# Effect of Perinatal Low Protein Diet on the expression of P-Glycoprotein and Organic Cation Transporter Novel-Type-2 in the Heart

By

Ali Alnakhli

An Undergraduate Thesis Submitted to

Oregon State University

In partial fulfillment of
the requirements for the
degree of

Baccalaureate of Science in BioResource Research,
Toxicology, and Biotechnology

06/15/2012

APPROVED:

| | |
|---|---|
| Ganesh Cherala, Pharmacy Department | Date |
| Gitali Indra, Pharmacy Department | Date |
| Katharine G. Field, BRR Director | Date |



I understand that my project will become part of the permanent collection of the Oregon State University Library, and will become part of the Scholars Archive collection for BioResource Research. My signature below authorizes release of my project and thesis to any reader upon request.

| | |
|---|---|
| Ali Alnakhli | Date |

**Introduction:**

Many studies in recent literature have shown a significant link between the abnormalities of a newly born individual and the type of environment that their mothers encountered during pregnancy. More specifically, over the last two decades, a discipline of research commonly known as "fetal programming" has linked abnormalities at birth to quality of life during adulthood[1]. Low birth weight (LBW), a surrogate measure of adverse in *utero* environment, is strongly linked to high incidence of chronic diseases such as hypertension, diabetes, high cholesterol, and obesity later in life[2].

Many animal models were developed to study the link between perinatal environment and incidence of diseases. The low birth weight rodent model resulting from a low protein diet administered to pregnant rats is a commonly used animal model[3]. Research carried out using the above animal model suggested changes in the physiology and morphology of multiple organs, including kidney and liver. The molecular changes in these organs are attributed to the long-term permanent effects of the perinatal environment. These changes can also affect drug pharmacokinetics. According to "*Pharmacokinetics in Drug Discovery"* by Ana Ruiz-Garcia, pharmacokinetics is the study of drug kinetics, which includes absorption, distribution, metabolism, and excretion[4]. These processes are affected by morphological and physiological changes in organs[3]. Therefore, morphological and physiological changes could alter the expression and consequently the location and number of drug metabolizing enzymes and transporters, which are the main functional units in drug pharmacokinetics in the organs. Overall, altered drug pharmacokinetics could result in unfavorable pharmaco- therapeutic outcomes, and hence it is essential to understand these processes.

Previous and ongoing research in our laboratory has examined the effect of changes in kidney, liver, and brain, on the expression of drug metabolizing enzymes and transporters. Significant changes were reported in these organs that could adversely affect drug pharmacokinetics. However, it is important to understand the contribution of other organs including heart. Heart is a major organ that maintains blood supply to the entire body. Animal data has shown both morphological and physiological changes in the heart. For instance, a study has proved that intrauterine growth retardation causes a major risk of prolongation in the interval between the Q wave and the T wave that represent depolarization and repolarization of the left and right ventricles in the heart's electrical cycle, which is a result of a drug factored cardiac toxicity, in neonates[5]. As mentioned above, low birth weight subjects are highly susceptible to cardiovascular diseases during adulthood. Therefore, understanding drug behavior in the heart can lead us to discovering ways to optimize treatment of cardiovascular diseases in those subjects.

For this purpose, optimizing treatment of cardiovascular diseases requires a careful observation of drug trafficking in cardiomyocytes. As an example from the QT interval prolongation study, cardiac toxicity occurred as a result of an accumulated Cisapride[5]. Hence, our research is focusing on two of the most important drug transporters in the heart in order to observe drug behavior in low birth weight subjects.

First, P-glycoprotein (P-gp) is one of the ATP-binding cassette (ABC) transporter proteins, which are abundant in many tissues in the body. P-gp is expressed in multiple organs including liver, intestines, heart, kidney, and blood-brain barrier. In heart, P-gp actively effluxes drugs out of cardiac myocytes, consequently, it plays an important role

in minimizing cardiotoxicity. P-gp transports many well-known drugs such as Verapamil, Adriamycin, Digoxin, and Vinblastine.

Secondly, organic cation transporter novel-type-2 (Octn2) is a part of the organic cation transporter proteins family, which is also found on cell membranes of liver, kidney, heart, intestines tissues, and other organs. Octn2 serves as an Na+ coupled carnitine transporter, which has a significant role on maintaining carnitine homeostasis in cells. Deficit expression of Octn2 can lead to many complications, including cardiomyopathy and cardiotoxicity.

## Materials and Methods:

### *Diets*

Modified versions of the AIN76A purified diet (Control), and the corresponding isocaloric low protein formulation (LPD) were purchased in pellet form from Purina Test Diets (Richmond, IN). The purified control and LPD diets contained 19% and 8% crude protein in the form of casein, respectively. Detailed compositions of both diets are available in the literature [14].

### *Experiment with dams*

The study was approved by the Institutional Animal Care and Use Committee of the Oregon Health & Science University, Portland, OR. Virgin female Sprague-Dawley

rats (Charles River Laboratories, Inc., Wilmington, MA) were mated by housing one male rat with two 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 two diet groups. Each group consisted of 5-7 pregnant rats, and these rats received their assigned diet throughout pregnancy and lactation (Figure 1).

*Experiments with offspring*

Upon birth, pups were sexed, and litter size was noted. 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. Offspring from both groups were weaned on day 28 after birth and were housed in isosexual groups according to perinatal diet treatment. Pups from litters in both groups were weaned onto a nonpurified diet. It is therefore important to note that different dietary treatments were administered only during gestation and lactation. One male and female offspring from each litter in both groups were randomly chosen and sacrificed using $CO_2$ asphyxiation on day 150 after birth. Kidneys, intestines, heart, and brain were collected from all sacrificed animals, weighed, snap-frozen in liquid nitrogen and stored at -80°C.

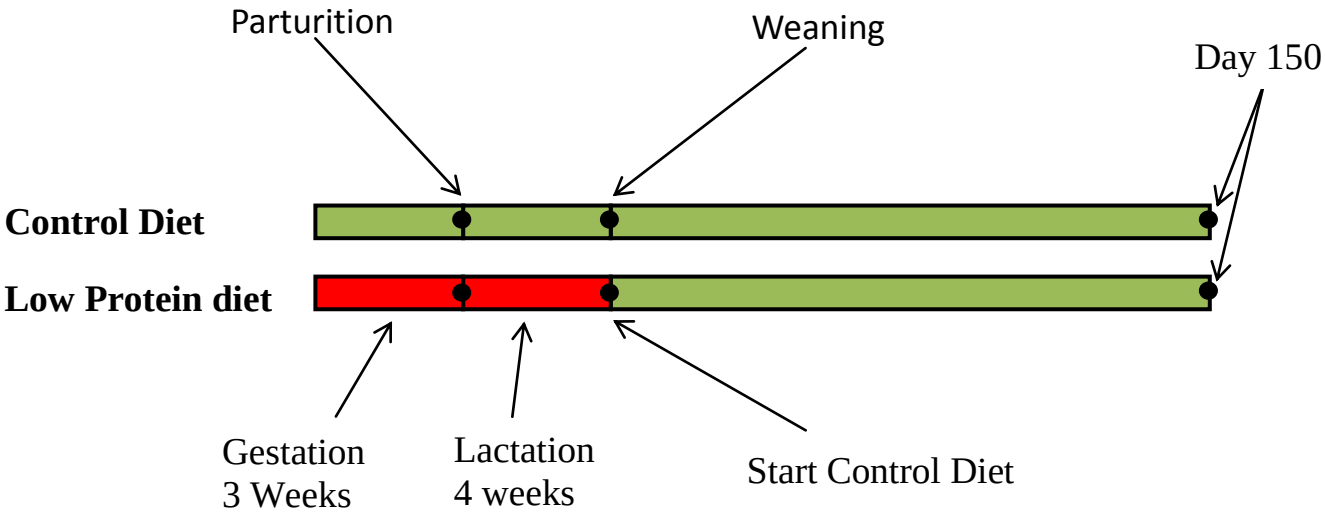


Figure 1. A graphical representation of the animal model used in the experiment. On one hand, maternal control diet is given to produce control subjects after parturition, and the diet is kept for the pups until sacrificing on day 150. On the other hand, a maternal low protein diet is given to produce low-birth weight subjects after parturition, and the diet is kept for pups until the end of lactation when they are weaned. Then, low-birth weight animals are given control diet from end of lactation until day 150, when they are sacrificed.

*RNA Extraction*

The tissue (100-200mg) was homogenized using a mortar and pestle over liquid nitrogen, and total RNA was isolated using TRIZOL according to the manufacturer's protocol and stored at -80˚C. Purity and concentration of the product was quantified using A260/230 and A260/280 values, respectively (Nanodrop 1000, Thermo-Scientific, Wilmington, DE).

*cDNA Synthesis*

cDNA was generated using iScript cDNA synthesis kit (BioRad, Hercules, CA). Briefly, 1 µg of total RNA was added to a reaction solution containing iScript reaction mix, iScript reverse transcriptase, and nuclease free water to a final volume of 20 µL. The reaction was carried out in the BioRad MyiQ thermocycler. Concentration of nucleic acid in the cDNA reaction was measured using A260/280 value (Nanodrop 1000).

*Real Time Polymerase Chain Reaction (RT-PCR)*

cDNA (10-20 ng) was added to a reaction mixture containing iQ SYBR Green supermix (BioRad, Hercules, CA) and forward and reverse primers (250nM concentration; Table 1) to a final volume of 20 µL. PCR amplification was performed in the BioRad MyiQ Thermocycler as follows: 2 minutes at 95˚C, followed by 40 cycles of 95˚C for 20 seconds and 50˚C for 30 seconds. Reactions were carried out in triplicate or duplicate. A positive template, randomly selected from control group, was included for each experiment.

Experiments were carried out to identify the suitable reference gene for RT-PCR analysis of P-gp and Octn2. The following references were examined: beta-actin (β-Actin), hypoxanthine phosphoribosyltransferase 1 (Hprt1), TATA-box-binding protein (Tbp), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and glucuronidase-beta (Gusb).

Table 1: Primer sequences for PCR study.

| Gene | Accession # | | Sequence |
|---|---|---|---|
| Gapdh | NM_017008 | Forward | GTGGTGCCAAAAGGGTCAT |
| | | Reverse | ATTTCTCGTGGTTCACACCCA |
| Hprt1 | XM_217584 | Forward | GCCCCAAAATGGTTAAGGTTG |
| | | Reverse | TCCACTTTCGCTGATGACACA |
| Gusb | NM_017015 | Forward | TCACTCGACAGAGAAACCCCA |
| | | Reverse | CTCTGGTTTCGTTGGCAATCC |
| Tbp | NM_001004198 | Forward | CGGTTTGCTGCAGTCATCAT |
| | | Reverse | GTGCACACCATTTTCCCAGA |
| Actb | NM_031144 | Forward | GCCAACACAGTGCTGTCTG |
| | | Reverse | CACATCTGCTGGAAGGTGG |
| P-gp | NM_012623.2 | Forward | CCGCATTCTGCCGAGCGTTACT |
| | | Reverse | AGACGTCATCTGTGAGCCGGGT |
| Octn2 | NM_019269 | Forward | GCCAGCATCATCCCCAATGGCTTTAC |
| | | Reverse | CCTTCATCTCCAACGGGATGCTGTG |

*Data Analysis*

The efficiency (E) of primer pairs was determined using a 10x serial dilution of a control cDNA sample[13]. The slope of the relationship between Log transformation of dilution factor and cycle threshold (Ct) values at each dilution factor was calculated. The 'E' of each primer pair was then calculated as 10^(-1/slope). Fold differences in the expression of a reference gene between groups were calculated as $E^{(\Delta Ct_{(control-LPD)})}$. Theoretical effects on target gene expression were calculated by the method adapted from Pfaffl method[6].

*Statistical Analysis*

For identification of reference gene, differences in the mean Ct values between groups were calculated using either Two-Way ANOVA or Kruskal-Wallis ANOVA on ranks test. Multiple pairwise comparisons were made with the Student-Newman-Keuls Test at $\alpha=0.05$ (SigmaPlot v11.0, San Jose, CA). The differences in the expression of P-gp or Octn2 were tested using student's t-test.

**Results:**

RNA Isolation

While nucleic acid contaminates absorb light at 280 nm wavelengths like proteins, and at 230 nm wavelengths like other organic/aromatic compounds such as phenol and TRIzol, nucleic acid can only absorb light at 260 nm wavelengths. Thus, RNA purity is assessed through the ratios 260/280 and 260/230 values. Optimum ratios range between $1.8\pm 0.3$. Isolated RNA samples from both groups and genders yielded acceptable ratios for both purity (A260/230) and concentration (A260/280). See Table 2 for RNA absorbance measurement data summary including 260/230 ratios, 260/280 ratios, and RNA concentration for all samples:

Table 2. RNA mean concentrations in (ng/µL), and RNA absorbance measurement data summary for 260/230 ratios, and 260/280 ratios.

| | 260/230 (Mean±S.D.) | 260/280 (Mean±S.D.) | Conc.(ng/µL) (Mean±S.D.) |
|---|---|---|---|
| LPD male | 1.77±0.17 | 2.18±0.10 | 316.06±163.16 |
| Control male | 2.03±0.23 | 2.16±0.10 | 329.07±165.54 |
| LPD female | 2.09±0.28 | 2.13±0.09 | 522.84±218.60 |
| Control female | 1.86±0.22 | 2.17±0.11 | 432.88±137.61 |

## cDNA Synthesis

Similar to RNA isolation, the concentration of cDNA synthesized is quantified using A260/280 absorbance ratio. Optimum ratios range between 1.8± 0.3. Synthesized cDNA samples from both groups and genders yielded acceptable ratios for concentration (A260/280). See Table 3 for DNA absorbance measurement data summary including 260/280 ratios, and DNA concentration for all samples:

Table 3. DNA mean concentrations in (ng/µL), and DNA absorbance measurement data summary for 260/280 ratios.

| | 260/280 (Mean±S.D.) | Conc.(ng/µL) (Mean±S.D.) |
|---|---|---|
| LPD male | 1.82±0.01 | 512.98±8.60 |
| Control male | 1.82±0.01 | 512.28±10.31 |
| LPD female | 1.82±0.01 | 514.86±8.80 |
| Control female | 1.82±0.01 | 512.85±15.70 |

## Identification of Reference Gene

Table 4: Effect of maternal LPD on heart mRNA expression of selected reference genes in day 150 old male and female offspring.

| Age | Gene | Females | | | Males | | |
|---|---|---|---|---|---|---|---|
| | | Control | LPD | Fold Diff.[1] | Control | LPD | Fold Diff. |
| Day 150 | *Gapdh* | 23.20±1.12 (4.83)[2] | 21.40±1.36 (6.36) | 2.83 * | 23.32±1.69 (7.26) | 23.46±1.72 (7.33) | -1.08 |
| | *Tbp* | 27.61±0.76 (2.74) | 26.75±0.99 (3.70) | 1.66 | 27.96±0.63 (2.25) | 28.25±2.58 (9.12) | -1.19 |
| | *Hprt1* | 22.40±0.63 (2.80) | 21.97±0.65 (2.96) | 1.29 | 28.88±9.63 (33.34) | 24.11±2.92 (12.12) | 16.75 |
| | *Gusb* | 27.76±0.36 (1.28) | 27.08±1.15 (4.24) | 1.54 | 27.98±0.33 (1.16) | 28.19±2.22 (7.88) | -1.14 |
| | *Actb* | 20.86±1.06 (5.09) | 20.03±0.43 (2.13) | 1.65 | 22.40±3.81 (17.03) | 21.97±2.23 (10.15) | 1.29 |

[1] Fold differences in the expression of gene in LPD group offspring, compared to the control group offspring; [2] The Ct values are expressed as mean±S.D. (%CV); * $p<0.05$.

In heart, expression of *Gapdh* was higher in LPD females (Table 4); however, there were no significant differences in the expression of other reference genes in females. Similarly, in males, none of the reference genes in the heart were affected in LPD group offspring (Table 4).

## Primer Efficiency

As described above (p. 7,8), the efficiency of the primers was calculated through a serial dilution. As β-actin primer pair was effeciently used as the positive control for RT-

PCR experiments, the efficincy percentage ({measured efficiency/2}*100) for β-actin was 92.5%. Similarly, following the same method of calculating the efficincy percentage, efficincy percentages for both P-gp and Octn2 are 77.5% and 92.6%, respectively.

## RT-PCR

There are substantial fold differences in the expression of mRNA in P-gp and Octn2 in both males and females at day 150. In P-gp, there is an increase of fold difference of ~1.8 in females while there is a decrease of ~2 in males, when compared to the control subjects. In Octn2, fold differences for both genders comparing to control animals increased. It was an increase of ~2.7 in females while it was an increase of ~1.5 in males. Figure(s) 2, 3 show graphical representations of these fold differences for P-gp and Octn2, respectively.

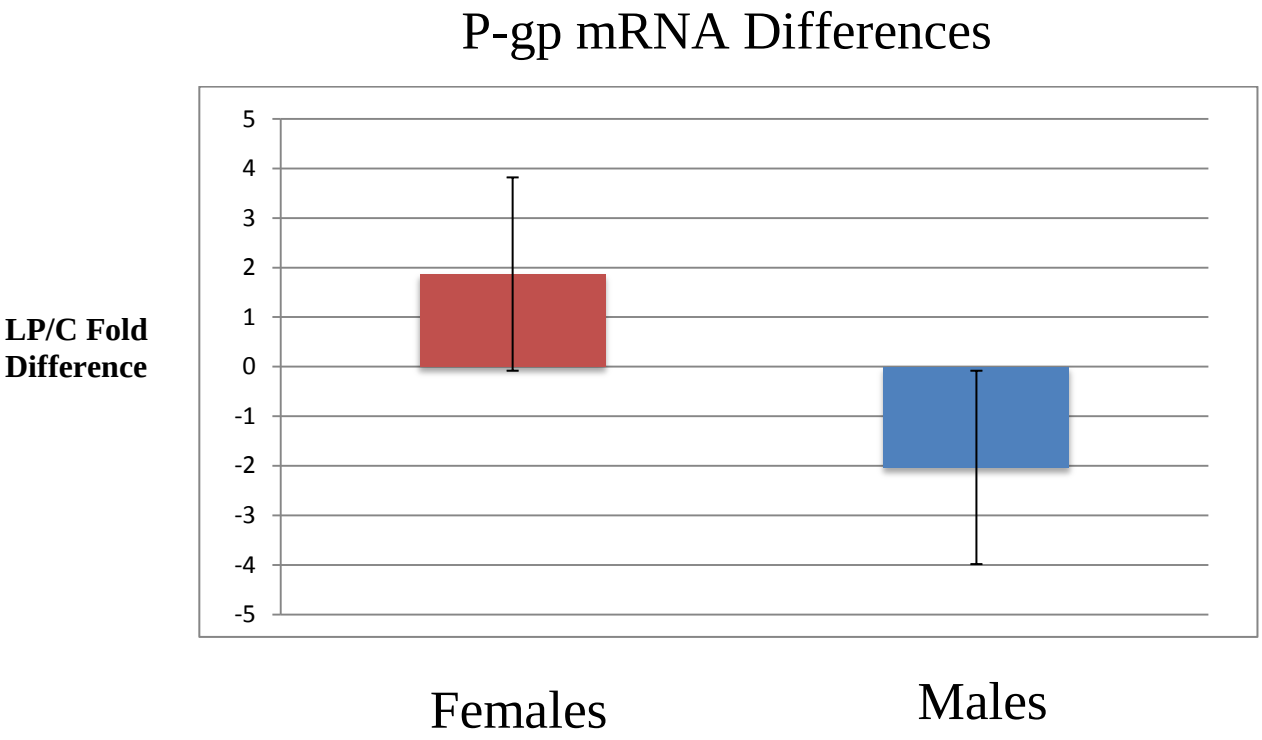


Figure 2: shows the relative expression of LBW P-gp mRNA as normalized control animals (LP/C). Positive values on the scale indicate fold increase, that is, more mRNA expressed in the LBW individuals than in the control individuals. Conversely, negative values indicate a relative fold decrease in mRNA.

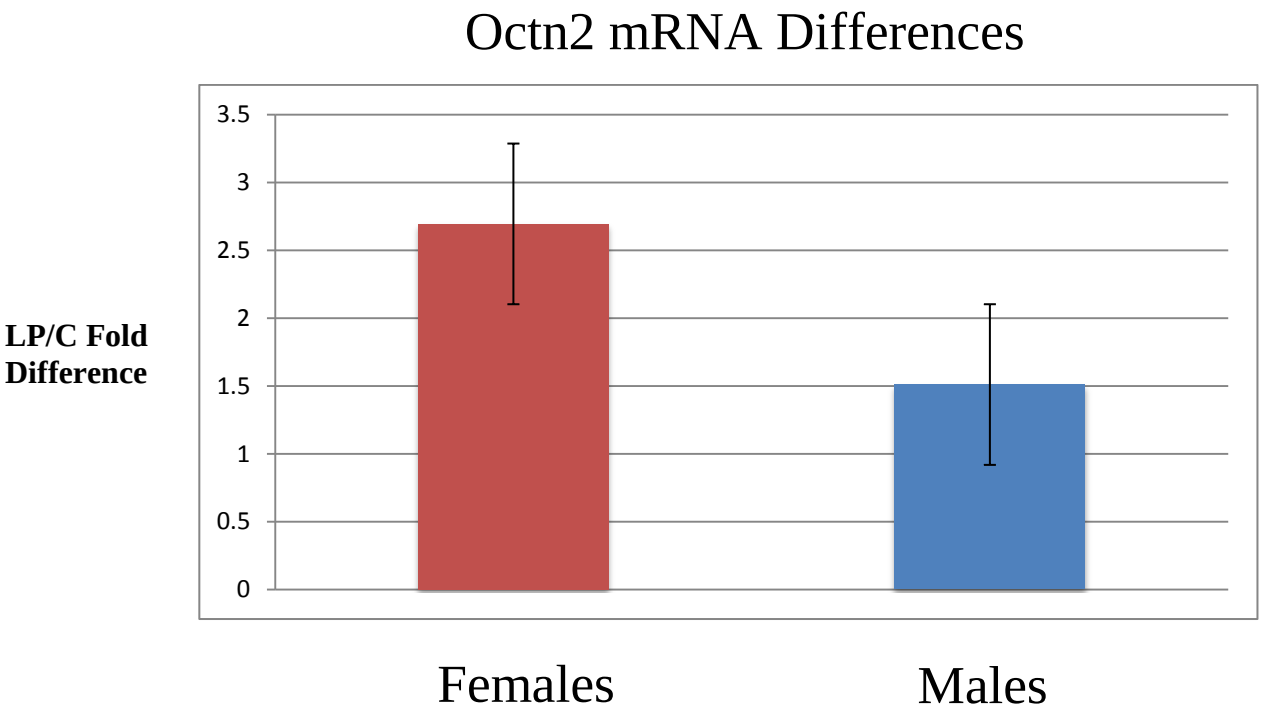


Figure 3: shows the relative expression of LBW Octn2 mRNA as normalized control animals (LP/C). Positive values on the scale indicate fold increase, that is, more mRNA expressed in the LBW individuals than in the control individuals. Conversely, negative values indicate a relative fold decrease in mRNA.

**Discussion:**

Investigations about low birth weight animals have led researchers to highlight the complications a low birth weight adult animal might experience. Low birth weight is a common outcome of adverse *in utero* environment leading to complications, which include high susceptibility to chronic diseases such as cardiovascular diseases, hypertension, diabetes, obesity, and high cholesterol. The link between low birth weight and adulthood diseases is a result of alterations in the physiology and morphology of multiple organs including heart. Given this context, the current study explores the expression of drug transporters P-gp and Octn2 in the heart. It was hypothesized that poor in *utero* environment would result in low birth weight offspring, subsequently leading to altered expression of cardiac P-gp and Octn2 in adult rat offspring. To investigate this hypothesis, the expressions of mRNA for both P-gp and Octn2 in cardiac tissue were measured using RT-qPCR.

In RT-qPCR, changes in the level of gene transcription are measured by normalizing target gene expression to that of a reference gene. While there are many reference genes to choose from, an ideal pick is one with reliable and ubiquitous expression in all cell types and tissues, and similar expression between experimental groups[7, 8, 9, 10]; it is important to identify reference genes which demonstrate little variation between experimental groups before differences in the gene(s) of interest can be quantified[11]. Denisenko *et al* observed that the expression of *Gapdh*, a commonly used reference gene, was consistently down regulated in the kidneys of microswine offspring from mothers fed a low protein diet (LPD) during pregnancy[12]. In addition to microswine model, rodent maternal LPD is also a commonly used animal model to

understand developmental origins of health and disease[13]. However, very limited data is available on the expression of commonly used reference genes in rodent maternal LPD model[14, 15].

In the present study, we examined the expression of the following reference genes in the heart of the offspring of rodent maternal LPD model: *Actb, Hprt1, Tbp, Gapdh,* and *Gusb*. The selection of reference genes was determined by their widespread use in molecular biology experiments. This study adhered to recently published MIQE guidelines for RT-qPCR experimentations[16], strengthening the validity of our findings. The study findings include that all reference genes, except for Gapdh in females, are unaltered in the hearts of low birth weight offspring. We chose beta-actin for RT-qPCR studies due to the relative high abundance and less variability of expression. Our findings underscore the importance of empirical determination of a reliable reference gene for RT-qPCR studies in the LPD model.

The mRNA expression of P-gp is less abundant in low birth weight males than control ones by about two folds. On the other hand, there is an increased expression of P-gp in low birth weight females comparing to control group. The above alterations in P-gp expression might result in gender-dependent outcomes: females would eliminate drug quicker, resulting in lower drug exposure in cardiac tissue, and might result in sub-optimal therapeutic outcomes, while the male offspring would accumulate drugs resulting in cardiotoxicity. The above speculation was made under the assumption that mRNA data linearly correlates with protein expression, which needs to be examined.

On the other hand, Octn2 expression is increase in both gender; 2.5 and 1.5 fold increases in females and male offspring, respectively, of low birth weight group

compared to control group. The increased transport of carnitine by Octn2 could result in greater utilization of fatty acids whose oxidation in cells including cardiac cells involves a rate-limiting step of carnitine availability. If this is true, such adaptation is probably advantageous for the survival of low birth weight offspring. However, the transport of drugs via Octn2 could result in cardiac accumulation leading to cardiomyopathy.

In summary, early life environment is an important determinant of health and disease during adulthood. Our focused research on cardiac drug transporter proteins suggests that the drug accumulation and pharmacotherapeutic outcomes could be determined partly by in utero conditions. Moreover, the gender specific alterations caution the generalization of above findings. Further studies are required to shed light on the outcomes associated with such alterations and understand the mechanistic basis.

# References

1. Zandi-Nejad K, Luyckx VA, Brenner BM. Adult hypertension and kidney disease: the role of fetal programming. Hypertension 2006;47: 502-508.
2. Harder T, Rodekamp E, Schellong K, et al. Birth weight and subsequent risk of type 2 diabetes: a meta-analysis. Am J Epidemiol. 2007;165(8): 849-857.
3. Cherala G, D'mello A, Shapiro B. Two Low Protein Diets Differentially Affect Food Consumption and Reproductive Performance in Pregnant and Lactating Rats and Long-Term Growth in Their Offspring. J. Nutr, 2006;136(11): 2827-2833.
4. Ruiz-Garcia A, Bermejo M, Moss A, Casabo VG. Pharmacokinetics in Drug Discovery. *J Pharm Sci*. 2007;97(2): 654-690.
5. Corvaglia L, Faldella G, Rotatori R, Lanari M, Capretti MG, Salvioli GP. Intrauterine Growth Retardation is a Risk Factor for Cisapride-Induced QT Prolongation in Preterm Infants. *CARDIOVASC DRUG THER*. 2004;18: 371-375.
6. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001;29(9):e45.
7. Catalan V, Gomez-Ambrosi J, Rotellar F, et al. Validation of endogenous control genes in human adipose tissue: relevance to obesity and obesity-associated type 2 diabetes mellitus. *Horm Metab Res.* 2007;39(7):495-500.

8. Chen J, Rider DA, Ruan R. Identification of valid housekeeping genes and antioxidant enzyme gene expression change in the aging rat liver. *J Gerontol A Biol Sci Med Sci.* 2006;61(1):20-27.
9. Ruan W, Lai M. Actin, a reliable marker of internal control? *Clin Chim Acta.* 2007;385(1-2):1-5.
10. Schmid H, Cohen CD, Henger A, Irrgang S, Schlondorff D, Kretzler M. Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies. *Kidney Int.* 2003;64(1):356-360.
11. de Kok JB, Roelofs RW, Giesendorf BA, et al. Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Lab Invest.* 2005;85(1):154-159.
12. Denisenko O, Lin B, Louey S, Thornburg K, Bomsztyk K, Bagby S. Maternal malnutrition and placental insufficiency induce global downregulation of gene expression in fetal kidneys. *Journal of Developmental Origins of Health and Disease.* 2011;2(2):124-133.
13. Zambrano E, Bautista CJ, Deas M, et al. A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. *J Physiol.* 2006;571(Pt 1):221-230.
14. Coupe B, Grit I, Darmaun D, Parnet P. The timing of "catch-up growth" affects metabolism and appetite regulation in male rats born with intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol.* 2009;297(3):R813-824.

15. Yates Z, Tarling EJ, Langley-Evans SC, Salter AM. Maternal undernutrition programmes atherosclerosis in the ApoE*3-Leiden mouse. *Br J Nutr*. 2009;101(8):1185-1194.
16. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;55(4):611-622.