (IUGR) on pharmacokinetics. The effect of IUGR on pharmacokinetics *in vivo* was evaluated using the loop diuretic furosemide as an example drug to determine differences in drug distribution and clearance, along with *in vitro* mechanistic studies to examine changes in drug metabolism, protein binding, and transport. Additionally we examined the effect of a chronic post-weaning high fat diet on the above mentioned processes to test the hypothesis that environmental mismatch between perinatal and post-weaning diets could exacerbate the effects of IUGR on pharmacokinetics.

In both studies, the maternal low protein diet produced IUGR offspring with a significant decrease in birth weight. These offspring had lower body weights and stunted lengths throughout the lifespan, but proportionally similar such that the body mass index (kg/m^2) was unaltered compared to normal birth weight (NBW) offspring. When challenged with the high fat diet offspring (HF) grew heavier and longer compared to their respective post-weaning lab chow controls (LC); this effect was not exacerbated by the IUGR phenotype. The additivity of these effects is similar to that observed in other models of maternal low protein diet coupled with either a post-weaning high fat or high fructose diet [1, 2].

In the 1^{st} study we observed significant decreases in furosemide urinary excretion and the diuretic effect. In the 2^{nd} study, in serum we observed the overall exposure profile in IUGR males was significantly reduced due to increases in both clearance and volume of distribution. Females appeared resistant to the IUGR phenotype, though

they trended in a similar direction. Contrary to our 1st study's findings, we did not observe any differences in furosemide urinary excretion or the extent of diuresis in the 2nd study. Mechanistically, in the 1st study we observed no changes in Western blots of Oat-1, Oat-3, and Nkcc2; in the 2nd study we observed a significant increase in Oat-1 by IUGR among males only. We observed a significant increase in our 1st study in hepatic UGT activity by IUGR among males, and no changes by IUGR among males or females in the 2nd study. The effects of the high fat diet trended in the opposite direction to that of IUGR, with increased drug exposure due to decreases in both clearance and volume of distribution with a longer half-life in both genders. While the *in vivo* pharmacokinetic parameters were changed by the high fat diet to similar degrees among males and females, the underlying mechanisms investigated were altered in gender-specific ways.

The results of this study suggest that maternal diet during perinatal period, post-weaning diet, and gender interact to yield sub-populations of rats with unique pharmacokinetic features. The nature of these interactions produces animals with similar body weights and BMIs but dissimilar pharmacokinetic profiles upon dosing by body-weight. These studies have important clinical implication the most important being the limitation of weight-based dosing which does not account for physiological differences in body composition resulting from adverse intrauterine stress. There appears to be a sex-dependent response between IUGR and HF diet with regard to body composition. IUGR-HF and NBW-LC rats grew to a similar size. While the males in these two groups had similar pharmacokinetic parameters, females in the two groups were different. Although similar in body size, furosemide pharmacokinetics

were significantly different between IUGR-HF and NBW-LC females. These observations underline two important limitations of weight-based dosing: 1) the effects of IUGR and the high fat diet on pharmacokinetics were not allometrically scaled by size, and 2) this model produce subjects who have similar BMI but do not have equivalent pharmacokinetics. Thus, one should be cautious about using BMI alone as a way to normalize data among the groups.

Additional experiments are needed to better understand this study. The following ideas are recommended for future work based on a review of the literature and the findings from the current study:

Characterization of adiposity

We expected to observe more profound differences in the IUGR phenotype when exposed to the chronic high fat diet. Others have shown that the perinatal low protein diet animal model produces offspring with low birth-weight, but offspring may have less sensitivity for catch-up growth and environmental mismatch [3-5]. BMI may be an insufficient method for estimating the changes in body composition by maternal low protein diet or high fat post-weaning diet. Other studies have utilized Dual-energy X-ray absorptiometry (DEXA) scans and tissue dissection to determine differences in adiposity and body composition [6, 7]. The use of DEXA scans, along with dissection of adipose and lean muscle tissue, in our model would allow for a more accurate estimation of adiposity and lean muscle mass.

Discordance between the 1st and 2nd studies

The discordance between our 1st and 2nd study with respect to furosemide urinary excretion could be an artifact of the differences in dosage and/or differences in

sampling time. In our 1st study a 2-mg/kg furosemide IP dose was used, and in the 2nd study a higher 10 mg/kg IP dose was chosen to better characterize the metabolite kinetics (given their relatively low concentrations), and to observe a larger pharmacodynamic effect. Both the urinary and serum data suggest that most of the data is excreted in the first few hours; therefore, cannulated bladders would be necessary in order to accurately estimate differences in renal excretion [8]. Also, given the significant contribution of biliary excretion to the overall clearance of furosemide, measuring drug concentration in bile via a cannulated common bile duct would allow a better estimate of hepatic clearance *in vivo* [9, 10].

Characterization of OATs and drug classes of interest

In order to better characterize the contribution of organic anion transporters (OATs), there are several experiments which could improve our characterization of their expression and activity. Understanding the localization and sub-cellular distribution of OATs (for example, within the kidney: S1 vs S2 segment, cortex vs. medulla, apical vs. basolateral membrane) would greatly improve our mechanistic understanding of furosemide transport. Immunohistochemistry, coupled with Western blotting of more specific tissue isolations (isolating renal cortex and medulla) would allow for more accurate characterization of amount and localization of transporter proteins[11]. Characterizing these transporters could also be connected with the pharmacokinetics of many other xenobiotics besides furosemide. *In vivo* studies of other compounds which utilize drug transporters, in particular the OATs, could utilize a diverse set of substrates from a variety of drug classes including antihypertensives, antibiotics, antiviral agents, nonsteroidal anti-inflammatory drugs, and statins [12].

The results of this study suggest that maternal diet during perinatal period, post weaning diet, and gender interact to yield sub-populations of rats with unique pharmacokinetic features. The nature of these interactions produces animals with similar body weights and BMIs but dissimilar pharmacokinetic profiles upon dosing by body weight. These studies have important clinical implication the most important being the limitation of weight-based dosing which does not account for physiological differences in body composition resulting from adverse intrauterine stress. While furosemide is a drug with a wide therapeutic window, the mechanisms underlying its kinetics are shared with other drugs such as beta-lactams whose therapeutic windows are far narrower, and where subtle changes in renal clearance can have a dramatic clinical effect. Future studies are needed to determine if the changes observed with furosemide are conserved in other similar biopharmaceutical classification system (BCS) class III and IV compounds; if so, a strong case could then be made to include birth weight, a surrogate marker for IUGR, as a covariate for pharmacovigilance in clinical trials. Understanding the trajectory of one's growth from the womb to adulthood could explain variability in drug response by pharmacokinetic mechanisms.

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