Fall 11-28-2018

Concentrations of Purine Metabolites Are Elevated in Human Fluids from Adults and Infants and in Livers from Mice Fed Diets Depleted of Bovine Milk Exosomes and their RNA Cargos

Ana Aguilar Lozano
University of Nebraska - Lincoln, ana_aguilar0311@hotmail.com

Aguilar Lozano, Ana, "Concentrations of Purine Metabolites Are Elevated in Human Fluids from Adults and Infants and in Livers from Mice Fed Diets Depleted of Bovine Milk Exosomes and their RNA Cargos" (2018). *Nutrition & Health Sciences Dissertations & Theses*. 81.
http://digitalcommons.unl.edu/nutritiondiss/81

CONCENTRATIONS OF PURINE METABOLITES ARE ELEVATED IN HUMAN FLUIDS FROM ADULTS AND INFANTS AND IN LIVERS FROM MICE FED DIETS DEPLETED OF BOVINE MILK EXOSOMES AND THEIR RNA CARGOS

by

Ana Gabriela Aguilar Lozano

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Interdepartmental Area of Nutrition
Under the Supervision of Professor Janos Zempleni
Lincoln, Nebraska
November 2018

CONCENTRATIONS OF PURINE METABOLITES ARE ELEVATED IN HUMAN FLUIDS FROM ADULTS AND INFANTS AND IN LIVERS FROM MICE FED DIETS DEPLETED OF BOVINE MILK EXOSOMES AND THEIR RNA CARGOS

Ana Gabriela Aguilar Lozano, M.S.

University of Nebraska, 2018

Advisor: Janos Zempleni

Since miRNAs discovery, evidence keeps accumulating on their role in human physiology for homeostasis and implications during disease development. Upon the discovery that miRNAs could be encapsulated in exosomes and provide protection, evidence keeps accumulating on the possibility for miRNAs to be transferred between distant cells and elicit gene expression changes, and clinical trials are being developed to identify miRNAs in body fluids that could predict disease outcomes based on miRNA encapsulation in exosomes making them stable in body fluids. Recently, studies have shown that dietary miRNAs encapsulated in exosomes can be absorbed in mice and humans, and on the other hand, milk consumption has been associated with lower uric acid concentrations and distinct benefits for human health, therefore the aims of this work were to determine whether milk exosome and RNA-depleted (ERD) and exosome and RNA-sufficient (ERS) diets could alter the concentrations of purine metabolites in mouse livers, and to determine whether diets depleted of bovine milk alter the plasma concentration and urine excretion of purine metabolites in adults and infants, respectively. Effects upon ERD diet were observed, hepatic purine metabolites in ERD fed mice were significantly higher compared to ERD controls. Additionally, plasma concentrations and urine excretion of purine metabolites were significantly higher in dairy avoiders and in infants fed soy milk, and finally purine hepatic gene expression in mice was significantly different between ERD and ERS. Comprehensively, diets depleted of bovine milk exosomes and RNA cargos caused increases in hepatic purine metabolites in mice, and in plasma and urine from adults and infants compared to exosome-sufficient controls. These findings are important since purines play a role in intermediary metabolism and cell signaling, therefore there is a possible link between milk benefits and dietary exosomes and their miRNA cargo transfer. Further research will need to address the underlying mechanisms that drive the purine concentration and enzyme gene expression changes upon ERD diets.

ACKNOWLEDGEMENTS

I would like to start thanking Dr. Zempleni for the opportunity of pursuing a graduate degree at his laboratory in a fascinating line of research, for his time, support and thoroughness to prepare me for a future career. During this time, I learned numerous lessons for my academic and personal development. Also, I would like to thank Dr. Adamec, Dr. Chung, Dr. Cui and Dr. Natarajan for their support, guidance and for always being willing to share their valuable knowledge in diverse topics. My gratitude is also for David Giraud who always had patience to teach laboratory techniques, show all different equipment troubleshooting and always be available to support in the development of this research project. I also extend my gratitude to the NHS department staff for their guidance and support when needed.

Additionally, my appreciation goes to all my laboratory peers including Ryan Grove, Deborah Fratantonio, Sonal Sukreet, Ezra Mutai, Fang Zhou and Di Wu for taking time to teach me different techniques contributing to the development of this research project, in addition to their love and support.

I really appreciate the support from my friends in Lincoln and Mexico, their constant love, inspiration, encouragement and advise that have been key for me to complete this degree.

Finally, infinite gratitude to my father, mother and brother, for the various visits to Lincoln, endless support and love, for being great examples and for the constant encouragement to keep pursuing this degree.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

CHAPTER I

LIST OF TABLES

CHAPTER I

INTRODUCTION

miRNAs are endogenous short (22 nt) non-protein coding RNA sequences with a hairpin stem shape, that serve as post-transcriptional gene expression regulators. The synthesis of miRNAs is a coordinated process where firstly, miRNAs are transcribed from introns or exons by Pol II enzyme, and some miRNAs can share promoters with other messages, however most miRNAs genes are distant from protein-coding genes. Interestingly, several miRNAs are highly conserved between species and they are organized as poly-cistronic units. miRNAs are subsequently trimmed by Drosha (along with DGCR and RNase III endonuclease protein activity), which cleaves the transcribed pri-miRNA sequence, resulting in the liberation of a ~60-70 nt doubled-stranded hairpin named pre-miRNA, which maintains a 3´overhang nt. Exportin 5 with Ran-GTP activity subsequently transports the pre-miRNA to the cytoplasm. Once the pre-miR is in the cytosol, Dicer along with RNase III activity, cleaves the pre-miRNA ~22 nt from the end of the sequence by recognizing the double stranded part of the pre-miRNA, mainly based on the protein harboring affinity for the 5´phsophate and 3´ nt overhang of the stem loop base. This will result in an 11nt sequence mature miRNA (1–5).

To proceed with repressive activity, Dicer guides the miRNA into an Argonaute2 (Ago2) protein forming the RNA-induced silencing complex (RISC), which selects 1 strand form the miRNA:miRNA duplex to achieve mRNA repression, liberating the other miRNA strand for subsequent degradation. RISC will recognize mRNAs based on perfect or nearly perfect paring with the miRNA sequence form the RISC. The miRNA maintains a base pairing with the 3´UTR of the mRNA, with either a perfect match or near perfect pairing, the latter being more common. The requirements for pairing are mostly, for the miRNA 5´region (seen sequence) to bind at the 3´UTR of the message resulting in the formation of the mRNA:miRNA duplex (Figure1-2) (1–7).

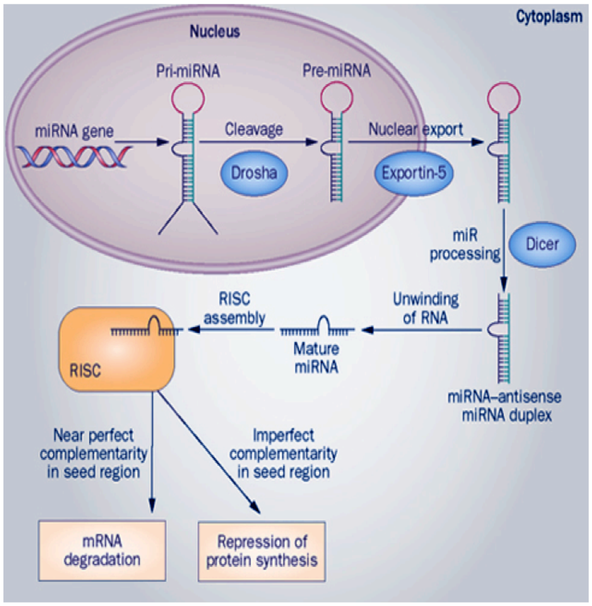


Figure 1. miRNAs biogenesis and function. Drosha, Exportin-5 and Dicer accordingly acting towards miRNA maturation (Adapted from O´Kelly, 2012).

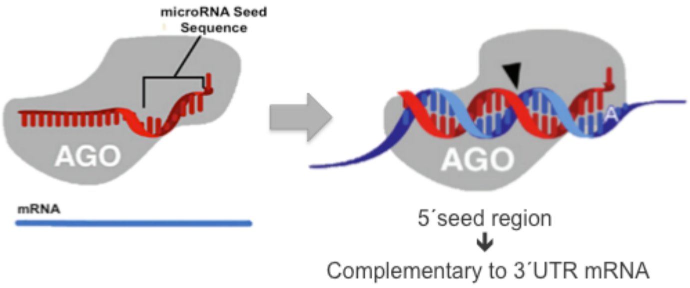


Figure 2. miRNA and mRNA complementarity. Duplex formation involves matching of the mRNA and miRNA seed region sequence (Adapted from Bartel, 2009).

Depending on the degree of complementarity, the message will be either cleaved or translation halted, since ribosomes will be blocked to perform their function. Hence, miRNAs activity will in most cases result in a decrease of the protein output (1–7). miRNAs expression occurs in most tissues, it is finely tuned and for some miRNAs, their expression is constrained to only one tissue or certain stage of the life cycle, providing them with an spatiotemporal expression pattern that results in an specific miRNA milieu providing a context for all cells to maintain homeostasis (1–3) Therefore, miRNAs are indispensable mediators for mostly all physiological processes throughout life cycle, including developmental processes, cell differentiation and cell cycle arrest, and their expression is modified upon situations such as nutrient deprivation, hypoxic environment, among many others (1–3) Importantly, it has been established that more than 60% of human protein coding genes are conserved targets for miRNAs, and 1-2% of predicted protein coding human genes correspond to miRNAs expression (1,6,8).

miRNAs can target many messages and mRNAs can be targeted by many miRNAs, this represents high complexity for effectively investigating possible miRNA:mRNA interactions and the phenotypic consequences of these interactions, hence computational and systems biology approaches are currently being developed and tuned to predict interactions and keep advancing in the research field. The basis of most prediction algorithms involve evolutionary conservation, based on mRNA 3´UTR being highly conserved among species due to selective pressure, and phenotypic analyses include network construction to identify possible disrupted hubs in response to specific miRNA:mRNA interactions (1,6,8–10). For instance, Boudreau et al. performed a transcriptome-wide discovery of miRNAs binding sites in brain, since miRNAs hold essential roles in neurological functions such as synaptic plasticity, and slight miRNAs disturbances result in repercussions in brain physiology. The study found by HITS-CLIP (High throughput sequencing – cross linking immunoprecipitation), an enriched interaction between miR-128 and MAPT gene, which encodes for TAU proteins involved in Alzheimer´s disease (11), suggesting that the loss-of-function of these miRNA could be implicated in the development of the neurological disease.

In addition, throughout human development, certain miRNA species are fundamental for processes like osteoblast differentiation, and studies have shown that miR-29b directly down regulates osteoblast inhibitors providing an spatiotemporal control of development and homeostasis during osteogenesis by miRNAs (12).

miRNAs have been subject of research for numerous diseases with the attempt of determining miRNA profiles that could be involved in the development of the disease to develop miRNA therapies or profiles that result from the disease to detect biomarkers, preventing advance of disease. Numerous clinical trials are currently researching miRNAs. During cancer progression, characteristic tumor pathways can be controlled by single or multiple miRNAs, commonly engaging reciprocal feedback interactions with mRNA targets, some miRNAs acting as tumor suppressors while others act as oncomiRs. Cancer cells actually develop mechanisms to dysregulate miRNA expression including the generation of mRNA isoforms with shortened 3 ´UTR (13,14). Examples include miR-200c inhibiting ZEB transcription factor which promotes EMT (15,16), general lower expression of miRNAs during hepatocellular carcinoma (17), the interplay between miRNAs and the development of the Warburg metabolic phenotype inducing a tumor promoting microenvironment (18), consistent up regulation of miR-21 in different types of cancers resulting in tumorigenesis (19), a 4-miRNA signature displayed during non-small-cell lung cancer (20), the p53-miR34 regulatory axis that harbors tumor suppressive activity (21), or in contrast, miR-155 which repress p53 activity resulting in tumor progression (22), among many others. In sum, cancer progression is deeply regulated by miRNAs.

Other diseases where miRNAs are players in the pathogenesis include autoimmune diseases, infections, metabolic conditions and vascular/cardiac illnesses (23–25). Currently, miRNAs have inherent therapeutic applications during endothelium dysfunction, for instance miR-30 family contribute in the protection against atherogenesis (24) and during diabetes, studies have shown that miR-375 and -144 disrupt insulin sensitivity during hyperglycemia (25).

Interestingly, Fabbri et al have shown a non-canonical mechanism were miRNAs encapsulated in exosomes can be transferred from cell-to-cell. Exosome encapsulated miR-21 and mir-29 can bind to Toll-like receptors in immune cells, leading to TLRs promotion of prometastatic inflammatory response, meaning that miRNAs can bind to proteins and modify their function (26).

Exosomes are cell-derived nanovesicles (40-100 nm) released by all cells, serving as strong cell-to-cell communicators. Exosomes emerge during the development of late endosomes to become MVBs, where the endosome membrane is invig。inated, forming intra luminal vesicles, resulting in the completed process of creating MVBs, which contain the intraluminal vesicles, namely exosomes. The process involves the accumulation of ubiquitinated proteins, which are transferred by the cytosolic endosomal-sorting complex required for transport (ESCRT). There are 4 different types of ESCRT involved in the formation of exosomes, ESCRT-0 sequesters ubiquitinated proteins, ESCRT-I coordinated with ESCRT-II start the budding of the endosomal membrane, and finally ESCRT-III is involved in the release of intraluminal vesicles to become exosomes (Figure 3) (27–33).

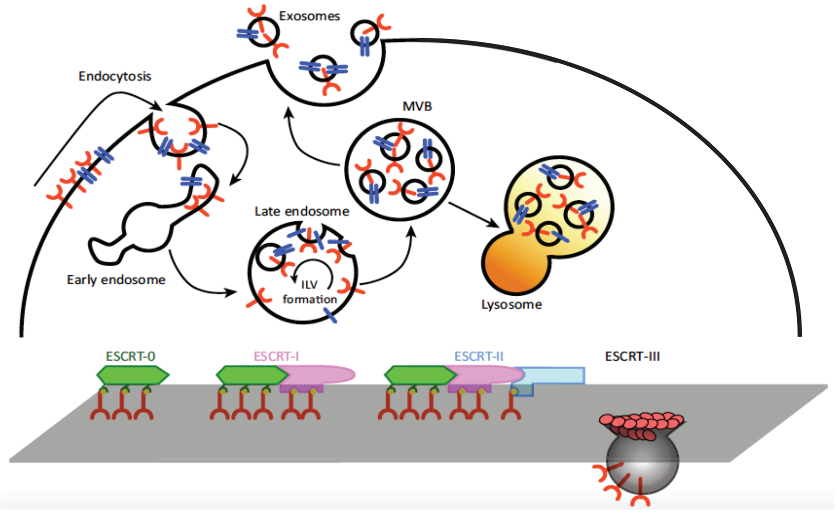


Figure 3. Exosomes assembly and release. Machinery for exosomes assembly involves ESCRT interaction with endosomes (Adapted from Cocucci, 2015).

During exosomes assembly, one of the most important encapsulated cargo correspond to miRNAs, since their transport influences numerous physiological and pathological functions, and interestingly there is a curvature sorting for miRNA content during budding of the membrane, this confers control for the physiological role the exosome will play (27,30,32).

Exosomes are composed by a lipid bilayer and their uptake by target cells is mediated usually by endocytosis or phagocytosis, implicating an specific exosome membrane glycosylation pattern to be interacting with the recipient cell membrane (27,30,33). Exosomes miRNA cargos are released within the recipient cell, leading to post-transcriptional gene regulation, hence exosomes can modify the physiological state of the recipient cell (28–30). Controlled miRNA encapsulation is relevant since all studied mRNA:miRNA interactions are influenced by cell communication since the exosomes lipid bilayer confer protection for miRNA transport across tissues against RNases and low-pH (30).

Furthermore, based on the possibility of transporting miRNA and other cargoes, exosomes are widely studied in the development of various diseases, for instance in cancer, tumors secrete exosomes contributing to metastasis by transferring miRNA species that favor tumorigenesis and studies have shown that cancer exosomes harbor miRNA biogenesis machinery, and exosomes are implicated in the tumor-genetic alterations within the tumor microenvironment (31,33–36).

Moreover, during inflammation exosomes can transport miR-155 which enhances inflammatory activity (37), while during type 2 diabetes, specific miRNA profiles have been found in exosomes, and during obesity, exosomes originated form adipose tissue can increase cytokine secretion and impair insulin signaling (32). Finally as miRNAs contained in exosomes have been found to regulate TLRs activity (26), Paschon et al described exosomes secreted by neuronal cells, activate TLRs resulting in inflammation that contributes to the development of brain disorders including Alzheimer's, Parkinson's disease and amyotrophic lateral sclerosis (38). Notably, since exosomes are present in many body fluids including urine, plasma and breast milk, exosomes can serve as accurate biomarkers for disease (27,39,40) .

Remarkably, based on exosome encapsulation, plant miRNAs are present in animal body fluids (41,42), Zhang et al. was the first to demonstrate a possible cross-kingdom gene regulation describing that miR168a abundant in rice and one of the most enriched exogenous miRNA in Chinese population sera, can cross the gastro-intestinal (GI) track and bind to low density lipoprotein receptor adaptor 1 (LDLRAP1) mRNA, inhibiting their expression in mice liver, resulting in a decrease of LDL removal from plasma (41). Moreover, exosomes allow for plant miRNAs to be stable in cooked foods and to resist low-pH and RNases activity in the GI track (41–44).

Dietary exogenous exosomal miRNAs are also present in animal sources such as pork, beef and chicken (45) and studies have shown that humans absorb egg-borne miRNAs, who are delivered to PBMCs where they affect gene expression (46).

In human breast milk, numerous studies have shown the presence of miRNAs, mainly encapsulated in exosomes. These miRNAs have been implicated in immunoprotective activities, growth and development, transcriptional regulation, metabolic response, cell proliferation and apoptosis, lung epithelial progenitor cell differentiation, and epithelial-to-mesenchymal transition, providing a new insight of how breast milk modulate infant growth and development (47–51). Interestingly, certain plant miRNA species have been identified in human breast milk exosomes (52,53). Several studies have described that encapsulation in exosomes allow for human milk miRNAs to resist harsh conditions through the intestinal barrier and exert effects in infant development and maturation (54).

Naturally, numerous miRNAs have also been identified in cow´s milk, mainly encapsulated in exosomes resulting in miRNA stability during milk processing and GI transport conditions, for instance temperature changes, low-pH and RNases activity (55–59). Studies in our laboratory have displayed that humans and mice can absorb cow´s milk miRNAs and the absorbed miRNAs are able to alter gene expression. More importantly, endogenous miRNA synthesis cannot compensate for the depletion of dietary miRNAs (60). Bovine milk exosomes are transported across the intestine by glycoprotein dependent endocytosis in Colon Carcinoma Caco-2 Cells and Rat Small Intestinal IEC-6 cells. Also, endocytosis transport is observed across endothelium in Human Vascular Endothelial (HUVEC) cells. Additionally, fluorophore-tagged milk exosomes can accumulate in liver spleen and brain after oral administration, demonstrating the host can absorb exosomes and their miRNAs. Finally, exosomal miRNAs can be uptaken by human macrophages, and for instance in mice, absorbed exosomes can exert immunoregulatory effects (55,59,61–64).

Currently, 417 distinct miRNAs have been detected in bovine milk exosomes (65), which could interact with host mRNA targets. Furthermore, oral delivery of bovine milk exosomes displayed osteocytes increase in mice (66), while in C_2C_{12} myotubes, increases in skeletal muscle protein synthesis and anabolism was observed upon whey protein derived exosomes treatment, possibly lead by exosomal miRNAs (67).

Moreover, *In silico* predictions have identified numerous exogenous miRNAs encapsulated in exosomes as transportable candidates in human circulation that harbor identical sequences with humans, providing them the possibility of targeting mRNAs regulating gene expression (68), based on this plethora of dietary miRNAs from diverse food sources that could have diverse mRNA targets, a dietary microRNA Database (DMD) was developed and is currently available for public use (69).

Altogether, these observations lead to conclude that milk-derived exogenous miRNAs can be qualified as novel nutrient components and could be relevant for the pathogenesis of certain diseases (41,42,60,66,67,70). This statement challenges the paradigm that miRNAs can have physiological effects only when synthetized endogenously, since exosomes and their RNA cargos may also be obtained from dietary sources such as bovine milk.

Importantly, human breast milk is the optimum nutritional source for infants, and compared to commercial formulas, human breast milk provides superior benefits related to adequate growth, cognitive development, insulin sensitivity, immunological function, antioxidant activities, bone mineral content, and lower risk biomarkers for future obesity during the first months of life. In addition, a different metabolite profile was detected by serum biochemistry in infants and gene expression in breast milk cells can impact infant´s development (71–77).

Moreover, bovine milk and dairy products are globally consumed foods by children and adults. Notably, epidemiological studies have established a negative association between milk intake and development of type 2 diabetes, hypertension and cardiovascular disease, metabolic syndrome and a positive correlation between milk intake and cognitive function, particularly memory improvement (78,79) Interestingly, supporting evidence has demonstrated that milk consumption can decrease serum uric acid concentrations (80).

Uric acid is a purine metabolite, the metabolism of purines involve 2 biosynthetic and one catabolic pathways, purine *de novo* and salvage synthesis is mediated by numerous signals and various critic transcription factors, including mTOR, Myc, p53, among others and it also involves a purinosome assembly (81,82). Additionally it is known that purines are also regulated by the hepatic circadian rhythm (83). Purines participate in numerous critical cellular functions and biological processes thus they must be quantitatively controlled during quiescent or proliferative cell conditions (84). Defects in purine metabolism can result in disease development in infants and adults (85).

It is known that purine continuous and persistent increased concentrations can lead to the aberrant activation of signaling molecules involved in pathogenic cascades, and alterations in the purine metabolic pathway can lead to the development of several diseases. For instance during cancer, purines can serve as signaling molecules involved in cancer progression, as such certain adenosine receptors activation leads to immunosuppressive mechanisms that drive to tumor growth (86–88), and it is known that adenosine is a signaling molecule in angiogenesis and in some cell conditions, increased adenosine results in p53 activation (89,90).

Moreover, implications of deregulated purine metabolism and purinergic signaling have been associated to neurological function, cardiovascular health, metabolic syndrome, non-alcoholic fatty liver disease, type 2 diabetes, chronic renal disease and fertility impairment (91,92). Particularly, high concentrations of UA have been associated with the development of diabetes, metabolic syndrome, and cancer (93–96).

Additionally, studies determined that ATP and adenosine impairs hippocampal synaptic plasticity, thus learning and memory process (97,98). Furthermore, a differential pattern of purine expression was detected in the precuneus of Alzheimer's patients, with an increased expression of AK1 and NT5C and decreased expression of AK4 and NME6 (99). Finally, cognitive impairment during aging, including deficits in learning and memory, is led by a metabolic drift characterized by altered purine biosynthesis and degradation (100).

A metabolomic and transcriptomic approach to identify a differential phenotype in response to bovine milk exosome and their RNAs depletion could help confirm miRNA transfer among different species through diet and identify functional consequences, such as increase in purine metabolite concentrations.

References

1. Bartel DP. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. Cell. 2004;116:281–97.
2. Chuang JC, Jones PA. Epigenetics and microRNAs. Pediatr Res. 2007;61:17–23.
3. Cai Y, Yu X, Hu S, Yu J. A Brief Review on the Mechanisms of miRNA Regulation. Genomics, Proteomics Bioinforma. 2009;7:147–54.
4. Yates L a, Norbury CJ, Gilbert RJC. Minireview The Long and Short of MicroRNA. Cell. 2013;153:516–9.
5. Vidigal JA, Ventura A. The biological functions of miRNAs: Lessons from in vivo studies. Trends Cell Biol. Elsevier Ltd; 2015;25:137–47.
6. Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions. Cell. 2009;136:215–33.
7. Djuranovic S, Nahvi A, Green R. miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. Science (80-). 2012;336:237–40.
8. Friedman RC, Farh KKH, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19:92–105.
9. Agarwal V, Bell GW, Nam J-W, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife. eLife Sciences Publications, Ltd; 2015;4:e05005.
10. Helwak A, Kudla G, Dudnakova T, Tollervey D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. Cell. Cell Press;

2013;153:654–65.

11. Boudreau RL, Jiang P, Gilmore BL, Spengler RM, Tirabassi R, Nelson JA, Ross CA, Xing Y, Davidson BL. Transcriptome-wide discovery of microRNA binding sites in Human Brain. Neuron. Elsevier Inc.; 2014;81:294–305.
12. Li Z, Hassan MQ, Jafferji M, Aqeilan RI, Garzon R, Croce CM, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem. 2009;284:15676–84.
13. Bracken CP, Scott HS, Goodall GJ. A network-biology perspective of microRNA function and dysfunction in cancer. Nat Rev Genet. Nature Publishing Group; 2016;17:719–32.
14. Sundarbose K, Kartha R, Subramanian S. MicroRNAs as Biomarkers in Cancer. Diagnostics. 2013;3:84–104.
15. Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, Brabletz T. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 2008;9:582–9.
16. Korpal M, Kang Y. The emerging role of miR-200 family of microRNAs in epithelial-mesenchymal transition and cancer metastasis. RNA Biol. 2008;5:115–9.
17. Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, Shimotohno K. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene. 2006;25:2537–45.
18. Arora A, Singh S, Bhatt AN, Pandey S, Sandhir R, Dwarakanath BS. Interplay

between metabolism and oncogenic process: Role of microRNAs. Transl Oncogenomics. 2015;7:11–27.

19. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: Biomarkers, functions and therapy. Trends Mol Med. Elsevier Ltd; 2014;20:460–9.

20. Hu Z, Chen X, Zhao Y, Tian T, Jin G, Shu Y, Chen Y, Xu L, Zen K, Zhang C, et al. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J Clin Oncol. 2010;28:1721–6.

21. Peng Y, Croce CM. The role of MicroRNAs in human cancer. Signal Transduct Target Ther. 2016;1:15004.

22. Vannini I, Fanini F, Fabbri M. Emerging roles of microRNAs in cancer. Curr Opin Genet Dev. 2018.

23. Tsai L, Yu D. MicroRNAs in common diseases and potential therapeutic. Clin Exp Phamarcology Physiol. 2010;37:5269.

24. Araldi E, Suárez Y. MicroRNAs as regulators of endothelial cell functions in cardiometabolic diseases. Biochim Biophys Acta. 2016;1861:2094–103.

25. Santovito D, Egea V, Weber C. Small but smart: MicroRNAs orchestrate atherosclerosis development and progression. Biochim Biophys Acta - Mol Cell Biol Lipids. Elsevier B.V.; 2016;1861:2075–86.

26. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. Pnas. 2012;109:E2110–E2116.

27. Yáñez-Mó M, Siljander PRM, Andreu Z, Zavec AB, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles. 2015;4:1–60.
28. Tkach M, Théry C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. Cell. 2016;164:1226–32.
29. Cocucci E, Meldolesi J. Ectosomes and exosomes: Shedding the confusion between extracellular vesicles. Trends Cell Biol. Elsevier Ltd; 2015;25:364–72.
30. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. Nature Publishing Group; 2007;9:654.
31. Neviani P, Fabbri M. Exosomic microRNAs in the Tumor Microenvironment. Front Med. 2015;2:1–6.
32. Huang-Doran I, Zhang CY, Vidal-Puig A. Extracellular Vesicles: Novel Mediators of Cell Communication In Metabolic Disease. Trends Endocrinol Metab. Elsevier Ltd; 2017;28:3–18.
33. Escrevente C, Keller S, Altevogt P, Costa J. Interaction and uptake of exosomes by ovarian cancer cells. BMC Cancer. 2011;11:1–10.
34. Fanini F, Fabbri M. Cancer-derived exosomic microRNAs shape the immune system within the tumor microenvironment: State of the art. Seminars in Cell and Developmental Biology. 2017. p. 23–8.
35. Melo SA, Sugimoto H, O’Connell JT, Kato N, Villanueva A, Vidal A, Qiu L, Vitkin E, Perelman LT, Melo CA, et al. Cancer Exosomes Perform Cell-

Independent MicroRNA Biogenesis and Promote Tumorigenesis. Cancer Cell. Elsevier Inc.; 2014;26:707–21.

36. Zhao H, Yang L, Baddour J, Achreja A, Bernard V, Moss T, Marini JC, Tudawe T, Seviour EG, San Lucas FA, et al. Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism. Elife. 2016;5:1–27.

37. Alexander M, Hu R, Runtsch MC, Kagele DA, Mosbruger TL, Tolmachova T, Seabra MC, Round JL, Ward DM, O'Connell RM. Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. Nat Commun. 2015;6:1–15.

38. Paschon V, Takada SH, Ikebara JM, Sousa E, Raeisossadati R, Ulrich H, Kihara AH. Interplay Between Exosomes, microRNAs and Toll-Like Receptors in Brain Disorders. Mol Neurobiol. 2016;53:2016–28.

39. Wang K, Zhang S, Weber J, Baxter D, Galas DJ. Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res. 2010;38:7248–59.

40. Lasser C, Alikhani VS, Ekstrom K, Eldh M, Paredes PT, Bossios A, Sjostrand M, Gabrielsson S, Lotvall J, Valadi H. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. J Transl Med. England; 2011;9:9.

41. Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y, Li J, Bian Z, Liang X, Cai X, et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: Evidence of cross-kingdom regulation by microRNA. Cell Res. 2012;22:107–26.

42. Fabris L, Calin GA. Circulating free xeno-microRNAs - The new kids on the block. Mol Oncol. 2016;10:503–8.

43. Philip A, Ferro VA, Tate RJ. Determination of the potential bioavailability of plant microRNAs using a simulated human digestion process. Mol Nutr Food Res. 2015;59:1962–72.

44. Zhang H, Li Y, Liu Y, Liu H, Wang H, Jin W, Zhang Y, Zhang C, Xu D. Role of plant MicroRNA in cross-species regulatory networks of humans. BMC Syst Biol. BMC Systems Biology; 2016;10:1–10.

45. Otsuka K, Yamamoto Y, Matsuoka R, Ochiya T. Maintaining good miRNAs in the body keeps the doctor away?: Perspectives on the relationship between food-derived natural products and microRNAs in relation to exosomes/extracellular vesicles. Mol Nutr Food Res. 2018;62:1700080.

46. Zempleni J, Baier SR, Howard KM, Cui J. Gene regulation by dietary microRNAs. Can J Physiol Pharmacol. 2015;93:1097–102.

47. Kosaka N, Izumi H, Sekine K, Ochiya T. MicroRNA as a new immune-regulatory agent in breast milk. Silence. 2010;1:1–8.

48. Zhou Q, Li M, Wang X, Li Q, Wang T, Zhu Q, Zhou X, Wang X, Gao X, Li X. Immune-related MicroRNAs are Abundant in Breast Milk Exosomes. Int J Biol Sci. 2012;8:118–23.

49. Alsaweed M, Hartmann PE, Geddes DT, Kakulas F. MicroRNAs in breastmilk and the lactating breast: Potential immunoprotectors and developmental regulators for the infant and the mother. International Journal of Environmental Research and

Public Health. 2015. 13981-14020 p.

50. Munch EM, Harris RA, Mohammad M, Benham AL, Pejerrey SM, Showalter L, Hu M, Shope CD, Maningat PD, Gunaratne PH, et al. Transcriptome Profiling of microRNA by Next-Gen Deep Sequencing Reveals Known and Novel miRNA Species in the Lipid Fraction of Human Breast Milk. PLoS One. 2013;8:1–13.
51. Alsaweed M, Lai CT, Hartmann PE, Geddes DT, Kakulas F. Human milk miRNAs primarily originate from the mammary gland resulting in unique miRNA profiles of fractionated milk. Sci Rep. Nature Publishing Group; 2016;6:1–13.
52. Lukasik A, Brzozowska I, Zielenkiewicz U, Zielenkiewicz P. Detection of plant miRNAs abundance in human breast milk. Int J Mol Sci. 2018;19.
53. Lukasik A, Zielenkiewicz P. In silico identification of plant miRNAs in mammalian breast milk exosomes - A small step forward? PLoS One. 2014;9.
54. Tomé-Carneiro J, Fernández-Alonso N, Tomás-Zapico C, Visioli F, Iglesias-Gutierrez E, Dávalos A. Breast milk microRNAs harsh journey towards potential effects in infant development and maturation. Lipid encapsulation can help. Pharmacol Res. 2018;132:21–32.
55. Izumi H, Tsuda M, Sato Y, Kosaka N, Ochiya T, Iwamoto H, Namba K, Takeda Y. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. J Dairy Sci. 2015;98:2920–33.
56. Chen X, Gao C, Li H, Huang L, Sun Q, Dong Y, Tian C, Gao S, Dong H, Guan D, et al. Identification and characterization of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products. Cell

Res. Nature Publishing Group; 2010;20:1128–37.

57. Cui J, Zhou B, Ross SA, Zempleni J. Nutrition, microRNAs, and Human Health. Adv Nutr. 2017;8:105.

58. Benmoussa A, Lee CHC, Laffont B, Savard P, Laugier J, Boilard E, Gilbert C, Fliss I, Provost P. Commercial Dairy Cow Milk microRNAs Resist Digestion under Simulated Gastrointestinal Tract Conditions. J Nutr. 2016;146:2206–15.

59. P Rani, M. Vashisht, N. Golla, S. Shandilya, S. Onteru DS. Milk miRNAs encapsulated in exosomes are stable to human digestion and permeable to intestinal barrier in vitro. J Funct Foods. 2017;v. 34:9-439–2017 v.34.

60. Baier SR, Nguyen C, Xie F, Wood JR, Zempleni J. MicroRNAs are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 kidney cell cultures, and mouse livers. J Nutr. United States; 2014;144:1495–500.

61. Wolf T, Baier SR, Zempleni J. The Intestinal Transport of Bovine Milk Exosomes Is Mediated by Endocytosis in Human Colon Carcinoma Caco-2 Cells and Rat Small Intestinal IEC-6 Cells. J Nutr. 2015;145:2201–6.

62. Kusuma RJ, Manca S, Friemel T, Sukreet S, Nguyen C, Zempleni J. Human vascular endothelial cells transport foreign exosomes from cow's milk by endocytosis. Am J Physiol - Cell Physiol. 2016;310:C800–7.

63. Manca S, Upadhyaya B, Mutai E, Desaulniers AT, Cederberg RA, White BR, Zempleni J. Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. Sci Rep. Nature Publishing Group UK;

2018;8:11321.

64. Pieters BCH, J.Arntz O, Bennink MB, Broeren MGA, Van Caam APM, Koenders MI, Van Lent PLEM, Van Den Berg WB, De Vries M, Van Der Kraan PM, et al. Commercial cow milk contains physically stable extracellular vesicles expressing immunoregulatory TGF-β. PLoS One. 2015;10:1–14.

65. Sun J, Aswath K, Schroeder SG, Lippolis JD, Reinhardt TA, Sonstegard TS. MicroRNA expression profiles of bovine milk exosomes in response to Staphylococcus aureus infection. BMC Genomics. BioMed Central; 2015;16:806.

66. Oliveira MC, Arntz OJ, Blaney Davidson EN, van Lent PLEM, Koenders MI, van der Kraan PM, van den Berg WB, Ferreira AVM, van de Loo FAJ. Milk extracellular vesicles accelerate osteoblastogenesis but impair bone matrix formation. J Nutr Biochem. 2016;30:74–84.

67. Mobley CB, Mumford PW, McCarthy JJ, Miller ME, Young KC, Martin JS, Beck DT, Lockwood CM, Roberts MD. Whey protein-derived exosomes increase protein synthesis and hypertrophy in C2- C12 myotubes. J Dairy Sci. Elsevier; 2017;100:48–64.

68. Shu J, Chiang K, Zempleni J, Cui J. Computational characterization of exogenous microRNAs that can be transferred into human circulation. PLoS One. 2015;10:1–19.

69. Chiang K, Shu J, Zempleni J, Cui J. Dietary microRNA database (DMD): An archive database and analytic tool for food-borne microRNAs. PLoS One. 2015;10:1–13.

70. Rani P, Yenuganti VR, Shandilya S, Onteru SK, Singh D. miRNAs: The hidden bioactive component of milk. Trends Food Sci Technol. 2017;65:94–102.

71. Shoji H, Shimizu T. The role of human breast milk on biological metabolism in infants. Pediatr Int. 2018;0–3.

72. Wu TC, Huang IF, Chen YC, Chen PH, Yang LY. Differences in serum biochemistry between breast-fed and formula-fed infants. J Chinese Med Assoc. 2011;74:511–5.

73. Andres A, Cleves MA, Bellando JB, Pivik RT, Casey PH, Badger TM. Developmental Status of 1-Year-Old Infants Fed Breast Milk, Cow's Milk Formula, or Soy Formula. Pediatrics. 2012;129:1134–40.

74. Andres A, Casey PH, Cleves MA, Badger TM. Body fat and bone mineral content of infants fed breast milk, cow's milk formula, or soy formula during the first year of life. J Pediatr. Elsevier Ltd; 2013;163:49–54.

75. Twigger AJ, Hepworth AR, Tat Lai C, Chetwynd E, Stuebe AM, Blancafort P, Hartmann PE, Geddes DT, Kakulas F. Gene expression in breastmilk cells is associated with maternal and infant characteristics. Sci Rep. Nature Publishing Group; 2015;5:1–14.

76. Smilowitz JT, O'Sullivan A, Barile D, German JB, Lonnerdal B, Slupsky CM. The human milk metabolome reveals diverse oligosaccharide profiles. J Nutr. United States; 2013;143:1709–18.

77. Pecoraro L, Agostoni C, Pepaj O, Pietrobelli A. Behind human milk and breastfeeding: not only food. Int J Food Sci Nutr. Taylor & Francis; 2018;69:641–

6.

78. Visioli F, Strata A. Milk, Dairy Products, and Their Functional Effects in Humans: A Narrative Review of Recent Evidence. Adv Nutr An Int Rev J. 2014;5:131–43.

79. Camfield DA, Owen L, Scholey AB, Pipingas A, Stough C. Dairy constituents and neurocognitive health in ageing. Br J Nutr. 2011;106:159–74.

80. Choi HK, Liu S, Curhan G. Intake of purine-rich foods, protein, and dairy products and relationship to serum levels of uric acid: The third national health and nutrition examination survey. Arthritis Rheum. 2005;52:283–9.

81. Ben-Sahra I, Hoxhaj G, Ricoult SJH, Asara JM, Manning BD. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. Science. United States; 2016;351:728–33.

82. French JB, Jones SA, Deng H, Pedley AM, Kim D, Chan CY, Hu H, Pugh RJ, Zhao H, Zhang Y, et al. Spatial colocalization and functional link of purinosomes with mitochondria. Science. 2016;351:733 LP-737.

83. Fustin JM, Doi M, Yamada H, Komatsu R, Shimba S, Okamura H. Rhythmic Nucleotide Synthesis in the Liver: Temporal Segregation of Metabolites. Cell Rep. The Authors; 2012;1:341–9.

84. Lane AN, Fan TWM. Regulation of mammalian nucleotide metabolism and biosynthesis. Nucleic Acids Res. 2015;43:2466–85.

85. Vidotto C, Fousert D, Akkermann M, Griesmacher A, Müller MM. Purine and pyrimidine metabolites in children’s urine. Clin Chim Acta. 2003;335:27–32.

86. Gessi S, Merighi S, Sacchetto V, Simioni C, Borea PA. Adenosine receptors and

cancer. Biochim Biophys Acta - Biomembr. 2011;1808:1400–12.

87. Allard B, Beavis PA, Darcy PK, Stagg J. Immunosuppressive activities of adenosine in cancer. Curr Opin Pharmacol. 2016;29:7–16.

88. Di Virgilio F. Purines, purinergic receptors, and cancer. Cancer Res. United States; 2012;72:5441–7.

89. Long JS, Crighton D, O'Prey J, MacKay G, Zheng L, Palmer TM, Gottlieb E, Ryan KM. Extracellular adenosine sensing-a metabolic cell death priming mechanism downstream of p53. Mol Cell. Elsevier Inc.; 2013;50:394–406.

90. Gama V, Deshmukh M. Adenosine: Essential for life but licensed to kill. Mol Cell. Elsevier; 2013;50:307–8.

91. Kanbay M, Jensen T, Solak Y, Le M, Roncal-Jimenez C, Rivard C, Lanaspa MA, Nakagawa T, Johnson RJ. Uric acid in metabolic syndrome: From an innocent bystander to a central player. Eur J Intern Med. 2016;29:3–8.

92. Bilodeau-Goeseels S, Sasseville M, Guillemette C, Richard FJ. Effects of adenosine monophosphate-activated kinase activators on bovine oocyte nuclear maturation in vitro. Mol Reprod Dev. United States; 2007;74:1021–34.

93. Lippi G, Montagnana M, Franchini M, Favaloro EJ, Targher G. The paradoxical relationship between serum uric acid and cardiovascular disease. Clin Chim Acta. Netherlands; 2008;392:1–7.

94. Viazzi F, Garneri D, Leoncini G, Gonnella A, Muiesan ML, Ambrosioni E, Costa F V, Leonetti G, Pessina AC, Trimarco B, et al. Serum uric acid and its relationship with metabolic syndrome and cardiovascular risk profile in patients

with hypertension: insights from the I-DEMAND study. Nutr Metab Cardiovasc Dis. Netherlands; 2014;24:921–7.

95. Rock KL, Kataoka H, Lai J-J. Uric acid as a danger signal in gout and its comorbidities. Nat Rev Rheumatol. United States; 2013;9:13–23.

96. Johnson RJ, Nakagawa T, Sanchez-Lozada LG, Shafiu M, Sundaram S, Le M, Ishimoto T, Sautin YY, Lanaspa MA. Sugar, Uric Acid, and the Etiology of Diabetes and Obesity. Diabetes. 2013;62:3307 LP-3315.

97. Düster R, Prickaerts J, Blokland A. Purinergic signaling and hippocampal long-term potentiation. Curr Neuropharmacol. Bentham Science Publishers; 2014;12:37–43.

98. Shrager Y, Bayley PJ, Bontempi B, Hopkins RO, Squire LR. Spatial memory and the human hippocampus. Proc Natl Acad Sci. 2007;104:2961 LP-2966.

99. Ansoleaga B, Jove M, Schluter A, Garcia-Esparcia P, Moreno J, Pujol A, Pamplona R, Portero-Otin M, Ferrer I. Deregulation of purine metabolism in Alzheimer's disease. Neurobiol Aging. United States; 2015;36:68–80.

100. Ivanisevic J, Stauch KL, Petrascheck M, Benton HP, Epstein AA, Fang M, Gorantla S, Tran M, Hoang L, Kurczy ME, et al. Metabolic drift in the aging brain. Aging (Albany NY). United States; 2016;8:1000–20.

CHAPTER I

Concentrations of Purine Metabolites are Elevated in Fluids from Adults and Infants and in Livers from Mice Fed Diets Depleted of Bovine Milk Exosomes and their RNA Cargos

Ana Aguilar-Lozano,[4] Scott Baier,[4] Ryan Grove,[5] Jiang Shu,[6] David Giraud,[4] Amy Leiferman,[4] Kelly E. Mercer, [7] Juan Cui,[6] Thomas M. Badger,[7] Jiri Adamec,[5] Aline Andres, [7] Janos Zempleni[4]

[4]*Department of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, NE*

[5]*Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, NE*

[6]*Department of Computer Science & Engineering, University of Nebraska-Lincoln, Lincoln, NE*

[7]*Arkansas Children's Nutrition Center and Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR*

Corresponding Author: Janos Zempleni; jzempleni2@unl.edu; 316C Leverton Hall, Lincoln, NE 68583, USA

Co-corresponding Author: Aline Andres; alineandres@uams.edu; Arkansas Children's Nutrition Center and Department of Pediatrics, Little Rock, Arkansas 72202, USA

Running Title: Purine alterations in mammals fed milk exosome-defined diets

Funding: This material is based upon work that is supported by the National Institute of Food and Agriculture (NIFA), U.S. Department of Agriculture, under award numbers 2015-67017-23181 and 2016-67001-25301, National Institutes of Health grant 1P20GM104320, Egg Nutrition Center, the Gerber Foundation, the University of Nebraska Agricultural Research Division (Hatch Act), and USDA multistate group W3002 (all to J.Z.), a Food for Health grant by the University of Nebraska President's Office (J.C. and J.Z.). Funding for the clinical study was from the USDA-Agricultural Research Service Project 6026-51000-010-05S A.A and T.B). J.Z. serves as a consultant for PureTech Health, Inc.

Key words: Bovine milk; exosomes; metabolomics; purines; RNA

Abstract

Background: Humans and mice absorb bovine milk exosomes and their RNA cargos.

Objectives: Objectives were to determine whether milk exosome and RNA-depleted (ERD) and exosome and RNA-sufficient (ERS) diets alter the concentrations of purine metabolites in mouse livers, and to determine whether diets depleted of bovine milk alter the plasma concentration and urine excretion of purine metabolites in adults and infants, respectively.

Methods: C57BL/6 mice were fed ERD (providing 2% of the microRNA cargos compared to ERS) and ERS diets starting at age 3 weeks; livers were collected at age 7 weeks. Plasma and 24-h urine samples were collected from healthy adults who consumed (DC) or avoided (DA) dairy products. Spot urine samples were collected from healthy infants fed human milk (HM), milk formula (MF) or soy formula (SF) at age 3 months. Purine metabolites were analyzed in livers, plasma and urines; mRNAs and microRNAs were analyzed in female mice livers.

Results: Nine hepatic purine metabolites in ERD fed mice were 1.76 ± 0.43 times the concentrations in ERS ($P < 0.05$). Plasma concentrations and urine excretion of purine metabolites in DA was up to 1.62 ± 0.45 times the concentrations in DC ($P < 0.05$). The excretion of 13 purine metabolites in urine from SF infants was up to 175 ± 39 times the excretion in HM and MF ($P < 0.05$). mRNA expression of 5’-Nucleotidase, Cytosolic IIIB and adenosine deaminase in mice fed ERD was 0.64 ± 0.52 and 0.60 ± 0.28 times the expression in mice fed ERS.

Conclusion: Diets depleted of bovine milk exosomes and RNA cargos caused increases in hepatic purine metabolites in mice, and in plasma and urine from adults and infants compared to exosome-sufficient controls. Findings are important, because purines play a role in intermediary metabolism and cell signaling.

Key words: Bovine milk; exosomes; metabolomics; purines; RNA

1. Introduction

Virtually every living cell produces and secretes a class of nanoparticles named exosomes (1, 2). Exosomes deliver cargos such as lipids, proteins and various species of RNA to adjacent or distant recipient cells. Exosome uptake by recipient cells is mediated by endocytosis (3). The delivery of exosome cargos elicits changes in gene expression and metabolism in recipient cells (1, 2). Among exosome cargos, microRNAs are of particular interest, because more than 60% of human genes have conserved microRNA-binding sites and loss of microRNA maturation is embryonic lethal (4, 5). MicroRNAs have been implicated in most physiological and pathological conditions (6, 7).

We have provided strong evidence that exosomes and their RNA cargos do not originate exclusively in endogenous synthesis, but may also be obtained from dietary sources such as bovine milk. The following lines of evidence were presented in support of this paradigm-shifting theory. First, the plasma concentrations of milk-borne microRNAs increased following a milk meal in humans, whereas the concentration of a microRNA not detectable in milk (miR-1) did not increase (8, 9). Recently, we confirmed these findings by using a nucleotide-specific PCR protocol, RNase H2 PCR, which distinguishes microRNAs derived from endogenous synthesis and those obtained from bovine milk (10). Second, human and rodent intestinal cells, human immune cells and human vascular endothelial cells accumulate bovine milk exosomes by endocytosis and secrete microRNA across the basolateral membrane (11-13). These observations are consistent with reports suggesting that orally administered milk exosomes accumulate in peripheral tissues and that only about half of the extracellular RNAs in human plasma are of human origin (14, 15). Third, *in vitro* evidence suggests that microRNAs are protected against degradation by RNases and low pH through encapsulation in exosomes, and partially protected against degradation in the TNO intestinal model (16, 17). Some voices of concern remain, doubting that the absorption of dietary microRNAs is quantitatively meaningful (reviewed in (18) and discussed below). However, evidence is accumulating that RNAs from dietary sources other than milk are also bioavailable (reviewed in (18); additional recent evidence in (19)).

Of note, when mice were fed an AIN-93G-based diet, modified to be defined by its content of bovine milk exosomes for four weeks, plasma microRNA levels were ≈60% lower in mice fed exosome RNA-depleted (ERD) diet compared to mice fed the exosome RNA-sufficient (ERS) diet (8). The observation that endogenous microRNA synthesis cannot compensate for dietary depletion raises the important question as to what the phenotypes of dietary RNA depletion might be. Our first attempts at identifying phenotypes of milk RNA depletion included an unbiased liver metabolomics screen of mice fed ERD and ERS diets, which suggested that hepatic adenosine and inosine were among the metabolites for which the concentrations were higher in ERD mice than in ERS mice. Here, we followed up on this initial observation and conducted a series of studies, specifically targeting purine metabolites with the following objectives. 1) Conduct a comprehensive analysis of hepatic purine metabolites in mice fed ERD and ERS diets. 2) Assess the effects of ERD and ERS diets on the expression of enzymes in purine metabolism in murine livers. 3) Assess the effects of milk intake on purine metabolites in human adults and infants. This study is significant since it provides a new insight into hepatic purine metabolism pathways impacted by dietary milk-derived microRNA in mice and shows, for the first time, marked purine metabolic shifts also take place in infants fed milk-free formula. Purines play a role in numerous pathways in energy metabolism and cell signaling (20).

2. Materials and methods

2. 1 Mouse feeding studies

C57BL/6 mice (Jackson Laboratories), age 3 weeks were randomly assigned to two diet groups at weaning (21), housed in groups, 12:12 light cycle and at ~22°C. Absence of sex effects on liver metabolites was demonstrated by referencing against 5 male C57BL/6 mice determined hydrophilic-interaction chromatography-multiple reaction monitoring-tandem mass spectrometry (HILIC-MRM-MS/MS, **Supplemental Table 1**). Diets were based on the AIN-93G formula, and modified by their content of exosomes and their RNA cargos from bovine milk (8, 22). One group was fed a milk exosome and RNA-depleted (ERD) diet, whereas the other group was fed a milk exosome and RNA-sufficient (ERS) diet, both for 4 weeks. Briefly, ERD and ERS diets are based on the AIN-93G formulation (8, 22). In the diets, lyophilized milk powder (and soy protein) substitutes for milk casein in the AIN-93G formulation to eliminate dairy exosomes present in the AIN-93G formulation. The milk added to the diets provides the equivalent of 0.5 L milk consumed by a human adult per day, adjusted by body weight in mice. The milk used to prepare the powder for the ERD diet is ultrasonicated for 1.5 h and incubated for 1 h at 37°C prior to lyophilization; the milk used to prepare the powder for the ERS is not ultrasonicated.

Ultrasonication leads to a transient disruption of exosome membranes and a >98% depletion of RNA cargos in exosomes, 20% decrease in exosome count ($9.1 \times 10^{12} \pm 7.1 \times 10^{11}$ exosomes/mL in ERS milk *vs.* $7.3 \times 10^{12} \pm 3.5 \times 10^{11}$ exosomes/L in ERD milk; $P < 0.05$, n = 3) and >60% decrease in intestinal exosome transport rates (23). Diet ingredients other than milk are not ultrasonicated, *i.e.,* nutrients other than exosomes and their RNA cargos, including purines are the same in either sonicated milk or non-sonicated milk, used for ERD and ERS diets accordingly (see below). Differential purine content from the two diets was tested by HPLC, three samples were analyzed, using separate batches of both ERD and ERS milk. Mice had free access to diets and water for the entire study period, but no differences in food and water intake were noted in the two groups (not shown). At age 7 weeks, mice were euthanized using carbon dioxide during postabsorption and livers were flash frozen in liquid nitrogen and immediately stored at -80°C until analysis. Body composition was assessed by dual-energy X-ray absorptiometry at time of study termination prior to liver extraction. Studies were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee (protocol 1229).

2.2 Human feeding studies

Cross-sectional studies were conducted in infants and adults. Spot urine samples were collected from infants fed human milk (HM, *n* = 45), milk formula (MF, *n* = 41) or soy formula (SF, *n* = 46) at age 3 months. The key to group assignments was broken after purine and creatinine analyses were complete. The infant population and formulas fed have been described elsewhere ((24). The Beginnings Study, Clinical Trial # NCT00616395). In addition, two cohorts of apparently healthy adults were recruited (**Supplemental Table 2**). Dairy consumers (DC) were persons who regularly consume a self-chosen diet providing at least 0.50 L milk per day and perhaps other dairy, whereas dairy avoiders (DA) were persons who regularly consume diets free of animal milk and less than two weekly servings of dairy (1 serving = 0.25 L) other than milk. Dairy consumption was assessed by questionnaire. DA were instructed to avoid dairy other than milk altogether for at least two weeks before sample collection. Exclusion criteria shared by both groups included history of metabolic disease including lactose intolerance, body weight of less than 55 kg, history of intestinal surgery, smoking and use of prescription and over-the-counter medications less than four weeks before sample collection. Blood samples and 24-h urine samples were collected after an overnight fast. EDTA tubes were used to avoid inhibition of PCR reactions (10). Plasma was collected by centrifugation and stored at -80ºC until analysis**.** Urine samples were briefly centrifuged to remove debris and stored at -80ºC until analysis. Studies were approved by the Institutional Review Board at the University of Nebraska-Lincoln (protocol NU ID 14778). We estimated the power of human studies to detect changes in purine metabolism based on the differential concentration of seven purine metabolites between ERD and ERS diets, including adenosine, guanosine, inosine, xanthine, adenine and hypoxanthine. Fourteen infants allow to detect a 40% change in urinary purine metabolites, and six adults allow to detect a 60% change of purine metabolites in urine and plasma at $1-\beta = 0.8$ and $\alpha = 0.05$. For both human cohorts, there were no apparent sex effects on purine metabolism.

2.3 Analyses of purine metabolites

Mouse livers polar and non-polar metabolites were extracted with a mixture of chloroform, methanol and water and analyzed by liquid chromatography-mass spectrometry as described previously (25) for non-targeted metabolomics. Metabolites were identified using the METLIN database (26). Multivariate analyses were performed using Metaboanalyst 3.0 (27). Mouse livers and human plasma and urine metabolites were extracted using methanol, dried, and stored at -80°C until analyzed as previously described (28), only differing by the use of a 5-µm Luna NH2 column (150 x 2 mm) at a flow rate of 0.4 mL/min. Prior to mass spectrometry, samples were re-suspended in 40 µL of 85% acetonitrile and 20 µL were injected. Targeted metabolite quantification was performed using a QTRAP 6500 mass spectrometer (AB Sciex LLC) coupled to a Shimadzu Nexera X2 HPLC system (Shimadzu Co., Japan). Peak analysis was performed using Analyst Software (AB Sciex LLC). Multiple reaction monitoring transition data were collected for each purine metabolite (28). Select purines in livers, plasma and urine samples in the different diets were also analyzed by HPLC (inosine and hypoxanthine) as described previously with minor modifications (29), the Amplex® Red Xanthine Assay Kit (Life Technologies) and the BioVision Uric Acid Colorimetric/Fluorometric Assay Kit (Biovision Inc.) following the manufacturer´s instructions. The concentrations of purine metabolites in urine were normalized using creatinine, which was measured by using a colorimetric assay (30). Adult urine uric acid test was measured in samples collected in a 24-h period, whereas purines from infant were analyzed in spot urine samples which require normalization by creatinine.

2.4 RNA sequencing analysis

Total RNA and microRNAs were extracted from mouse livers using spin columns (Qiagen, Inc.) and shipped to BGI, Inc. (Hong Kong, China) for sequencing using the Illumina HiSeq 2500 platform with protocols of 125-bp read length, paired end and 50-bp read length, single end, respectively. Data quality control was performed using FastQC (31). For RNA, adaptor sequences and reads containing ambiguous bases or having average quality score less than 30 were removed. The remaining high-quality reads were aligned to the reference sequences in murine RNA [GRCm38, mm10] by using RSEM (32) and Tophat (33). Cufflinks and Cuffdiff was used to obtain the transcripts and quantify according to the sum of normalized reads units displayed as Fragments Per Kilobase Million (FPKM) (34). DESeq2 R was used to determine differentially expressed genes, based mainly on fold change, p value and q-value (FDR, False Discovery Rate) (35). For microRNA, expression was quantified using CAP-miSeq, and miRBase (Version 21) was used as reference library for annotation (36, 37). We have carefully filtered out the low-quality reads and strictly mapped the qualified reads to all known mature sequence, precursor sequences and genomes of mouse. Raw sequence data were deposited in the NCBI-BioProject database under accession No. PRJNA428903 for RNA-Seq data and PRJNA428904 for microRNA-Seq data.

Spin columns for RNA purification may be contaminated with microbial RNAs and produce false positive results in microRNA analysis (38). We confirmed hepatic microRNA-Seq data by real-time quantitative PCR (RT-qPCR), using microRNAs isolated using spin columns that were purified by treatment with 0.5% sodium hypochlorite (38). We selected three microRNAs that represented the top (miR-340-5p), middle (miR-99b-5p) and bottom (miR-362-3p) tertile of microRNA abundance in microRNA-Seq analysis. In addition, miR-340-5p has a nucleotide sequence complementary to mRNAs implicated in purine metabolism. mRNA-microRNA interactions were predicted by using miRWalk (39).

2.5 Statistical analysis

Multivariate statistical analysis, pathway enrichment analysis, and topology analysis approaches were used in order to compare high-throughput data between ERD and ERS using Metaboanalyst 3.0 (27). Homogeneity of variances was confirmed by using F-test. Kolmogorov-Smirnov test was used to confirm normal distribution. Differences between two groups were determined by unpaired t-test. If variances were heterogeneous, we used Welch´s test for pairwise comparisons. One-way ANOVA and Tukey´s posthoc test was used for comparisons among more than two groups. Homogeneity of variances was confirmed using Barlett’s test. Effects of diets were considered statistically significant if $p < 0.05$. Data in figures and tables are reported as mean ± SEM and mean ± SD, respectively. Prism 6 (Graph-Pad), R Studio and SPSS were used to perform statistical analyses.

3. Results

3.1 ERD and ERS milk contained feeding in mice

ERD and ERS diets contained the same concentrations of the purine metabolites hypoxanthine and inosine (**Supplemental Table 3**). Physical activity, food consumption, feeding frequency, water consumption and respiratory exchange rate was the same in mice fed ERD or ERS diets (23). Body weight, lean body weight and fat mass were the same in the two diet groups at age 7 weeks (**Supplemental Figure 1**).

3.2 Hepatic purine metabolites

When the metabolome in mouse livers was analyzed using non-targeted mass spectrometry, seven purine metabolites were among the 25 metabolites (purines and non-purines) affected the most by ERD or ERS diets (**Figure 1A**). In principal component analysis, mouse livers were clustered separately by dietary treatment (**Figure 1B**), which was primarily driven by differential abundance of purine metabolites. When non-targeted metabolite analysis was followed up with a targeted analysis of purine metabolites by LC-MS, the concentrations of nine measured hepatic purine metabolites were significantly higher in ERD mice compared to ERS mice (**Figure 1C**). Metabolite set enrichment analysis of hepatic metabolites suggested that purine metabolism was among the pathways most affected by ERD diet in mice (**Supplemental Figure 2**). When individual purines were analyzed by using colorimetric assays, HPLC and HILIC-MRM-MS/MS, the hepatic concentrations of xanthine and guanosine triphosphate were significantly higher in mice fed ERD than in mice fed ERS (**Supplemental Figure 3**), whereas the concentrations of seven other purine metabolites in ERD mice were not significantly different from ERS mice ($0.07 < P < 0.46$).

3.3 Liver transcriptomics

Two hundred seventy-two mRNAs were differentially expressed, with criteria of at least 1.5-fold difference, a p-value less than 0.05 and a q-value less than 0.05 ($|\log2FC| \geq 0.8$, $P < 0.05$, $Q < 0.05$), in livers from mice fed ERD or ERS diets (**Figure 2A**). The mRNAs that were differentially expressed included adenylate kinase 1 and cAMP-specific 3', 5'-cyclic phosphodiesterase 4D, which were 6,216% and 175% more abundant, respectively, in ERD than in ERS. The unbiased RNA-seq analysis was supplemented with a targeted in-depth analysis of mRNA, which detected 9 enzymes in purine metabolism for which the expression of mRNA was significantly different between mice fed ERD and ERS diets (**Table 1**). Forty-nine microRNAs were expressed at a lower level in livers from mice fed the ERD diet compared to mice fed the ERS diet ($|\log2FC| \geq 1.0$, $P < 0.05$) (**Figure 2B**). Five of these differentially expressed microRNAs (miR-338-5p, miR-340-5p, miR-17-5p, miR-362-3p and miR-3087-3p) have putative binding sites in mRNAs implicated in purine metabolism, including *Polr3k, Polr3f, Dck, Pde4b, Pde7b, Pfas and Prps2* **(Supplemental Table 4)**.

MicroRNA analysis by RT-qPCR revealed the same expression patterns that were observed in microRNA-Seq analysis. The expression of miR-340-5p was higher in livers from mice fed ERS compared to ERD (Ct values 26.7 ± 0.3 vs. 27.8 ± 0.6). The expression of miR-99b-5p was not different between the two diet groups (Ct values 20.3

± 0.2 vs. 20.3 ± 0.4), and miR-362-3p was not detectable (Ct > 30.0) **(Supplemental Table 5).**

3.4 Human purine metabolites

Concentrations of purine metabolites followed the same pattern in the urine of dairy-avoiding (DA) human adults compared to the liver patterns observed in the ERD mice. When purines were analyzed by colorimetric assays, HPLC and HILIC-MRM-MS/MS, urinary concentrations of AMP and plasma concentrations of GMP were significantly higher in DA than in DC (**Figure 3**). While 21 purine metabolites excretion and/or concentrations were also elevated in DA, these differences were not significantly different from those in DC (**Supplemental Figure 4**). The excretion of creatinine was not statistically different in DA and DC ($P > 0.10$).

When infant urine samples were analyzed by HPLC and colorimetric assays, uric acid and inosine were significantly higher in group SF and lower in group HM and MF (**Figure 4**). The concentrations of 12 out of 21 purine metabolites analyzed in infant urine samples by using HILIC-MRM-MS/MS were significantly higher in SF compared to HM and MF (**Table 1**). The excretion of creatinine was not statistically different among SF, HM and MF ($P > 0.10$).

4. Discussion

Evidence is accumulating that exosomes and their RNA cargos in milk are bioavailable (8-13, 40). In a previous study, consumption of the ERD diet caused an about 60%

decrease in miR-29b and miR-200c concentrations in murine plasma compared with mice fed the ERS diet (8). Phenotypes associated with the dietary depletion of milk exosomes and their RNA cargos remain elusive. To the best of our knowledge, this is the first report linking the dietary depletion of milk exosomes with a physiologically important metabolic phenotype, namely: (1) a shift in hepatic purine biochemical pathways, and (2) an increase in the concentrations of purine metabolites in tissues and body fluids compared to controls fed milk exosome-sufficient diets.

Changes in purine metabolism have exceptional biological significance because of the essential roles of purines in major pathways in intermediary metabolism and in cell signaling (20). The roles of ATP and adenosine in purinergic receptor signaling in cognitive performance is of particular interest, and ongoing research in our laboratory suggests that spatial learning and memory is impaired in mice fed ERD compared to ERS controls (41). It is widely acknowledged that purinergic receptor signaling plays important roles in spatial learning and memory (42, 43). Herein, we made the novel observation of significantly higher urinary purine metabolites in soy-fed infants. Further studies are needed to fully determine if there are direct linkages between limited postnatal milk intake, purine biochemistry and brain development.

It is particularly noteworthy that the same purine metabolic phenotype was consistently observed in three independent experimental situations, *i.e.*, mice fed ERD or ERS diets, DA versus DC human adults, and infants fed SF, HM or MF. While it can be challenging to model human disease and nutrition on animal studies (44, 45), this study established cause-and-effect in animal feeding studies and translated the findings in animal studies to human adults and infants.

The diets used in mouse feeding studies are based on the AIN-93G formula and are defined by their RNA content in the milk exosomes added to the diet (8, 22). We formally excluded differences in dietary purines as confounders in this study. Recently, we demonstrated that ERD and ERS differ by their content of milk exosomes and that the exosomes in the ERD diet are depleted of RNA compared to the exosomes in the ERS diet (S. Sukreet and J. Zempleni, unpublished observation). The amount of bovine milk exosomes added to the diets is the equivalent of exosomes in 0.5 L milk consumed by a human adult. The diets consumed by the two human cohorts in this study were less stringently controlled than the murine diets and yet the same patterns in purine metabolism was evident in humans.

Five independent laboratories, including ours, have demonstrated that milk exosomes are transported by intestinal cells, immune cells and vascular endothelial cells and are bioavailable in mice (11-14, 40, 46, 47). These observations have gone undisputed. In contrast, concerns were raised by Laubier *et al.* (48), Auerbach *et al.* (49), Title *et al.* (50) and Kang *et al.* (51) as to whether the low concentrations of microRNAs in bovine milk exosomes can elicit phenotypes in non-bovine species. Laubier *et al.* fostered wild-type pups to transgenic mice that overexpressed miR-30b and failed to see a substantial increase in tissue levels of miR-30b in pups (48). The failure to observe an increase in miR-30b in pup tissues was probably due to the fact that the miR-30b in overexpression dams was not encapsulated in milk exosomes, thereby compromising miR-30b stability and bioavailability (11, 16-18). Auerbach *et al.* reported a failure to detect bovine miR-29b and miR-200c in human plasma following a milk meal (49). Subsequent studies suggest that the integrity of the samples used in that study was compromised and the RNA was degraded (10). Title *et al.* detected only trace amounts of miR-375 in the plasma of miR-375 knockout mouse pups fostered to wild-type dams (50). Our studies suggest that miR-375 in milk, unlike many other microRNAs, is subject to "first passage elimination" in intestinal mucosa and liver and therefore its concentrations in circulation and peripheral tissues are low ((52, 53), S. Manca and J. Zempleni, unpublished). Kang *et al.* conducted a meta-analysis of published RNA-Seq datasets and concluded that the abundance of dietary microRNAs in body fluids is very low and possibly due to assay artifacts (51). Their analysis is biased by applying considerably lower levels of stringency when mapping human microRNAs (3 mismatches allowed) compared to dietary microRNAs (1 mismatch), by disregarding the abundance of microRNAs in foods, by withholding details of data normalization protocols across datasets, and by dismissing the possibility that local concentrations of dietary microRNAs at the site of absorption might be high.

Some uncertainties remain. For example, we do not know how exosomes and their cargos alter purine metabolism. The binding of microRNAs to binding sites in mRNAs implicated in purine metabolism is a plausible mechanism, but the evidence in support of this theory is circumstantial and based on *in silico* predictions. Alternative scenarios include the docking of exosomes to the cell surface triggering cell signaling cascades and exosome-dependent changes in the gut microbiome and microbial metabolites (54-56).

Another limitation of our study is the expression of mRNAs and microRNAs was assessed only in female but not in male livers. Note that we did not observe any effects of sex on the hepatic concentrations of purine metabolites in mice. This observation is consistent with a previous report suggesting that sex does not affect the hepatic expression of mRNAs encoding enzymes in purine metabolism in mice (57).

Ongoing and future work in our laboratory focuses on the bioavailability and distribution of microRNAs implicated in brain function and the roles of milk exosomes and their RNA cargos in spatial learning and memory. For example, we have observed that spatial memory is impaired in young female mice born to parents fed the ERD diet and continued on the parental diet compared with ERS controls. We speculate that altered purinergic receptor signaling plays a role in loss of spatial learning and memory in mice fed the ERD diet compared with ERS controls since adenosine and ATP play crucial roles in purinergic receptor signaling (42). Urine patterns of purine-relevant metabolites in low milk-consuming adults and infants were consistent with those predicted from basic research studies in mice fed ERD. This strongly supports the idea that delivery of RNA cargoes from milk exosomes is a fundamental mammalian process with potentially profound impacts on physiology and development.

Acknowledgments

The authors acknowledge the use of the Biomedical and Obesity Research Core in the Nebraska Center for the Prevention of Obesity Disease through Dietary Molecules (NIH

1P20GM104320). Authors would like to acknowledge Sean H. Adams for collaborating with editorial comments

References

1. Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borras FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 2015;4:27066.
2. Abels ER, Breakefield XO. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell Mol Neurobiol* 2016;36:301.
3. Svensson KJ, Christianson HC, Wittrup A, Bourseau-Guilmain E, Lindqvist E, Svensson LM, Morgelin M, Belting M. Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. *J Biol Chem* 2013;288:17713-24.
4. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. *Nat Genet* 2003;35:215-7.
5. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009;19:92-105.
6. Vojtech L, Woo S, Hughes S, Levy C, Ballweber L, Sauteraud RP, Strobl J, Westerberg K, Gottardo R, Tewari M, et al. Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. *Nucleic Acids Res* 2014;42:7290-304.
7. Danielson KM, Das S. Extracellular Vesicles in Heart Disease: Excitement for the Future? *Exosomes Microvesicles* 2014;2:1.

8. Baier SR, Nguyen C, Xie F, Wood JR, Zempleni J. MicroRNAs are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow's milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 kidney cell cultures, and mouse livers. *J Nutr* 2014;144:1495-500.
9. Shu J, Chiang K, Zempleni J, Cui J. Computational characterization of exogenous microRNAs that can be transferred into human circulation. *PLoS One* 2015;10:e0140587.
10. Wang L, Sadri M, Giraud D, Zempleni J. RNase H2-Dependent Polymerase Chain Reaction and Elimination of Confounders in Sample Collection, Storage, and Analysis Strengthen Evidence That microRNAs in Bovine Milk Are Bioavailable in Humans. *J Nutr* 2018;148:153-9.
11. Wolf T, Baier SR, Zempleni J. The intestinal transport of bovine milk exosomes is mediated by endocytosis in human colon carcinoma caco-2 cells and rat small intestinal IEC-6 cells. *J Nutr* 2015;145:2201-6.
12. Izumi H, Tsuda M, Sato Y, Kosaka N, Ochiya T, Iwamoto H, Namba K, Takeda Y. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. *J Dairy Sci* 2015;98:2920-33.
13. Kusuma Jati R, Manca S, Friemel T, Sukreet S, Nguyen C, Zempleni J. Human vascular endothelial cells transport foreign exosomes from cow's milk by endocytosis. . *Am J Physiol Cell Physiol* 2016;310:C800-C7.
14. Munagala R, Aqil F, Jeyabalan J, Gupta RC. Bovine milk-derived exosomes for drug delivery. *Cancer Lett* 2016;371:48-61.

15. Wang K, Li H, Yuan Y, Etheridge A, Zhou Y, Huang D, Wilmes P, Galas D. The complex exogenous RNA spectra in human plasma: an interface with human gut biota? *PLoS One* 2012;7:e51009.

16. Izumi H, Kosaka N, Shimizu T, Sekine K, Ochiya T, Takase M. Bovine milk contains microRNA and messenger RNA that are stable under degradative conditions. *J Dairy Sci* 2012;95:4831-41.

17. Benmoussa A, Lee CH, Laffont B, Savard P, Laugier J, Boilard E, Gilbert C, Fliss I, Provost P. Commercial dairy cow milk microRNAs resist digestion under simulated gastrointestinal tract conditions. *J Nutr* 2016;146:2206-15.

18. Zempleni J, Aguilar-Lozano A, Sadri M, Sukreet S, Manca S, Wu D, Zhou F, Mutai E. Biological activities of extracellular vesicles and their cargos from bovine and human milk in humans and implications for infants. *J Nutr* 2017;147:3-10.

19. Luo Y, Wang P, Wang X, Wang Y, Mu Z, Li Q, Fu Y, Xiao J, Li G, Ma Y, et al. Detection of dietetically absorbed maize-derived microRNAs in pigs. *Sci Rep* 2017;7:645.

20. Garrett RH, Grisham CM. Biochemistry. Fort Worth, TX: Saunders College Publishing; 1995.

21. Couzin-Frankel J. When mice mislead. *Science* 2013;342:922-3, 5.

22. Reeves PG, Nielsen FH, Fahey GC, Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993;123:1939-51.

23. Leiferman A, Shu J, Grove R, Cui J, Adamec J, Zempleni J. A diet defined by its content of bovine milk exosomes and their RNA cargos has moderate effects on gene expression, amino acid profiles and grip strength in skeletal muscle in C57BL/6 mice. *J Nutr Biochem* 2018 (in press)
24. Andres A, Casey PH, Cleves MA, Badger TM. Body fat and bone mineral content of infants fed breast milk, cow's milk formula, or soy formula during the first year of life. *J Pediat* 2013;163:49-54.
25. Nygren H, Seppanen-Laakso T, Castillo S, Hyotylainen T, Oresic M. Liquid chromatography-mass spectrometry (LC-MS)-based lipidomics for studies of body fluids and tissues. *Methods Mol Biol* 2011;708:247-57.
26. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G. METLIN: a metabolite mass spectral database. *Ther Drug Monit* 2005;27:747-51.
27. Xia J, Wishart DS. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. *Curr Protoc Bioinformatics* 2016;55:14 0 1- 0 91.
28. Yuan M, Breitkopf SB, Yang X, Asara JM. A positive/negative ion-switching, targeted mass spectrometry-based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. *Nat Protoc* 2012;7:872-81.
29. Burdett TC, Desjardins CA, Logan R, McFarland NR, Chen X, Schwarzschild MA. Efficient determination of purine metabolites in brain tissue and serum by high-performance liquid chromatography with electrochemical and UV detection. *Biomed Chromatogr* 2013;27:122-9.

30. Husdan H, Rapoport A. Estimation of creatinine by the Jaffe reaction. A comparison of three methods. *Clin Chem* 1968;14:222-38.

31. FastQC [database on the Internet] 2017 [cited 8/24/2017]. Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

32. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 2011;12:323.

33. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 2009;25:1105-11.

34. Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol* 2013;31:46-53.

35. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550.

36. Sun Z, Evans J, Bhagwate A, Middha S, Bockol M, Yan H, Kocher JP. CAP-miRSeq: a comprehensive analysis pipeline for microRNA sequencing data. *BMC Genomics* 2014;15:423.

37. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res* 2014;42:D68-D73.

38. Heintz-Buschart A, Yusuf D, Kaysen A, Etheridge A, Fritz JV, May P, de Beaufort C, Upadhyaya BB, Ghosal A, Galas DJ, et al. Isolation of nucleic acids from low biomass samples: detection and removal of sRNA contaminants. *BMC Biology* 2018;16:52.

39. Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat Methods* 2015;12:697.

40. Chen T, Xie MY, Sun JJ, Ye RS, Cheng X, Sun RP, Wei LM, Li M, Lin DL, Jiang QY, et al. Porcine milk-derived exosomes promote proliferation of intestinal epithelial cells. *Sci Rep* 2016;6:33862.

41. Mutai E, Zhou F, Zempleni J. Depletion of dietary bovine milk exosomes impairs sensorimotor gating and spatial learning in C57BL/6 mice. *FASEB J* 2017;31:150.4 [peer-reviewed meting abstract].

42. Duster R, Prickaerts J, Blokland A. Purinergic signaling and hippocampal long-term potentiation. *Curr Neuropharmacol* 2014;12:37-43.

43. Burnstock G, Krugel U, Abbracchio MP, Illes P. Purinergic signalling: from normal behaviour to pathological brain function. *Prog Neurobiol* 2011;95:229-74.

44. McGonigle P, Ruggeri B. Animal models of human disease: challenges in enabling translation. *Biochem Pharmacol* 2014;87:162-71.

45. Baker DH. Animal models in nutrition research. *J Nutr* 2008;138:391-6.

46. Agrawal AK, Aqil F, Jeyabalan J, Spencer WA, Beck J, Gachuki BW, Alhakeem SS, Oben K, Munagala R, Bondada S, et al. Milk-derived exosomes for oral delivery of paclitaxel. *Nanomedicine* 2017;13:1627-36.

47. Liao Y, Du X, Li J, Lonnerdal B. Human milk exosomes and their microRNAs survive digestion in vitro and are taken up by human intestinal cells. *Mol Nutr Food Res* 2017;61:11.

48. Laubier J, Castille J, Le Guillou S, Le Provost F. No effect of an elevated miR-30b level in mouse milk on its level in pup tissues. *RNA Biol* 2015;12:26-9.

49. Auerbach A, Vyas G, Li A, Halushka M, Witwer K. Uptake of dietary milk miRNAs by adult humans: a validation study. *F1000Res* 2016;5:721.

50. Title AC, Denzler R, Stoffel M. Uptake and function studies of maternal milk-derived microRNAs. *J Biol Chem* 2015;290:23680-91.

51. Kang W, Bang-Berthelsen CH, Holm A, Houben AJ, Muller AH, Thymann T, Pociot F, Estivill X, Friedlander MR. Survey of 800+ data sets from human tissue and body fluid reveals xenomiRs are likely artifacts. *RNA* 2017;23:433-45.

52. Pond SM, Tozer TN. First-pass elimination. Basic concepts and clinical consequences. *Clin Pharmacokinet* 1984;9:1-25.

53. Zempleni J, Baier SR, Hirschi K. Diet-responsive microRNAs are likely exogenous. *J Biol Chem* 2015;290:25197.

54. Purushothaman A, Bandari SK, Liu J, Mobley JA, Brown EE, Sanderson RD. Fibronectin on the Surface of Myeloma Cell-derived Exosomes Mediates Exosome-Cell Interactions. *J Biol Chem* 2016;291:1652-63.

55. Muller L, Mitsuhashi M, Simms P, Gooding WE, Whiteside TL. Tumor-derived exosomes regulate expression of immune function-related genes in human T cell subsets. *Sci Rep* 2016;6:20254.

56. Zhou F, Paz AH, Sadri M, Fernando CS, Zempleni J. A diet defined by its content of bovine milk exosomes alters the composition of the intestinal microbiome in C57BL/6 mice. *FASEB J* 2017;31:965.24 [peer-reviewed meeting abstract].

57. Gatti DM, Zhao N, Chesler EJ, Bradford BU, Shabalin AA, Yordanova R, Lu L, Rusyn I. Sex-specific gene expression in the BXD mouse liver. *Physiol Genomics* 2010;42:456-68.

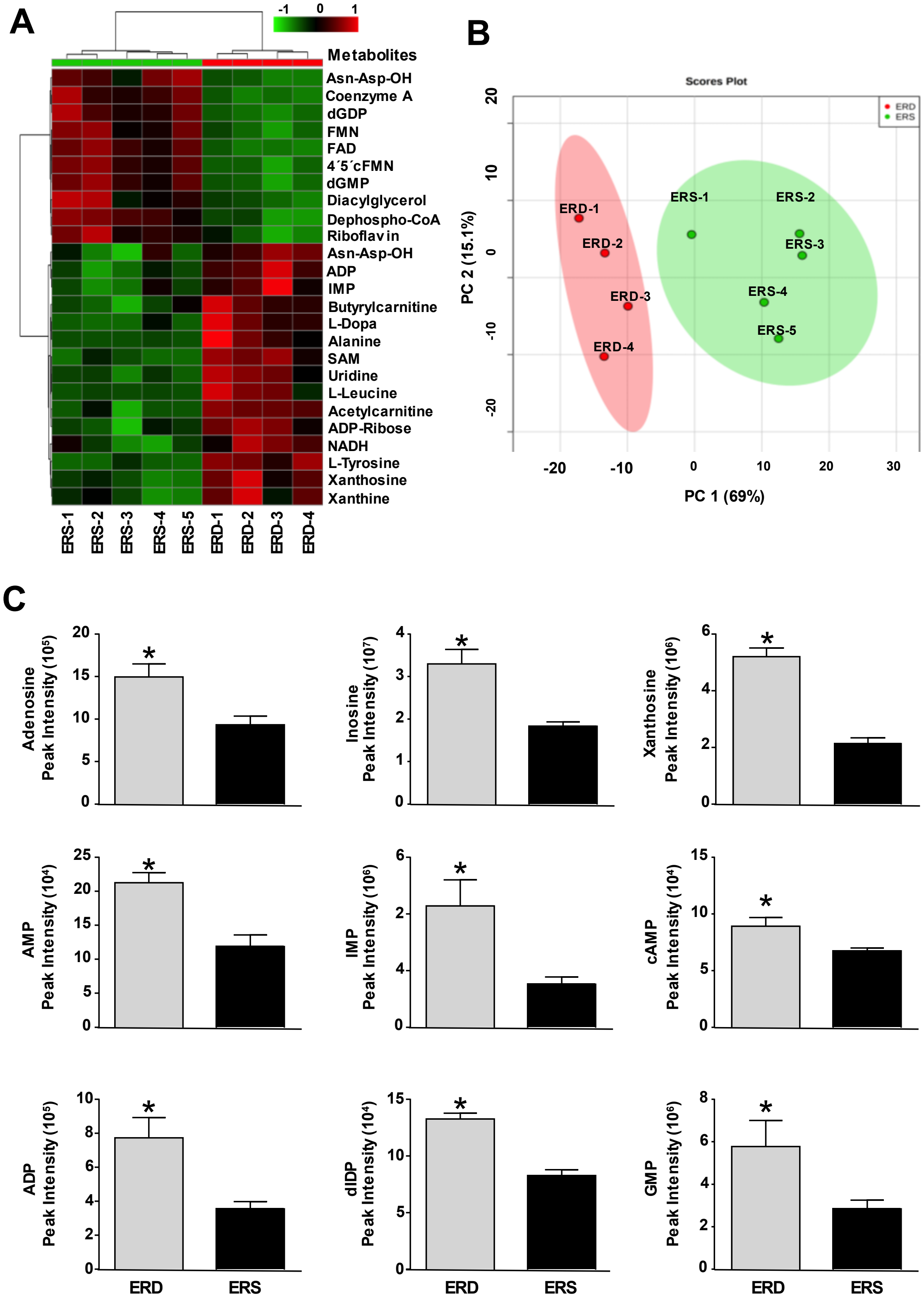

A
-1 0 1
Metabolites
Asn-Asp-OH
Coenzyme A
dGDP
FMN
FAD
4´5´cFMN
dGMP
Diacylglycerol
Dephospho-CoA
Riboflavin
Asn-Asp-OH
ADP
IMP
Butyrylcarnitine
L-Dopa
Alanine
SAM
Uridine
L-Leucine
Acetylcarnitine
ADP-Ribose
NADH
L-Tyrosine
Xanthosine
Xanthine
ERS-1
ERS-2
ERS-3
ERS-4
ERS-5
ERD-1
ERD-2
ERD-3
ERD-4
B
Scores Plot
ERD
ERS
PC 2 (15.1%)
PC 1 (69%)
20
10
0
-10
-20
-20
-10
0
10
20
30
ERD-1
ERD-2
ERD-3
ERD-4
ERS-1
ERS-2
ERS-3
ERS-4
ERS-5
C
Adenosine Peak Intensity (10^5)
Inosine Peak Intensity (10^7)
Xanthosine Peak Intensity (10^6)
AMP Peak Intensity (10^4)
IMP Peak Intensity (10^6)
cAMP Peak Intensity (10^4)
ADP Peak Intensity (10^5)
dIDP Peak Intensity (10^4)
GMP Peak Intensity (10^6)
*
ERD
ERS

FIGURE 1 Hepatic metabolome in female mice fed ERD or ERS diets for 4 weeks. (A) Heat map with top 25 differential liver metabolites between ERD and ERS diets. (B) Principal Component Analysis (PCA) leveraging 191 polar metabolites indicates clear separation of mice by their diet treatment group. PC 1 and PC2 explain 69% and 15%, respectively, of the variance. Clusters defined by ERD and ERS diets are shown, with each symbol representing the Scores Plot value for each mouse. Ovals depict 95% of confidence interval (C) Diet-dependent purine metabolites in mouse livers. Values are means ± SEM. $^{a}P < 0.05$ *vs* ERS (*n* = 4-5 mice per group in all panels). Abbreviations: ERD, exosome and RNA-depleted; ERS, exosome and RNA-sufficient; PC, principal component; PCA, principal component analysis.

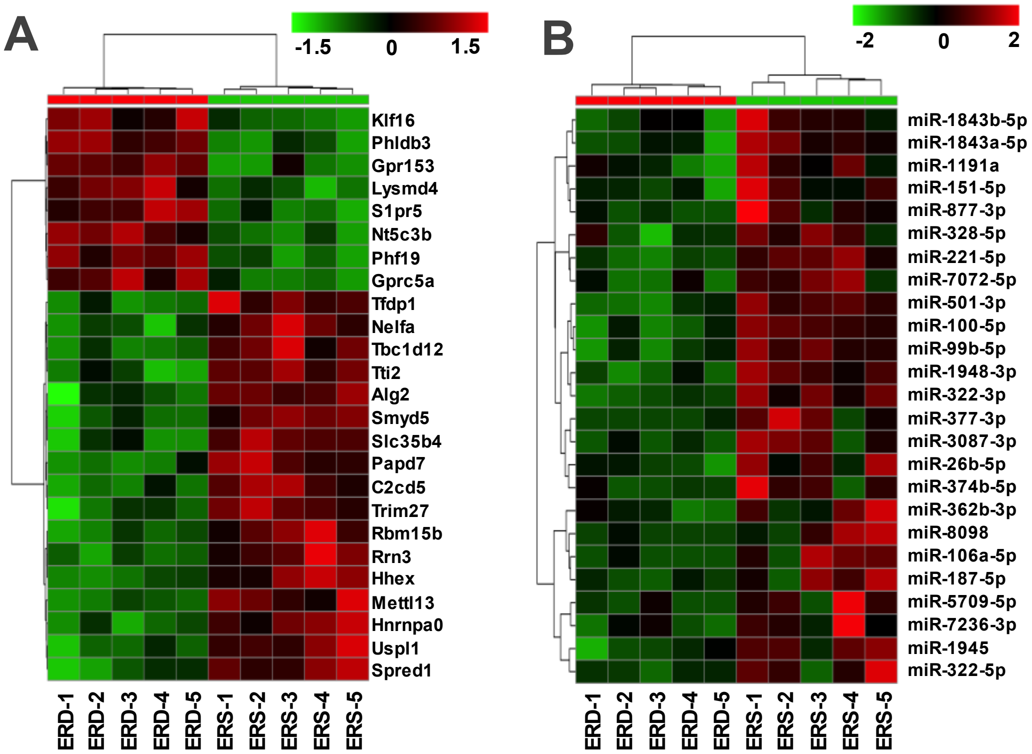


FIGURE 2 Transcriptomic analysis in female mice fed ERD and ERS for 4 weeks. Panels depict mRNA (A) Heat map with top 25 differentially expressed transcripts in livers between ERD and ERS diets. (B) Differentially abundant microRNAs in mouse livers between ERD and ERS diets. [a]$P < 0.05$ *vs* ERS ($n = 5$ mice per group in all panels). Abbreviations: ERD, exosome and RNA-depleted; ERS, exosome and RNA-sufficient.

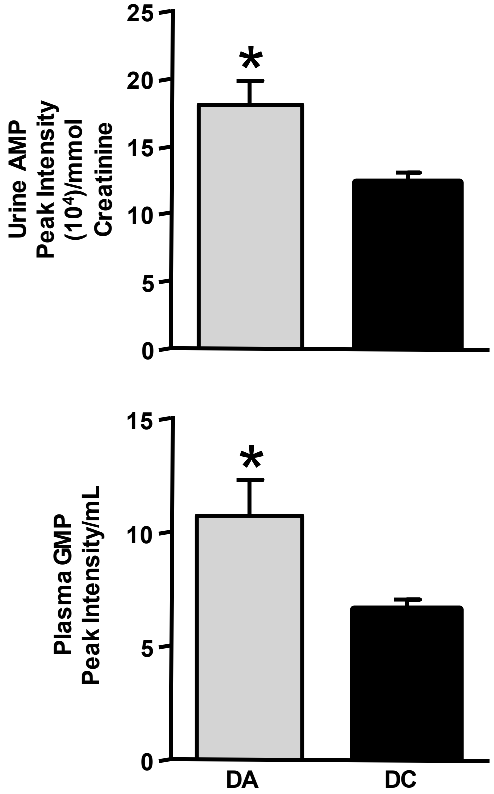


FIGURE 3 Urinary concentration of AMP and plasma concentration of GMP in adult human DA and DC. Values are means ± SEM, corrected for creatinine. $^{a}P < 0.05$ *vs* DC ($n = 6$ adults per group). Abbreviations: AMP, adenosine monophosphate; DA, dairy avoider; DC, dairy consumer; GMP, guanosine monophosphate.

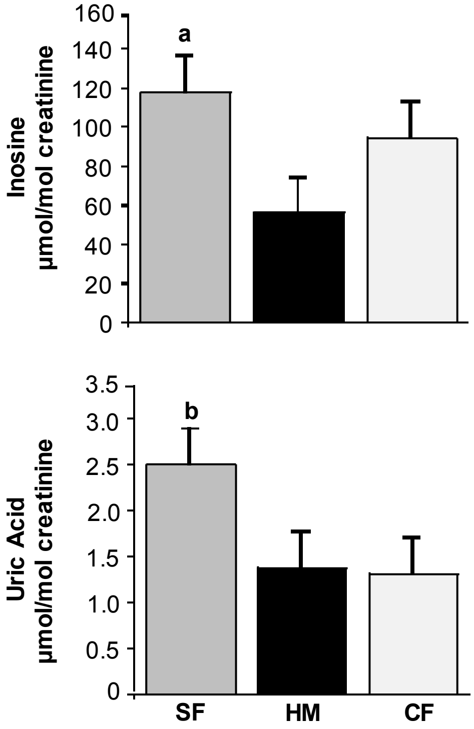


FIGURE 4 Purine metabolites in urine samples from infants, ages 3 months, fed SF, MF or HM. Values are means ± SEM. [a]$P < 0.05$ *vs* groups HM and SF, [b]$P < 0.05$ *vs* groups HM-MF and SF (*n* = 46 for SF, 40 for HM and 40 for MF). Abbreviations: HM, human milk; MF, milk formula; SF, soy formula.

TABLE 1 Differential expression of mRNAs involved in purine metabolism in livers from mice fed ERD or ERS diets for four weeks. [1]

mRNA	ERD	ERS
	FPKM	
Ak1[2]	9.0 ± 19.6	0.1 ± 0.1
Pde4d	1.2 ± 0.8	0.7 ± 0.1
Nt5c3b	2.4 ± 0.3*	1.5 ± 0.1
Ada	1.0 ± 0.2*	0.6 ± 0.2
Pole	0.3 ± 0.1*	0.2 ± 0.1
Nudt16l1	12.1 ± 0.8*	10.3 ± 1.4
Nme6	19.3 ± 3.3*	25.4 ± 3.2
Entpd7	1.1 ± 0.3*	1.8 ± 0.3
Enpp3	9.0 ± 1.4*	12.5 ± 2.8
Polr1b	5.0 ± 0.4*	6.1 ± 0.5
Ak4	5.5 ± 0.4*	7.6 ± 1.9

[1] Values are means ± SD (*n* = 5 mice per group). Values represent FPKM.

[2] Abbreviations: *Ada*, adenosine deaminase; *Ak1*, adenylate kinase 1; *Ak4*, adenylate kinase 4; *Enpp3*, ectonucleotide pyrophosphatase/phosphodiesterase family member 1/3; *Entpd7*, ectonucleoside triphosphate diphosphohydrolase 7; FPKM, fragments per kilobase million; *Nme6*, nucleoside diphosphate kinase 6; Nt5c3b, 5'-nucleotidase, cytosolic IIIB; Nudt16l1, (nucleoside diphosphate linked moiety X)-type motif 16-like 1; *Pde4d*, phosphodiesterase 4D, CAMP-specific; Pole, DNA polymerase epsilon subunit 1; *Polr1b*, DNA-directed RNA polymerase I subunit RPA2.

[*] Significantly different between dietary treatment, $P < 0.05$ *vs* ERS.

TABLE 2 Purine metabolites in infants fed SF, HM or MF.[1]

Metabolite	Multiplier[2]	SF	HM	MF
			Peak Intensity	
Adenosine	1,000,000	242 ± 129^{b}	135 ± 71	167.3 ± 78.2
ADP[3]	1,000	34 ± 27^{b}	7 ± 5	10.6 ± 6.6
AICAR[3]	10,000	92 ± 293	3 ± 2	10.2 ± 10.0
Allantoin	100,000	13 ± 14	9 ± 10	7.7 ± 6.3
AMP[3]	100,000	21 ± 39^{b}	1 ± 1	1.6 ± 1.5
ATP[3]	100,000	12 ± 26	0.1 ± 0.1	0.0 ± 0.0
cAMP[3]	100,000	88 ± 79^{b}	35 ± 25	51 ± 33
GDP[3]	1,000	57 ± 52^{b}	32 ± 22	31 ± 26
GMP[3]	1,000	60 ± 52^{b}	10 ± 9	22 ± 26
GTP[3]	1,000	69 ± 51^{b}	9 ± 9	34 ± 33
Guanine	1,000,000	68 ± 38^{b}	35 ± 20	45 ± 21
Guanosine	10,000	29 ± 22^{b}	14 ± 8	20 ± 9
Hypoxanthine	1,000,000	84 ± 73^{a}	75 ± 51	91 ± 47
IMP[3]	10,000	21 ± 25^{b}	3 ± 3	6 ± 6
Inosine	1,000,000	14 ± 9^{b}	12 ± 7	14 ± 10
PRPP[3]	100,000	32 ± 21^{b}	14 ± 10	28 ± 16
Uric acid	1,000,000	166 ± 179^{b}	40 ± 35	63 ± 52
Xanthine	1,000,000	83 ± 50^{a}	77 ± 48	81 ± 67
Xanthosine	1,000,000	17 ± 23^{b}	5 ± 3	7 ± 8
XMP[3]	100	77 ± 79^{a}	12 ± 13	31 ± 19

---

[1] Values are means ± SD (*n* = 13 for SF, 14 for HM and 13 for MF). Values represent peak intensities normalized by the total number of peaks and per mmol creatinine.

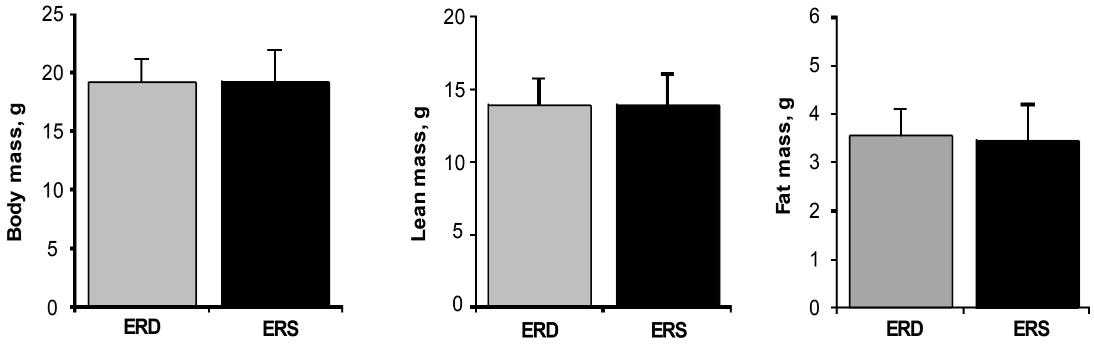
[2] Multiplication factor to be used to calculate the peak counts.

[3] Abbreviations: ADP, adenosine diphosphate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP,

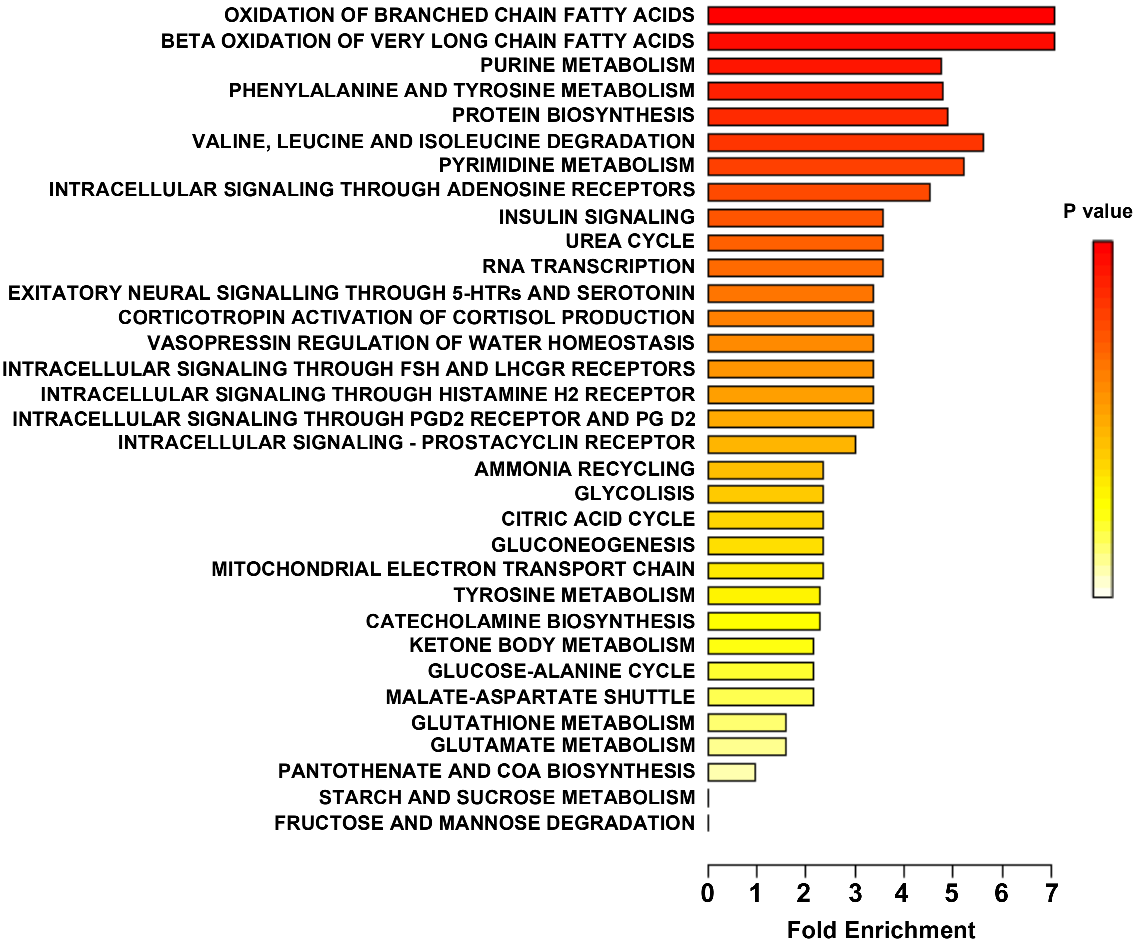
cyclic adenosine monophosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; IMP, inosine monophosphate; PRPP, 5-phospho-α-D-ribosyl 1-pyrophosphate; XMP, xanthosine monophosphate.

[a]$P < 0.05$ *vs* groups HM and SF.

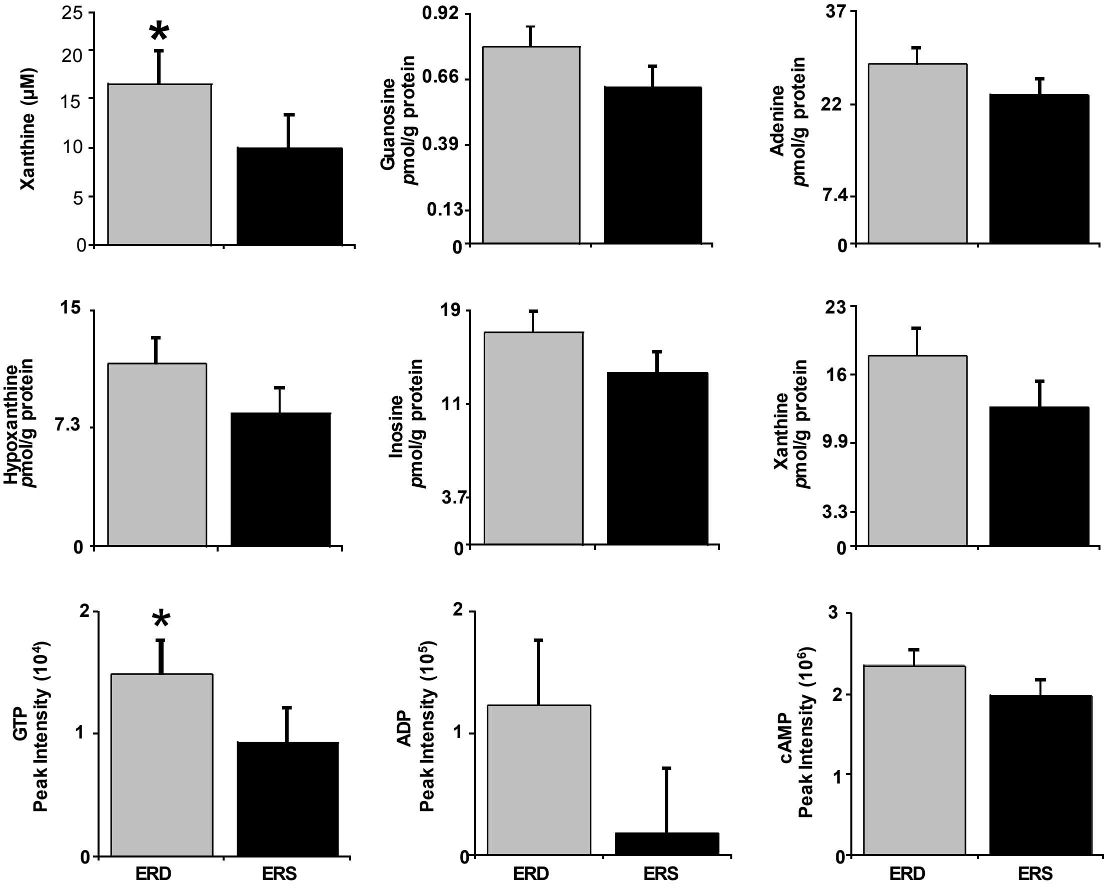
[b]$P < 0.05$ *vs* groups HM-MF and SF.



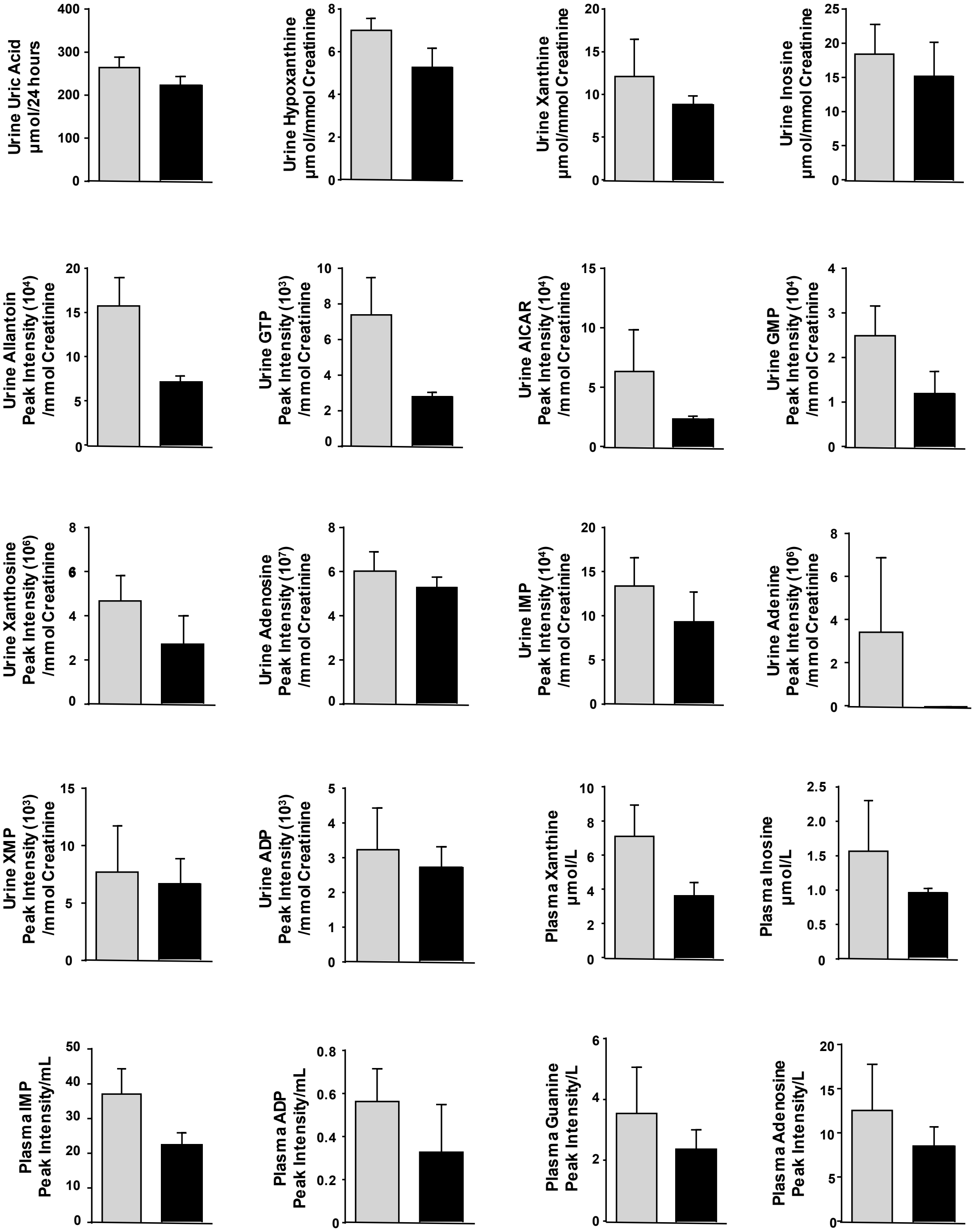
SUPPLEMENTAL FIGURE 1 Body weight, fat mass and lean body mass in female mice, age 7 weeks, fed ERD or ERS for 4 weeks. Values are means ± SEM (*n* = 5 mice per group). Abbreviations: ERD, exosome and RNA-depleted; ERS, exosome and RNA-sufficient.



SUPPLEMENTAL FIGURE 2 Metabolite set enrichment analysis of hepatic metabolites in female mice fed ERD or ERS diets for 4 weeks (*n* = 4-5 mice per group). Abbreviations: ERD, exosome and RNA-depleted; ERS, exosome and RNA-sufficient.



SUPPLEMENTAL FIGURE 3 Effects of exosome and RNA-defined diets on hepatic concentrations of targeted purine metabolites in mice. Values are means ± SEM. $*P < 0.05$ *vs* ERS ($n = 5$ mice each in groups ERD and ERS). Abbreviations: ADP, Adenosine diphosphate; cAMP, Cyclic Adenosine monophosphate; ERD, exosome and RNA-depleted; ERS, exosome and RNA-sufficient; GTP, guanosine triphosphate.



SUPPLEMENTAL FIGURE 4 Purine metabolites in urine and plasma of DA and DC. Values are presented as means ± SEM. (0.07 < *P* < 0.47; *n* = 6 adults per group). Abbreviations: DA, dairy avoiders; DC, dairy consumers.

SUPPLEMENTAL TABLE 1 Purine metabolites in female and male mice livers fed ERD and ERS diets.

Metabolite	Multiplier[2]	Female ERD and ERS	Male ERD and ERS
		Peak intensities	
Adenosine	10,000,000	93 ± 37	84 ± 53
Xanthosine	10,000,000	15 ± 3	15 ± 6
AMP	10,000,000	37 ± 13	32 ± 20
IMP	1,000,000	27 ± 11	25 ± 18
cAMP	10,000	22 ± 4	27 ± 9
GMP	10,000	22 ± 15	19 ± 10
Xanthine	10,000,000	44 ± 7	42 ± 6
Guanosine	100,000	13 ± 1	15 ± 6
Xanthine	10,000,000	44 ± 7	42 ± 6
GTP	1,000	12 ± 5	14 ± 5

[1] Values are means ± SD (*n* = 10 mice per group). Values represent peak intensities normalized by the total number of peaks.

[2] Multiplication factor to be used to calculate the peak counts.

SUPPLEMENTAL TABLE 2 Cohorts of dairy consumers (DC) and dairy avoiders (DA).

Variable	DC	DA
n (M/F[a])	6 (3/3)	6 (4/2)
Age (years)	32 ± 9.4	36.8 ± 14.6
BMI (kg/m^2)	22.8 ± 1.6	23.5 ± 3.9

[a] Abbreviations: F, female; M, male

SUPPLEMENTAL TABLE 3 Purine metabolites in milk used for preparing the ERD and ERS diets in mouse feeding studies.

Metabolite	ERD	ERS
	μmol/L	
Hypoxanthine	1.08 ± 0.28[1]	0.87 ± 0.22
Inosine	5.44 ± 1.04	6.06 ± 0.79

[1] Values are means ± SD (*n* = 3 batches per group). ERD and ERS diets contain 200 mL of milk in 1 kg diet. Three samples were analyzed, using separate batches of both ERD and ERS milk.

SUPPLEMENTAL TABLE 4 Hepatic expression of microRNAs (Tab 1) mRNA (Tab 2) in murine livers, and predictions of microRNA-mRNA interactions (Tab 3).

Supplemental_Table_4.xlsx

SUPPLEMENTAL TABLE 5 Abundance of hepatic miRNAs in mice fed the ERD and ERS diets for four weeks

miRNA	ERD	ERS
	Relative Abundance	
miR-340-5p	0.73 ± 0.10[*]	1.02 ± 0.26
miR-99b-5p	1.80 ± 0.92	1.05 ± 0.44
miR-362-3p	not detectable	

[1] Values are means ± SD (n = 5 mice per group) of RT-qPCR analysis.

[*] Significant differences between the two diets, $P < 0.05$ *vs* ERS.

FUTURE OUTLOOK

The theory of dietary exogenous miRNAs having physiological effects in the consumer is of great importance and hence at great debate. The diverse studies conducted in our lab support this theory and consistently evidence that bovine exosomes and their miRNA cargos are transferred into the host system and in mice, certain phenotypes can be observed.

The results from this thesis strongly support the theory by displaying differences in purine metabolite concentration and enzymatic gene expression, not only in mice, but also in adults and infants upon dairy avoidance. Nevertheless, there are multiple approaches that could keep strengthening the theory and complement it by determining the possible specific interactions that could be occurring between the host and the exogenous bovine milk exosomes and their RNA cargos.

Utilizing high-throughput approaches to understand the differential network of interactions occurring between ERD and ERS could help to determine which components and specific miRNAs could be driving to the phenotypic purine change among the two diets. Determining these important nodes could lead to link the nodes with different conditions that are triggered upon the possible disruption of these nodes, for instance, cognitive impairment, cancer development, possible infertility, among others. Numerous studies could be developed to determine these possible interactions in an effective manner, such as stable isotope studies and CLASH or HITS-CLIP.

As for humans, epidemiological studies could be performed to determine the link between milk intake, decrease in purine catabolism, and the purine implications in the development of neurological diseases, diabetes, cardiovascular conditions and cancer. Assessing the chronic gene expression changes upon consuming dairy products could translate into possible recommendations with proven rationale for milk intake, such as a beneficial chronic modification in gene expression.

In infants, performing experiments to deeply analyze the different diets with high-throughput and subsequent confirmatory experiments could enable to determine the possible candidates in human breast milk and cow´s milk that lead to lower profiles of purine metabolites.

Subsequently, with models and experiments that could link the amount of exosomes or their miRNAs with the concentration for purine metabolites in infants urines could strengthen the theory that exosomes and their miRNA cargoes from human or bovine milk are beneficial for human health. These correlations could enable the research field to fortify cow's milk and soy formula with this components that could drive the differential metabolomic phenotype to avoid possible future disease development of this well-known disease driven by chronic increased purine metabolite concentrations.

ANNEX

Hepatic non-targeted metabolome in female mice fed ERD or ERS diet for 4 weeks									
Sample	1	2	3	4	1	2	3	4	5
Phenotype	ERD	ERD	ERD	ERD	ERS	ERS	ERS	ERS	ERS
Metabolite	**Peak Intensities (10^5)**								
(-)-beta-Phellandrene	3.1	2.0	2.8	2.3	1.9	1.6	1.4	1.1	1.1
(R)-S-Lactoylglutathione	4.0	1.4	3.1	3.5	2.1	0.7	1.2	0.8	1.5
1,4-beta-D-Glucan	26.6	15.5	24.8	31.1	19.3	13.0	12.4	13.1	15.6
2-Methylbutyroylcarnitine	3.6	3.6	2.3	3.3	1.2	1.5	1.9	1.4	1.3
2-Methylbutyrylglycine	0.8	0.5	0.6	0.8	0.7	0.5	0.5	0.4	0.4
dGMP	9.1	10.6	10.4	12.4	14.7	19.7	20.7	19.5	24.9
C22H40O11	7.3	7.2	6.1	5.7	10.8	20.7	15.1	16.7	13.7
4-(4-Deoxy-alpha-D-gluc-4-enuronosyl)-D-galacturonate	1.5	1.3	1.6	2.0	1.0	1.2	0.9	0.9	1.1
5-Methylcytidine	4.2	2.6	3.3	2.1	1.8	1.7	1.4	1.2	1.2
6-Methylthioguanine	0.3	0.3	0.3	0.3	0.4	0.6	0.5	0.4	0.4
Acetylcarnitine	23.1	17.6	17.1	20.2	5.8	5.6	6.3	7.8	4.2
Adenosine	18.6	16.5	11.9	12.8	8.9	8.0	13.2	7.0	9.7
AMP	2.5	1.9	2.3	1.9	1.7	0.9	0.9	1.0	1.5
Adenosine monophosphate	247	119	99	136	116	94	93	52	74
ADP	11.3	6.9	6.1	6.6	4.5	3.4	4.2	2.1	3.6
ADP-ribose	161	110	126	116	69	61	68	51	61
Alanine	0.3	0.5	0.3	0.2	0.0	0.0	0.0	0.0	0.0
Asn-Asp-OH	12.5	5.7	7.2	9.0	5.9	2.3	3.0	2.3	3.9
Asn-Met-OH	2.6	2.8	2.7	2.5	6.1	5.8	7.4	4.8	5.0
Asp-Asp-OH	0.5	0.2	0.3	0.4	0.2	0.2	0.1	0.1	0.1
Asparaginyl-Proline	3.9	2.7	3.6	2.8	2.4	2.2	2.0	1.6	1.7
Asparaginyl-Tyrosine	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.2
Butyrylcarnitine	1.1	1.2	0.9	1.0	0.3	0.3	0.6	0.2	0.2
cAMP	1.1	0.7	0.8	1.0	0.7	0.7	0.7	0.6	0.6
Coenzyme A	8.5	7.2	5.1	6.5	14.8	20.5	19.5	12.8	19.6
Cyclamic acid	3.3	1.9	1.5	1.7	1.4	1.2	0.9	0.5	0.6
Cysteinylglycine	0.6	0.4	0.5	0.7	0.5	0.5	0.4	0.4	0.4
HO AKG	0.8	1.0	1.3	1.6	2.7	4.1	3.6	3.1	2.8
D-4'-Phosphopantothenate	2.1	1.8	3.7	2.3	1.3	1.0	0.7	1.2	2.0
D-Cysteine	0.4	0.4	0.3	0.3	0.3	0.1	0.2	0.1	0.2
dGDP	3.1	3.6	3.1	4.2	7.0	10.6	9.8	7.2	9.9
Dephospho-CoA	25	26	28	23	36	42	33	37	55
Diacylglycerol	0.5	0.4	0.4	0.3	0.6	1.0	0.7	0.9	0.7
dIDP	1.4	1.2	1.3	1.4	0.9	1.0	0.7	0.8	0.8
FAD	32	26	23	27	37	48	51	47	64

FMN	4.0	4.2	3.9	4.9	5.2	7.0	7.3	6.5	7.3
Gamma-Glutamylglutamine	4.0	2.2	2.9	3.0	2.2	1.1	1.4	1.1	1.7
gamma-L-Glutamyl-L-cysteinyl-beta-alanine	4.1	1.7	3.2	2.9	2.1	1.5	1.4	1.1	1.0
GDP-L-fucose	4.8	1.6	1.9	3.0	2.0	0.9	1.1	0.4	0.5
Genistein 7-O-glucoside-6''-malonate	0.3	0.6	0.5	1.0	0.9	1.1	0.9	1.4	2.0
Genistin 6''-O-acetate	1.0	0.5	0.5	1.2	0.4	0.3	0.3	0.3	0.4
Glutamylphenylalanine	0.4	0.3	0.3	0.3	0.2	0.1	0.2	0.1	0.2
Glutathione	109	55	77	87	63	27	38	30	45
Glycerol tripropanoate	14.8	16.7	14.4	11.0	12.0	13.1	10.2	10.6	9.6
Glycogen	1.0	0.7	0.5	0.8	0.2	0.2	0.1	0.5	0.3
Guanosine monophosphate	94	44	44	51	42	25	31	17	27
Indoleglycerol phosphate	1.5	1.0	1.3	1.0	0.8	0.7	0.6	0.4	0.3
Inosine	378	280	398	257	199	164	163	183	210
IMP	69.7	30.1	37.0	35.6	23.1	14.6	16.3	7.9	16.0
Inosine-5'-carboxylate	9.2	6.5	8.7	7.0	5.5	4.5	4.0	4.6	4.8
Inosinic acid	1.4	0.9	1.4	1.4	1.0	0.8	0.8	0.8	0.9
L-Aspartyl-L-phenylalanine	0.3	0.3	0.4	0.3	0.3	0.1	0.2	0.2	0.2
L-Dopa	1.0	1.2	1.0	0.9	0.4	0.2	0.2	0.1	0.2
L-gamma-glutamyl-L-isoleucine	6.9	7.7	8.2	7.0	4.0	4.0	3.9	3.2	3.2
L-Leucine	1.9	1.8	1.5	0.5	0.1	0.1	0.1	0.1	0.2
L-Phenylalanine	10.5	11.7	10.4	1.9	0.2	0.2	0.2	0.2	0.2
L-Tyrosine	2.5	2.9	2.8	4.1	0.1	0.0	0.0	0.0	0.9
L(-)-Nicotine pestanal	1.1	1.3	1.2	0.3	0.0	0.0	0.0	0.0	0.0
Leucyl-Aspartate	1.4	1.5	1.4	1.1	0.7	0.6	0.8	0.5	0.4
Luteolinidin	1.4	1.6	1.6	1.5	2.5	4.2	3.6	3.5	2.9
N-a-AcCitrulline	7.6	4.5	5.9	5.1	4.2	3.6	3.0	2.5	2.3
N-Acetylmuramoyl-Ala	6.2	4.7	3.5	3.1	4.7	8.8	6.3	7.2	5.8
N-Acetylvaline	0.8	1.1	0.7	0.5	0.2	0.0	0.1	0.0	0.1
N-Hydroxy-L-tyrosine	0.5	0.3	0.4	0.5	0.4	0.2	0.3	0.3	0.4
N2-Succinyl-L-ornithine	0.5	0.5	0.4	0.2	0.2	0.1	0.1	0.1	0.1
NADH	8.4	4.8	6.7	7.2	2.8	4.2	3.8	3.2	4.4
NAD	6.4	4.6	3.6	5.1	6.4	10.6	10.8	6.8	9.4
Nonanoic acid, 3-amino-, (R)	0.5	1.0	0.5	0.1	0.0	0.1	0.1	0.0	0.0
O-Phospho-4-hydroxy-L-threonine	0.6	0.6	0.5	0.4	0.3	0.2	0.3	0.2	0.3
Ophthalmic acid	4.0	4.0	3.2	4.5	9.8	4.3	10.8	8.3	5.5

Oxidized GSH (+)	253	96	120	185	115	42	56	41	65
Phenylalanyl-Gamma-glutamate	1.0	1.1	0.8	1.0	0.7	0.7	0.9	0.6	0.7
Phenylethylamine	0.9	0.6	0.8	0.7	0.7	0.5	0.5	0.5	0.5
Phenylpropiolic acid	0.6	0.7	0.7	1.7	0.0	0.0	0.0	0.0	0.1
Phosphoribosyl formamidocarboxamide	2.0	1.2	1.4	1.5	1.3	1.0	0.9	0.9	0.9
PhosphoribosylformiminoAICAR-phosphate	0.6	0.3	0.3	0.4	0.2	0.2	0.3	0.1	0.1
Quercetin 3-(2'',3'',4''-triacetylgalactoside)	1.1	0.8	1.0	1.8	0.3	0.3	0.2	0.2	0.3
Riboflavin	1.8	2.5	1.9	2.0	2.6	3.1	2.7	3.2	3.6
4',5'-cFMN	10.2	12.1	12.6	15.3	19.5	26.3	26.6	24.9	31.7
S(Hydroxyphenylacetothiohydroximoyl)-L-cysteine	1.3	1.4	1.6	1.5	1.1	0.7	0.7	0.6	0.8
SAM	10.9	7.7	7.4	7.0	2.6	2.5	2.9	3.3	4.5
URIDINE	1.5	1.3	1.2	0.9	0.4	0.4	0.3	0.4	0.3
Xanthine	5.6	5.4	6.8	7.0	2.2	3.1	2.6	3.2	4.3
Xanthosine	49	45	58	56	15	22	18	22	30

Purine Transcriptomic analysis in female mice livers									
Sample	1	2	3	4	1	2	3	4	5
Phenotype	ERD	ERD	ERD	ERD	ERS	ERS	ERS	ERS	ERS
Gene	**FPKM**								
Ada	0.73	0.89	1.17	1.19	0.41	0.53	0.82	0.49	0.87
Adcy1	0.09	0.08	0.11	0.19	0.14	0.24	0.01	0.32	0.08
Adcy10	0.00	0.02	0.04	0.05	0.19	0.06	0.10	0.02	0.05
Adcy2	0.06	0.10	0.14	0.04	0.06	0.09	0.04	0.02	0.01
Adcy3	0.04	0.01	0.05	0.15	0.06	0.18	0.17	0.06	0.09
Adcy4	2.32	2.12	2.73	2.35	1.85	2.36	2.49	2.12	1.82
Adcy5	0.29	0.35	0.23	0.56	0.35	0.34	0.33	0.21	0.28
Adcy6	3.74	3.59	4.28	3.84	2.96	5.57	3.35	2.98	1.87
Adcy7	1.45	2.16	1.84	1.44	1.42	1.87	1.38	1.36	1.26
Adcy9	3.23	3.43	3.66	2.93	3.17	3.04	3.37	2.80	2.91
Adk	432	503	438	425	516	390	447	448	653
Adprm	7.54	11.64	10.11	7.54	8.61	8.00	10.38	7.03	9.87
Adsl	5.45	5.09	5.22	5.44	5.46	5.18	5.59	6.00	5.86
Adss	25.75	23.77	23.50	24.06	27.83	28.86	27.23	24.10	38.05
Adssl1	11.78	14.55	10.47	11.44	19.38	7.99	14.79	10.15	13.21
Ak1	0.13	0.13	0.34	0.49	0.27	0.07	0.20	0.06	0.11
Ak2	155	162	162	144	173	137	161	148	162
Ak3	183	190	187	171	188	180	192	158	215
Ak4	5.34	5.10	5.31	6.19	9.94	5.06	7.72	6.45	8.71
Ak5	0.02	0.00	0.05	0.02	0.00	0.00	0.09	0.12	0.02
Ak6	12.33	12.93	11.56	16.59	16.37	12.31	11.33	14.89	19.45

Ak7	0.00	0.05	0.00	0.07	0.00	0.00	0.06	0.12	0.07
Ak8	0.00	0.00	0.06	0.00	0.09	0.06	0.09	0.00	0.07
Ampd2	13.75	13.01	15.43	12.29	14.03	21.42	13.15	12.90	11.56
Ampd3	0.67	0.31	0.46	0.68	0.50	0.50	0.33	0.42	0.39
Aprt	17.87	15.38	20.47	14.53	19.14	19.27	20.07	16.93	21.64
Atic	7.39	6.25	6.20	7.15	10.34	4.72	8.87	10.27	11.08
Cant1	9.69	8.29	8.82	7.24	8.27	9.45	8.75	9.09	6.82
Dck	1.36	2.28	1.13	1.87	1.20	1.18	1.49	2.25	1.84
Dguok	20.01	17.58	17.85	17.11	17.63	22.14	19.03	17.30	16.17
Enpp1	2.67	2.69	2.79	3.47	3.70	2.06	3.17	3.88	3.51
Enpp3	9.65	7.92	7.88	11.12	11.64	10.20	9.79	14.50	16.15
Enpp4	1.71	1.40	2.20	2.33	1.27	1.53	1.49	1.83	1.87
Entpd1	1.67	1.54	1.32	1.70	1.88	0.80	1.34	1.32	1.50
Entpd2	0.83	0.80	1.16	1.22	1.00	0.95	0.98	0.86	1.47
Entpd5	34.53	31.88	36.86	36.16	33.40	29.21	40.80	34.78	41.17
Entpd6	3.94	4.39	5.46	4.32	5.45	5.14	5.14	4.50	4.34
Entpd8	46.11	54.37	53.62	40.10	38.75	61.74	42.00	39.78	35.77
Fhit	9.18	4.44	5.96	6.07	4.61	6.61	8.97	5.12	10.83
Gart	10.38	12.55	11.36	9.77	11.92	9.91	10.17	11.41	11.59
Gda	7.73	7.75	7.82	7.00	7.42	5.09	7.22	7.03	7.30
Gmpr	0.69	0.31	0.57	0.48	0.63	0.38	0.56	0.37	0.14
Gmpr2	9.81	10.64	10.67	11.10	9.33	9.15	9.44	9.00	12.47
Gmps	12.70	11.26	9.48	14.79	15.82	9.41	13.29	16.35	18.97
Gucy1a2	0.16	0.10	0.12	0.21	0.13	0.14	0.14	0.17	0.21
Gucy2c	0.08	0.25	0.12	0.17	0.35	0.02	0.15	0.35	0.31
Gucy2g	0.07	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00
Guk1	25.12	23.42	25.83	26.17	24.01	24.79	28.40	28.69	27.99
Hddc3	1.68	3.81	3.07	1.44	2.00	2.48	3.06	0.97	3.35
Hprt	44.75	41.47	44.83	46.54	43.77	38.14	51.62	47.73	56.88
Impdh1	0.82	0.54	0.67	0.76	0.88	0.96	0.87	0.90	0.47
Impdh2	22.99	21.01	21.27	22.53	25.28	21.30	28.15	26.33	24.99
Itpa	12.74	11.10	13.15	10.87	11.55	7.81	14.31	14.23	17.77
Nme1	46.29	41.25	43.18	47.68	42.24	51.53	53.95	51.55	62.58
Nme2	288	273	278	248	236	340	242	248	282
Nme3	28.32	28.11	28.54	21.73	24.25	30.66	28.19	22.69	29.59
Nme4	0.63	1.65	1.78	0.96	1.51	1.56	2.39	1.20	1.48
Nme6	20.59	16.74	19.79	23.74	19.80	27.11	25.76	27.52	26.88
Nme7	3.01	2.32	1.67	3.85	3.39	1.70	3.94	5.16	3.97
Npr1	4.21	4.99	6.01	5.20	5.26	4.95	4.59	3.68	3.01
Npr2	17.72	21.29	19.77	20.56	14.13	24.30	14.88	18.35	11.30
Nt5c	23.98	31.42	22.08	24.06	33.60	27.16	24.84	23.48	28.07
Nt5c2	4.13	3.95	4.24	4.14	3.65	4.02	3.87	4.02	3.28
Nt5c3	12.14	10.75	11.98	11.58	12.71	12.67	14.02	12.77	14.58
Nt5c3b	2.25	2.04	2.39	2.63	1.61	1.47	1.47	1.72	1.33
Nt5e	13.65	12.98	11.44	13.81	15.99	11.64	15.45	17.20	18.35
Nt5m	11.07	11.18	10.84	9.06	9.92	12.01	10.87	9.11	8.52
Ntpcr	5.66	5.87	4.78	4.51	5.41	4.95	5.13	4.96	4.94
Nudt16	6.47	9.04	6.46	4.28	6.08	4.76	6.84	4.20	6.66

Nudt2	21.39	26.69	27.90	26.38	17.73	22.56	19.79	17.96	28.36
Nudt5	9.40	6.38	7.16	10.82	9.30	9.79	9.89	10.82	11.17
Nudt9	33.74	31.00	38.45	35.61	37.74	32.26	37.64	38.60	38.39
Paics	77.24	70.56	67.19	75.44	78.84	71.02	66.73	59.81	101.98
Papss1	6.07	4.97	5.20	5.59	4.55	5.41	4.78	6.94	5.72
Papss2	114	137	122	111	129	79	116	86	123
Pde10a	0.06	0.11	0.10	0.08	0.10	0.08	0.07	0.06	0.03
Pde1a	0.85	0.87	1.13	1.39	0.78	0.41	1.27	0.44	0.53
Pde1b	0.83	0.85	1.08	0.51	0.71	1.43	0.45	0.84	0.65
Pde1c	0.08	0.10	0.02	0.08	0.10	0.19	0.04	0.07	0.10
Pde2a	4.29	6.24	6.02	5.11	4.74	5.79	5.28	3.32	2.47
Pde3a	0.06	0.00	0.00	0.17	0.06	0.11	0.03	0.22	0.15
Pde3b	23.59	22.89	21.05	22.34	25.52	21.41	21.06	20.74	27.71
Pde4a	1.74	1.52	1.95	1.71	1.43	2.28	1.79	1.50	1.17
Pde4b	7.46	5.36	5.16	8.79	7.49	9.90	4.85	13.84	10.26
Pde4c	4.16	4.05	6.20	2.89	2.97	7.58	3.11	3.66	2.16
Pde4d	0.78	0.73	0.91	1.03	0.56	0.74	0.80	0.85	0.54
Pde5a	0.20	0.36	0.41	0.51	0.27	0.24	0.28	0.23	0.28
Pde6a	0.01	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00
Pde6c	0.60	0.52	0.55	0.62	0.45	0.22	1.32	0.09	0.33
Pde6d	8.98	7.88	11.59	9.99	7.92	9.84	10.81	7.11	8.06
Pde6g	1.16	1.09	1.36	0.56	0.73	0.78	0.93	0.48	0.55
Pde7a	1.74	1.36	1.52	1.77	1.61	1.35	1.56	1.34	1.66
Pde7b	0.59	0.36	0.37	0.83	1.07	0.37	0.34	0.55	0.18
Pde8a	9.49	7.21	6.27	10.49	12.43	7.05	7.51	10.05	8.43
Pde8b	0.53	0.72	1.21	0.68	0.45	1.05	0.62	0.62	0.72
Pde9a	21.54	21.64	19.00	19.05	19.11	18.42	16.22	18.28	22.80
Pfas	2.40	2.37	1.98	1.66	1.98	1.95	2.59	2.03	1.38
Pgm1	4.79	3.93	4.12	4.70	4.81	4.00	3.74	3.87	4.76
Pgm2	77.20	77.51	76.15	81.34	88.26	65.63	94.33	87.07	101.04
Pklr	126	157	186	120	273	127	133	147	222
Pkm	5.59	6.56	6.51	9.33	6.73	5.39	5.76	8.14	6.12
Pnp	29.12	25.32	25.66	30.66	30.16	15.83	30.06	28.60	36.92
Pnp2	0.51	0.32	0.04	0.23	0.16	0.17	0.52	0.11	0.15
Pnpt1	16.14	13.81	14.09	15.48	17.11	12.11	13.73	16.97	21.23
Pola1	0.51	0.45	0.62	0.41	0.35	0.48	0.58	0.57	0.83
Pola2	2.87	2.88	2.69	3.45	2.71	2.90	1.88	2.23	2.32
Pold1	1.19	1.27	1.49	1.60	1.51	1.31	1.14	1.07	0.94
Pold2	7.91	8.28	8.95	8.03	8.02	8.51	8.08	9.09	8.13
Pold3	4.97	3.81	5.32	5.23	4.34	5.38	4.78	4.79	5.17
Pold4	21.60	32.24	31.78	19.70	16.74	29.39	28.93	19.34	17.94
Pole	0.32	0.38	0.35	0.40	0.20	0.24	0.12	0.23	0.13
Pole2	0.35	0.25	0.20	0.54	0.06	0.50	0.14	0.05	0.47
Pole3	27.09	27.50	22.54	29.28	26.76	28.88	22.81	31.02	27.96
Pole4	10.55	8.59	10.51	10.62	9.52	10.19	11.61	9.61	10.13
Polr1a	4.09	4.74	4.54	4.75	4.27	4.67	4.13	4.91	5.20
Polr1b	5.18	5.69	4.62	4.62	6.58	6.32	5.83	6.35	5.36
Polr1c	14.12	12.89	11.56	13.07	13.64	11.51	15.31	13.89	18.45

Polr1d	67.66	64.91	63.64	65.65	65.13	57.66	68.97	70.85	68.06
Polr1e	1.30	1.75	2.17	1.85	2.39	1.73	2.71	2.50	3.19
Polr2a	6.01	7.87	8.30	8.18	8.80	7.78	6.93	7.72	5.05
Polr2b	6.88	7.19	5.20	10.17	9.20	4.67	7.71	10.41	11.85
Polr2c	13.17	12.14	14.82	12.61	12.82	15.65	15.33	15.33	14.57
Polr2d	6.60	5.98	9.35	8.17	8.45	6.15	6.85	8.85	8.68
Polr2e	28.28	33.51	33.02	27.85	33.00	34.38	31.39	30.24	35.70
Polr2f	90.82	107.34	115.10	81.73	78.27	124.02	85.07	79.82	97.97
Polr2g	12.64	11.91	12.70	12.45	12.57	8.90	16.74	16.55	16.57
Polr2h	11.07	12.73	10.13	11.13	11.82	12.82	17.14	12.15	17.18
Polr2i	46.88	43.17	52.40	43.42	48.45	54.69	48.27	45.30	47.49
Polr2j	95.29	90.50	98.10	79.45	82.01	80.06	72.25	73.80	82.93
Polr2l	23.20	25.96	25.68	19.44	19.00	26.86	26.23	18.98	20.81
Polr3a	4.44	4.07	4.49	4.88	4.06	5.35	4.10	5.00	4.68
Polr3b	2.56	2.69	2.52	3.08	3.01	2.70	2.55	2.61	2.72
Polr3c	12.47	10.15	10.96	16.52	12.36	10.86	14.64	13.53	11.51
Polr3d	7.19	8.97	7.26	9.05	11.58	8.99	10.72	12.79	11.23
Polr3e	4.06	3.73	3.09	3.28	3.86	4.66	4.63	4.96	5.35
Polr3f	5.04	4.96	4.45	4.42	5.50	5.14	4.76	5.13	5.34
Polr3g	4.11	4.88	5.38	3.65	4.06	3.95	3.77	3.19	3.58
Polr3gl	16.30	22.44	16.23	13.20	19.17	10.94	19.54	14.02	14.38
Polr3h	2.63	2.64	3.02	2.32	2.02	2.96	2.29	2.82	2.59
Polr3k	6.93	6.56	6.04	6.84	6.37	5.93	5.56	6.74	7.62
Ppat	7.83	4.84	5.58	7.18	7.44	4.41	5.89	6.00	7.77
Prim1	3.50	2.02	4.94	2.96	2.75	2.22	2.22	3.25	2.93
Prim2	1.24	1.75	1.21	1.44	1.66	1.38	1.03	1.42	1.41
Prps1	12.43	10.79	12.30	14.15	15.54	9.53	15.47	17.42	18.48
Prps1l1	0.10	0.00	0.09	0.00	0.00	0.12	0.00	0.03	0.00
Prps1l3	8.59	9.46	7.25	9.43	11.41	8.07	9.56	11.21	15.61
Prps2	6.04	4.32	5.70	4.91	5.72	3.14	5.09	5.57	5.63
Rrm1	2.94	2.40	2.11	3.33	2.83	1.89	2.07	2.80	2.83
Rrm2	3.45	1.86	2.80	3.09	6.04	2.59	3.23	3.22	4.68
Rrm2b	1.00	1.11	0.83	1.05	1.04	0.83	0.59	1.24	0.59
Twistnb	13.83	12.47	12.42	10.80	9.86	17.25	13.08	10.59	13.75
Uox	787	703	767	767	935	598	715	656	1093
Urad	14.35	15.80	13.20	15.08	14.67	10.10	14.61	20.91	19.00
Urah	354.77	362.98	380.07	356.94	363.75	330.83	421.90	384.78	397.00
Xdh	30.48	35.96	28.77	28.34	38.51	30.28	27.43	32.88	29.16
Znrd1	36.74	29.27	43.88	38.27	41.10	49.28	48.55	33.68	43.97

Purine Transcriptomic analysis in female mice livers										
Sample	1	2	3	4	5	1	2	3	4	5
Phenotype	ERD	ERD	ERD	ERD	ERD	ERS	ERS	ERS	ERS	ERS
miRNA	**FPKM**									
mmu-miR-106a-5p	0	0.12	0	0	0	0.27	0.26	0.18	0.34	0
mmu-miR-1945	0	0.18	0.19	0.25	0.33	0.53	0.6	0.5	0.25	0.5
mmu-miR-3087-3p	0	0.12	0	0.08	0	0	0.17	0.32	0.25	0.28

mmu-miR-377-3p	0	0	0	0	0.05	0	0.09	0.14	0.15	0.21
mmu-miR-8098	0	0.06	0	0	0	0.16	0.17	0.05	0.1	0
mmu-miR-344-3p	0.07	0.06	0	0.08	0.05	0	0	0	0.02	0
mmu-miR-5709-5p	0.07	0	0.19	0	0	0.53	0.26	0.23	0	0.28
mmu-miR-7236-3p	0.07	0.67	0.77	0.25	0.11	1.87	0.69	1.05	0.49	0.92
mmu-miR-877-3p	0.07	0	0.05	0	0	0.11	0.09	0.23	0.05	0.14
mmu-miR-669d-5p	0.14	0.09	0	0.12	0.16	0.05	0	0.07	0	0
mmu-miR-7072-5p	0.14	0	0	0.17	0	0.32	0.09	0.23	0.29	0.21
mmu-miR-187-5p	0.29	0.18	0.14	0.33	0.22	0.59	0.86	0.46	0.78	0.14
mmu-miR-328-5p	0.29	0.12	0	0.17	0.16	0.32	0.17	0.37	0.39	0.28
mmu-miR-376c-3p	0.29	0.55	0.24	0.92	0.71	0.11	0	0	0.05	0
mmu-miR-6911-3p	0.6	0.7	1.1	0.8	1.2	0.5	0.6	0.4	0.2	0.6
mmu-miR-29c-5p	1.6	1.7	1.7	1.7	1.8	1.8	1.7	2.1	1.8	2.2
mmu-miR-6516-5p	1.7	1.2	1.6	2.0	1.5	2.2	2.5	2.1	2.5	1.6
mmu-miR-193b-3p	2.2	2.4	1.9	1.8	2.5	1.7	1.2	1.4	1.2	1.1
mmu-miR-378b	2.7	3.8	3.3	1.7	4.7	1.6	2.4	2.0	1.6	2.3
mmu-miR-338-5p	3.3	2.6	2.6	3.2	2.7	2.1	1.8	2.4	1.3	2.1
mmu-miR-1191a	3.5	3.0	2.7	1.8	1.4	4.5	2.9	5.3	3.2	3.8
mmu-miR-96-5p	3.5	4.1	2.0	3.6	2.5	5.4	4.4	4.2	3.6	3.9
mmu-miR-192-3p	4.8	5.9	5.6	6.2	4.5	6.6	5.9	6.9	6.0	7.7
mmu-miR-342-5p	7.5	7.3	7.8	7.7	8.0	8.5	8.1	9.1	9.9	9.1
mmu-miR-221-5p	10	8	8	10	9	17	13	14	15	15
mmu-miR-362-3p	11	9	9	5	5	17	21	15	10	8
mmu-miR-322-3p	14	14	15	16	15	18	21	22	21	18
mmu-miR-582-3p	14	16	12	17	14	17	15	17	18	18
mmu-miR-501-3p	21	20	18	26	28	40	36	45	39	36
mmu-miR-340-3p	21	18	19	19	19	21	24	22	22	21
mmu-miR-374b-5p	21	20	20	20	20	20	22	25	23	22
mmu-miR-335-3p	24	28	24	19	20	26	29	33	30	29
mmu-miR-674-3p	26	21	26	31	18	29	36	41	32	34
mmu-miR-421-3p	33	32	34	28	30	34	36	37	36	37
mmu-miR-335-5p	33	28	32	33	33	30	43	41	41	39
mmu-miR-17-5p	37	36	33	30	36	44	46	43	37	36
mmu-miR-322-5p	37	37	35	38	38	40	46	42	36	41
mmu-miR-1948-3p	59	49	55	69	54	74	84	98	82	88
mmu-miR-142a-3p	60	56	65	54	48	71	63	75	72	64
mmu-miR-145a-3p	81	131	106	76	90	118	140	128	118	123
mmu-miR-148b-3p	83	76	81	81	72	106	100	98	90	97
mmu-miR-146b-5p	100	130	116	121	133	106	94	94	92	105
mmu-miR-671-3p	169	141	142	166	119	175	150	193	193	183
mmu-miR-1843b-5p	196	201	212	211	190	218	207	241	218	221
mmu-miR-28a-5p	206	197	203	189	160	209	234	219	216	207
mmu-miR-29c-3p	248	216	223	212	183	250	291	277	255	227
mmu-miR-100-5p	314	431	326	362	424	520	504	601	541	561
mmu-miR-1843a-5p	483	485	506	502	458	531	519	571	523	554
mmu-miR-103-3p	585	516	540	504	476	741	824	662	635	579
mmu-miR-107-3p	779	675	742	731	579	987	1062	835	857	852
mmu-miR-151-5p	896	892	872	902	829	908	951	1027	908	960

mmu-miR-99b-5p	1031	1381	1067	1294	1399	1641	1643	1992	1882	1711
mmu-miR-340-5p	1167	991	1105	1034	953	1302	1342	1185	1184	1099
mmu-miR-125a-5p	1904	2423	1988	1994	2355	2541	2181	2552	2804	2401
mmu-miR-26b-5p	2009	2001	1924	1911	1798	1976	2330	2304	2179	2036
mmu-miR-194-5p	2059	1923	1789	2016	1459	1959	2081	2214	2230	2123
mmu-miR-423-5p	2088	1651	1970	2114	2046	2349	2197	2400	2603	2202

Urine purine metabolites in DA vs DC							
Sample	1	2	3	4	1	2	3
Phenotype	DA	DA	DA	DA	DC	DC	DC
Metabolites	**Peak Intensities (10^4)**						
Gly	12.6	22.6	18.4	5.17	10	14.2	1.61
Ser	219	283	106	68	315	187	72.7
cystathione	30	80.6	58.1	97.5	15.5	27.1	44.3
Adenine	4.2	13800	3.88	7.59	4.52	3.56	16
guanine	19100	17900	12300	11600	11000	12700	16100
Guanosine	152	310	256	175	437	236	169
Adenosine	77600	72200	47600	43600	44100	52800	61100
AICAR	10.8	76.9	158	7.75	24.9	27.1	16.5
IMP	101	217	150	62.7	131	122	28.1
XMP	4.52	19.7	4.2	2.26	10.7	5.81	3.23
GMP	31.3	40.4	17.1	10.7	9.37	21.5	4.52
AMP	172	217	199	133	136	121	110
Inosine	1480	938	596	1690	1510	2420	878
hypoxanthine	14100	5630	7310	10000	13700	18400	13100
Xanthine	51600	24000	25600	118000	128000	150000	50300
Uric Acid	51500	65400	27700	12900	40300	13500	3850
Glu-6-P	243	310	160	48.3	58.8	62.3	11.5
Fru-6-P	22.3	52.3	19.4	0.807	5.81	2.26	4.2
Fru-1,6-biP	161	653	117	138	130	46.7	76.1
6-P-Gluconate	44.6	6.46	193	5.49	14.5	34.7	8.08
PRPP	0.646	0	3.55	0.969	170	270	1.62
xanthosine	7190	5940	3640	1890	5120	2360	552
GDP	2.58	5.81	3.88	0.323	5.82	2.91	3.55
GTP	6.14	13.2	7.11	2.91	2.58	3.23	2.58
ADP	0.646	5.81	4.52	1.94	3.88	1.94	2.26
ATP	0.969	0.323	0.645	0.323	0.323	1.61	0.646
cAMP	217	401	432	607	1830	1460	797
Allantoin	9.04	21	21.5	11.6	7.91	5.98	7.75
FMN	32.3	252	145	142	40.4	43.6	63

Plasma purine metabolites in DA vs DC								
Sample	1	2	3	4	1	2	3	4
Phenotype	DA	DA	DA	DA	DC	DC	DC	DC
Metabolites	**Peak Intensities**							
Adenine	0	2910	1620	0	0	3550	1290	4840

guanine	1490000	1120000	4100000	7520000	1180000	3360000	1410000	3620000
Guanosine	19400	47500	22100	122000	104000	9210	129000	8720
Adenosine	5230000	4070000	14400000	26700000	4450000	12300000	4940000	12400000
AICAR	23600	11600	22000	21000	13200	11100	30200	19900
IMP	40400	31300	20400	54600	28400	28400	17800	13600
XMP	323	646	969	4520	1940	646	646	969
GMP	7110	13900	12900	9040	7110	7430	6460	5490
AMP	230000	205000	212000	144000	185000	185000	183000	182000
FAD	5490	2910	5810	7750	3230	1940	3880	645
Inosine	234000	206000	336000	398000	440000	735000	347000	646000
hypoxanthine	1320000	880000	1720000	1340000	2020000	2450000	1660000	1140000
Xanthine	1240000	848000	897000	1370000	1640000	1480000	1280000	1400000
Uric Acid	5460000	4330000	4770000	4550000	1560000	6460000	4870000	24700000
Glu-6-P	7750	1940	1940	4850	3880	1450	2910	2260
Fru-1,6-biP	145000	40200	62700	33800	29700	44900	22100	33600
PRPP	1610	646	3550	2260	2910	323	7110	1940
xanthosine	12000	3230	7750	2910	808	15200	16300	33300
GDP	323	323	645	323	1290	323	323	323
GTP	646	323	646	2580	1610	1610	968	323
ADP	323	968	646	323	969	323	0	0
ATP	969	647	1290	646	969	323	646	1940
cAMP	7750	15800	11900	11300	4520	18100	8080	25200
Allantoin	323	969	323	645	1290	646	323	969
FMN	647	323	2260	2260	646	1290	1290	323

Metabolites in Infant Urine samples								
Sample	Phenotype	Ala	Arg	Asn	Asp	Cys	Gln	Glu
1	SF	218000	87400	17000	108000	112000	1320000	182000
2	SF	3800000	146000	37800	90100	211000	5100000	233000
3	SF	3160000	107000	11600	127000	138000	2190000	253000
4	SF	2440000	238000	120000	131000	203000	3650000	253000
5	SF	4620000	90100	13400	92200	122000	2550000	265000
6	SF	15500000	516000	214000	94500	368000	7000000	491000
7	SF	7580000	172000	83000	129000	262000	6670000	535000
1	HM	1380000	91600	12600	136000	63000	10300000	86100
2	HM	474000	316000	97300	107000	448000	14000000	311000
3	HM	3410000	134000	49400	108000	455000	20700000	393000
4	HM	1600000	159000	42500	131000	191000	33600000	422000
5	HM	859000	182000	54600	106000	212000	17800000	442000
6	HM	1880000	255000	74200	134000	224000	16500000	468000
7	HM	7000000	750000	307000	233000	879000	74100000	572000
1	CF	2200000	53600	16800	107000	141000	4780000	143000
2	CF	9450000	83400	24600	113000	247000	3490000	150000
3	CF	4890000	464000	4200	102000	95500	11700000	164000

4	CF	7060000	130000	9690	85600	125000	7980000	166000
5	CF	9210000	233000	63300	119000	604000	12000000	207000
6	CF	597000	50600	15200	110000	273000	4550000	231000
7	CF	8050000	250000	49400	138000	410000	14900000	407000
Sample	Phenotype	Gly	His	Ile	Lys	Met	Phe	Pro
1	SF	1290	2610000	40100000	169000	2380000	15100000	1330000
2	SF	7750	7350000	40100000	129000	3120000	19000000	3530000
3	SF	2260	2920000	40000000	131000	3580000	14900000	3090000
4	SF	5810	16600000	67600000	145000	2630000	34600000	1020000
5	SF	3230	3280000	42100000	150000	3080000	21200000	1970000
6	SF	27100	26200000	35500000	132000	2120000	28900000	11900000
7	SF	7430	9310000	29800000	235000	1520000	18200000	5120000
1	HM	2580	2250000	48600000	132000	3430000	18300000	1930000
2	HM	6140	18400000	74500000	85100	4260000	26700000	4090000
3	HM	15800	11300000	40400000	369000	2980000	26200000	12100000
4	HM	3230	6880000	34900000	253000	2860000	20800000	9380000
5	HM	11000	10900000	55000000	188000	2910000	26800000	9890000
6	HM	12600	16400000	43700000	95300	3220000	27900000	6760000
7	HM	30400	89100000	67700000	9040	5240000	38900000	20300000
1	CF	1290	1940000	23800000	160000	2020000	9940000	1500000
2	CF	8070	3410000	42200000	138000	2760000	18000000	5280000
3	CF	16200	2160000	53000000	260000	3810000	24200000	13600000
4	CF	5170	1870000	35300000	175000	2330000	23400000	7570000
5	CF	12900	11900000	50500000	128000	2260000	34400000	3230000
6	CF	4850	3310000	36000000	146000	2990000	23500000	2610000
7	CF	12600	6210000	57000000	156000	2750000	27900000	5040000
Sample	Phenotype	Ser	Thr	Trp	Tyr	Val	Adenine	guanine
1	SF	57100	123000	3670000	633000	30400	28300	16500000
2	SF	112000	443000	7280000	1650000	64000	0	24400000
3	SF	108000	171000	5140000	791000	39400	408000	22500000
4	SF	192000	567000	6930000	2650000	68500	219000	14800000
5	SF	29200	139000	9940000	758000	74300	0	23800000
6	SF	721000	2210000	19100000	3500000	72500	1280000	26100000
7	SF	230000	848000	11200000	1580000	82200	1340000	35200000
1	HM	136000	155000	2160000	763000	50100	818000	29700000
2	HM	586000	571000	9100000	2030000	82100	606000	36000000
3	HM	438000	1040000	27200000	1560000	110000	3120000	32700000
4	HM	386000	482000	25600000	2000000	83400	1790000	36300000
5	HM	407000	823000	20700000	2450000	77900	6010000	54900000
6	HM	507000	608000	14600000	2520000	120000	804000	44900000
7	HM	3150000	2370000	53300000	6150000	166000	2690000	8800000
1	CF	125000	134000	3900000	420000	65900	458000	24900000
2	CF	69800	318000	12200000	1330000	151000	527000	11400000
3	CF	61400	299000	17800000	1270000	180000	1270000	37900000
4	CF	3880	308000	13000000	1080000	168000	1380000	21400000
5	CF	242000	722000	19200000	2010000	191000	21400000	25100000
6	CF	2100	318000	21200000	1540000	118000	270000	35800000

7	CF	238000	631000	11900000	1320000	154000	1670000	30600000
Sample	Phenotype	Guanosine	Adenosine	AcetylCoA_pos	SuccinylCoA_pos	AICAR	IMP	XMP
1	SF	88200	65000000	141000	15500	32600	46500	2260
2	SF	107000	76800000	2130	12600	20500	38900	1940
3	SF	75600	72300000	8720	2910	53900	28800	969
4	SF	123000	65200000	31600	3230	43000	68500	4520
5	SF	114000	77400000	9880	24900	21300	37000	323
6	SF	132000	64500000	5620	14200	51700	71100	1940
7	SF	172000	119000000	8720	12000	4310000	112000	4840
1	HM	491000	107000000	19800	19700	39500	34600	645
2	HM	171000	127000000	7750	20000	41500	42300	1940
3	HM	167000	124000000	15900	24900	45700	30700	1940
4	HM	168000	147000000	13600	21300	32900	57200	969
5	HM	162000	218000000	51900	43000	26200	68300	2910
6	HM	143000	153000000	9300	29700	37500	53900	2910
7	HM	327000	33100000	58700	12000	48300	134000	4840
1	CF	110000	97300000	9880	8080	12600	70400	2580
2	CF	120000	35800000	10700	20000	86900	36800	4850
3	CF	136000	130000000	2910	23600	26800	57300	4840
4	CF	146000	74800000	2330	25200	77100	37500	2580
5	CF	122000	86800000	8140	19400	37600	50400	2260
6	CF	107000	134000000	10100	6780	53500	32600	323
7	CF	168000	104000000	13200	18100	60200	76300	1940
Sample	Phenotype	GMP	AMP	Urea	hydroxyproline	GSHpos	GSSGpos	NADpos
1	SF	7100	230000	231000000	297000	22600	113000	2580
2	SF	18100	213000	116000000	704000	57200	115000	2260
3	SF	7750	235000	128000000	1380000	28400	134000	1620
4	SF	22500	231000	187000000	351000	67500	148000	969
5	SF	12600	328000	170000000	493000	42200	103000	1290
6	SF	27600	328000	112000000	4540000	39100	112000	22300
7	SF	18400	420000	149000000	1660000	83000	71700	33300
1	HM	9370	234000	71800000	422000	19400	72400	969
2	HM	23300	184000	167000000	1300000	52700	132000	5170
3	HM	19100	175000	83200000	3870000	23600	69500	646
4	HM	13600	188000	127000000	1670000	24900	56900	646
5	HM	16100	251000	148000000	1800000	36800	63300	1290
6	HM	18900	192000	106000000	2130000	45200	131000	646
7	HM	28100	226000	116000000	7830000	63600	78800	1290
1	CF	10700	168000	102000000	158000	23600	80100	969

2	CF	6780	141000	99800000	750000	23900	163000	969
3	CF	11000	147000	134000000	961000	28800	120000	3550
4	CF	10300	173000	146000000	506000	22300	136000	1610
5	CF	16100	140000	107000000	988000	58000	149000	2260
6	CF	12000	167000	157000000	1040000	19400	132000	323
7	CF	19200	168000	80300000	1500000	34900	121000	645
Sample	Phenotype	NADHpos	NADPpos	NADPHpos	PropCoApos	acetoacetyl CoApos	MalCoApos	Ethanolamine
1	SF	3550	20000	157000	74600	8720	11600	3490000
2	SF	2260	67800	168000	97900	7110	47500	1740000
3	SF	1940	73300	56100	98200	2580	19700	2440000
4	SF	5810	23400	167000	41400	14900	15800	2280000
5	SF	646	50400	136000	83000	3230	13200	6990000
6	SF	3230	46500	119000	46700	59100	22600	27400000
7	SF	77500	71700	116000	64300	26200	26500	16300000
1	HM	1620	65300	9040	53000	17000	49700	4590000
2	HM	1290	52000	76900	255000	21300	25800	3770000
3	HM	323	72700	5170	112000	26200	69100	3420000
4	HM	1290	99200	9690	131000	10700	86600	3440000
5	HM	2580	74600	27800	88800	1620	30400	3220000
6	HM	969	87900	10300	142000	1940	72000	3400000
7	HM	1940	43900	28700	51200	4200	1610	4480000
1	CF	2580	43600	52800	48000	40000	27100	4290000
2	CF	2910	45500	9040	67200	3230	39400	4960000
3	CF	3550	83400	11000	43900	3880	72700	1810000
4	CF	1940	89200	11600	38800	11600	32900	3230000
5	CF	969	56900	16200	73300	1620	31700	4300000
6	CF	645	50400	24900	78800	2260	73700	14100000
7	CF	323	61400	9370	32100	9370	48100	3290000
Sample	Phenotype	Choline	Creatine	Nicotinimide	Ornithine	Citrulline	Carnitine	phosphrylcholine
1	SF	786000000	4520	47500000	337000	297000	515000	6980000
2	SF	274000000	1940	127000000	421000	1010000	16700000	5130000
3	SF	370000000	1610	156000000	423000	754000	15200000	4530000
4	SF	784000000	7110	38300000	328000	1330000	6990000	2590000
5	SF	514000000	2580	97800000	313000	419000	2840000	5900000
6	SF	390000000	12000	207000000	476000	1650000	69500000	9230000
7	SF	305000000	88900	107000000	340000	3750000	27200000	4350000
1	HM	640000000	4840	53300000	427000	5470000	8800000	6860000
2	HM	653000000	2260	46600000	546000	1410000	2840000	5620000
3	HM	372000000	8400	124000000	588000	2050000	27700000	10800000
4	HM	766000000	2260	40800000	581000	3100000	4880000	8510000
5	HM	431000000	5170	68700000	503000	3960000	4950000	11600000
6	HM	1.25E+09	4850	109000000	511000	1540000	9800000	12300000
7	HM	550000000	2910	81700000	684000	4960000	37600000	9820000
1	CF	673000000	2260	119000000	313000	532000	14100000	4590000
2	CF	1.01E+09	1940	67700000	380000	515000	34100000	5660000
3	CF	848000000	2580	55200000	411000	2230000	49900000	5480000
4	CF	866000000	1290	102000000	337000	1110000	39200000	5970000

5	CF	480000000	1940	34600000	342000	414000	68700000	6130000
6	CF	575000000	969	86400000	440000	966000	3890000	7040000
7	CF	1.06E+09	2910	104000000	458000	759000	35700000	6350000
Sample	Phenotype	acetylcarnitine	cystathione	glycerophosphocholine	thiamine	thiamine posphate	riboflavin	FAD
1	SF	6630000	20700	24400	397000000	6460	172000000	3230
2	SF	221000000	37500	108000	1.18E+09	12300	86300000	0
3	SF	145000000	4840	84300	528000000	5490	104000000	1940
4	SF	85500000	9690	72400	238000000	18400	175000000	1290
5	SF	45500000	27500	64600	768000000	10000	10800000	647
6	SF	176000000	66900	334000	417000000	28100	83100000	323
7	SF	162000000	1030000	106000	188000000	58500	88900000	2910
1	HM	104000000	7110	38400	2570000	22000	19200000	2580
2	HM	27300000	2590	191000	5140000	17100	20300000	969
3	HM	65100000	59600	215000	6220000	25800	9260000	1940
4	HM	26900000	14900	92400	24800000	10000	26200000	969
5	HM	26900000	28400	56800	11000000	31300	18800000	2580
6	HM	37100000	26500	116000	18000000	5490	107000000	969
7	HM	40200000	7910	170000	4660000	24900	8910000	0
1	CF	266000000	55600	47800	71900000	27100	168000000	0
2	CF	315000000	33600	134000	255000000	12900	112000000	2910
3	CF	153000000	51400	109000	104000000	22900	115000000	969
4	CF	232000000	6950	84200	102000000	11900	133000000	1620
5	CF	415000000	18900	337000	189000000	19100	166000000	1290
6	CF	35300000	33300	83700	409000000	14500	204000000	1620
7	CF	328000000	31700	215000	120000000	24200	226000000	0
Sample	Phenotype	3pSer	SAM	SAH_pos	Inosine	hypoxanthine	Xanthine	Uric Acid
1	SF	92400	71400	2260	3600000	18400000	11700000	25900000
2	SF	48500	225000	10500	6190000	28200000	18700000	37400000
3	SF	54300	124000	3230	4150000	43400000	20000000	30000000
4	SF	26200	125000	6780	3410000	16000000	19500000	27600000
5	SF	33600	70400	2260	4610000	29800000	11300000	28500000
6	SF	22300	14900	12300	11000000	45200000	44500000	49600000
7	SF	97200	122000	65300	4790000	37000000	63000000	38100000
1	HM	57800	19100	7270	12300000	55000000	35700000	16600000
2	HM	33300	158000	6300	8670000	28800000	62000000	45500000
3	HM	29400	274000	5820	12300000	99500000	71600000	29300000
4	HM	12000	110000	13200	11400000	91600000	66700000	31700000
5	HM	26500	82100	9040	7990000	51900000	71300000	37800000
6	HM	37800	179000	11600	7450000	37400000	51000000	42700000
7	HM	11000	430000	7430	27500000	157000000	116000000	20300000
1	CF	52000	70400	5170	3640000	34400000	21900000	24300000
2	CF	14900	146000	3550	3350000	36800000	19300000	31700000
3	CF	16800	43300	5490	8720000	73400000	20600000	13000000
4	CF	73000	73300	5810	3790000	55300000	19100000	20300000
5	CF	37800	345000	2260	6940000	34300000	38400000	46200000
6	CF	37800	59400	3550	4950000	38900000	28200000	19600000
7	CF	33400	166000	4200	10900000	55700000	51000000	37600000

Sample	Phenotype	Glu-6-P	Fru-6-P	Fru-1,6-biP	DHAP	G-3-P	bisphosphoglycerate	3-P-G
1	SF	13200	4200	187000	325000	194000	26700	341000
2	SF	18400	6620	203000	471000	323000	27800	445000
3	SF	27100	7110	298000	613000	281000	6300	447000
4	SF	19400	5330	594000	1070000	444000	11600	290000
5	SF	13200	5650	173000	317000	151000	9370	279000
6	SF	28400	6940	782000	632000	625000	33300	199000
7	SF	29200	7750	873000	1390000	1710000	44300	67100
1	HM	12100	1450	151000	43800	167000	1610	162000
2	HM	36200	5490	124000	47200	273000	9370	295000
3	HM	54900	4040	76900	149000	229000	2260	149000
4	HM	79800	1450	225000	27000	138000	969	97600
5	HM	119000	2910	289000	51400	432000	1620	67600
6	HM	28900	1940	325000	108000	179000	2910	195000
7	HM	223000	7430	266000	2260	358000	2910	145000
1	CF	18700	5490	128000	2100	190000	13200	384000
2	CF	14900	5820	146000	4840	244000	42000	317000
3	CF	12600	1620	194000	1290	118000	1290	41700
4	CF	12900	4040	70400	14200	307000	33400	126000
5	CF	15500	6460	166000	1940	249000	59400	269000
6	CF	7590	4850	98000	7920	106000	3880	601000
7	CF	20500	4520	410000	1130	288000	34200	134000
Sample	Phenotype	PEP	Pyruvate	AcetylCoA_neg	6-P-gluconolactone	6-P-Gluconate	Ribose-5-P	sedoheptulose-7-P
1	SF	559000	197000	646	582000	3230	46000	13000000
2	SF	936000	196000	646	720000	3230	102000	9110000
3	SF	540000	224000	969	781000	11300	16200	19100000
4	SF	415000	371000	647	819000	4200	52600	5120000
5	SF	633000	199000	15500	722000	68200	79000	5740000
6	SF	1540000	443000	3230	1530000	148000	108000	11400000
7	SF	1910000	229000	4200	2250000	247000	50700	7550000
1	HM	780000	188000	4200	1410000	7110	1940	26500000
2	HM	1570000	369000	968	1120000	10200	49400	28400000
3	HM	4050000	404000	646	1040000	3880	46400	40100000
4	HM	1800000	170000	2910	1090000	7110	21600	30400000
5	HM	2550000	181000	1290	1030000	9050	49900	44800000
6	HM	1510000	512000	323	925000	2100	26800	11600000
7	HM	3050000	347000	4840	2860000	4850	147000	95000000
1	CF	2070000	306000	646	1060000	67200	4200	23800000
2	CF	2470000	338000	323	0	65900	24400	12600000
3	CF	186000	91700	969	290000	1940	1290	31900000
4	CF	1220000	108000	646	643000	100000	3880	26100000
5	CF	2240000	274000	323	757000	64100	27500	6820000
6	CF	1560000	211000	1620	669000	33000	51200	14600000
7	CF	2330000	389000	323	932000	94200	13600	27200000
Sample	Phenotype	erythrose-4-P	Citrate	Aconitate	isocitrate	a-KG	SuccCoAneg	succinate

1	SF	122000	9040000	298000	1680000	5700000	323	1960000
2	SF	320000	7740000	608000	639000	14300000	2910	3600000
3	SF	454000	7640000	202000	382000	10300000	968	1870000
4	SF	268000	14400000	375000	266000	5900000	1290	1940000
5	SF	77800	4700000	361000	365000	13100000	1620	2070000
6	SF	205000	9060000	838000	2010000	11100000	1940	4420000
7	SF	185000	12200000	949000	3670000	10800000	3230	6200000
1	HM	77500	4110000	94500	42900000	1480000	1290	1960000
2	HM	174000	8370000	164000	9760000	4430000	1940	2000000
3	HM	82600	7180000	205000	15600000	8480000	2910	4240000
4	HM	125000	4140000	162000	13900000	7330000	2580	3030000
5	HM	94300	5720000	221000	32100000	1900000	2580	7780000
6	HM	63900	5230000	208000	16900000	735000	1940	3660000
7	HM	415000	5920000	382000	9140000	3100000	1940	1430000
1	CF	152000	11000000	113000	3430000	2150000	1290	1120000
2	CF	125000	7240000	196000	6180000	5850000	1620	2040000
3	CF	42300	341000	129000	112000	2000000	2580	2150000
4	CF	98900	3590000	108000	3980000	2340000	1940	1590000
5	CF	144000	7320000	300000	478000	8760000	2580	1730000
6	CF	151000	9790000	204000	996000	10500000	3230	1620000
7	CF	105000	11300000	269000	4670000	5570000	2580	2180000
Sample	Phenotype	Fumarate	malate	oxaloacetate	PRPP	xanthosine	GDP	GTP
1	SF	151000	108000	427000	682000	1990000	30700	31700
2	SF	406000	249000	1300000	1410000	3340000	42300	9210
3	SF	172000	109000	450000	1000000	2140000	9370	14900
4	SF	224000	199000	744000	660000	2300000	10300	13600
5	SF	233000	117000	619000	1050000	1780000	18700	9690
6	SF	421000	520000	1280000	1000000	3560000	8720	27800
7	SF	705000	339000	1480000	1720000	3070000	24100	37300
1	HM	304000	200000	465000	1720000	1350000	18400	19900
2	HM	690000	580000	846000	425000	4310000	16200	21300
3	HM	744000	976000	1260000	1110000	5120000	31000	14200
4	HM	767000	1460000	1400000	1280000	3530000	37100	17800
5	HM	1300000	544000	2000000	1020000	2990000	31300	11000
6	HM	536000	424000	825000	1340000	4660000	28700	16300
7	HM	1400000	469000	2270000	36600	11700000	34900	40100
1	CF	116000	490000	363000	1230000	1820000	7100	39100
2	CF	132000	330000	455000	1140000	2080000	7750	20000
3	CF	181000	124000	355000	1610000	1390000	9040	10700
4	CF	198000	115000	355000	1140000	1480000	16200	9370
5	CF	497000	553000	523000	1280000	2960000	7750	18100
6	CF	143000	170000	463000	1590000	1690000	6780	13200
7	CF	441000	435000	630000	1390000	4620000	12600	35200
Sample	Phenotype	ADP	ATP	cAMP	Allantoin	GSHneg	GSSGneg	Lactate
1	SF	3550	3870	1480000	348000	1620	0	1080000
2	SF	7430	2580	2540000	325000	4520	969	1910000
3	SF	4200	1620	1990000	213000	1940	969	1720000

4	SF	4200	9370	2410000	896000	9370	646	1520000
5	SF	9040	17800	1160000	56000	1290	646	1320000
6	SF	1940	27800	4100000	1360000	78200	646	2410000
7	SF	20400	128000	2550000	375000	47800	1610	1870000
1	HM	6620	2260	1160000	1400000	6780	0	5440000
2	HM	3070	646	3270000	1770000	12000	0	1740000
3	HM	3880	1940	3950000	350000	61700	323	2060000
4	HM	7430	1610	2970000	330000	2910	323	1780000
5	HM	8070	2910	2600000	205000	6780	0	2010000
6	HM	3550	1620	4020000	1270000	20400	0	1140000
7	HM	10700	1620	8250000	258000	11600	646	4780000
1	CF	3550	646	1110000	1020000	1940	0	947000
2	CF	2260	1940	2720000	61900	1610	0	1590000
3	CF	2910	968	2450000	10300	323	0	1080000
4	CF	3550	1940	1760000	82000	3880	0	1020000
5	CF	7430	969	2990000	487000	8070	323	1460000
6	CF	9370	969	1670000	507000	8080	323	950000
7	CF	5490	968	3760000	720000	15800	323	1260000
Sample	Phenotype	Acetoacetate	aminoadipic acid	kynurenic acid	trehalose-6-P	TPP	FMN	NADneg
1	SF	34600	1500000	103000000	2260	1610	26800	646
2	SF	32400	3040000	131000000	1940	1940	85600	1290
3	SF	71500	2260000	150000000	1940	1290	13600	646
4	SF	342000	4310000	120000000	2580	3880	13200	1940
5	SF	6780	2290000	99900000	1940	6780	229000	645
6	SF	16000	6230000	230000000	2260	1290	17400	323
7	SF	206000	3780000	141000000	6140	3550	166000	969
1	HM	50100	2160000	118000000	1290	27800	13200	646
2	HM	58100	5170000	139000000	5490	2910	39100	1610
3	HM	30700	5420000	221000000	2100	6140	37500	969
4	HM	28600	3830000	163000000	5810	2580	24900	646
5	HM	25800	5220000	228000000	14200	1610	43300	1620
6	HM	49100	5630000	105000000	5810	4200	15200	1290
7	HM	120000	7780000	176000000	7110	1940	112000	646
1	CF	29900	1360000	103000000	1620	2910	8070	1290
2	CF	83500	2820000	126000000	4200	2260	29400	2260
3	CF	6460	2450000	170000000	1610	1940	25800	1940
4	CF	36700	2030000	165000000	1940	2910	19400	645
5	CF	32600	3960000	68100000	3870	1620	18700	3230
6	CF	95000	3030000	110000000	1290	1620	21000	969
7	CF	5810	3890000	140000000	5810	6460	65900	1940
Sample	Phenotype	NADHneg	NADPneg	NADPHneg	propCoAneg	ButrylyCoA	acetoacetylCoAneg	malonylCoAneg
1	SF	968	323	2580	1620	646	323	6140
2	SF	969	0	1290	1290	1940	323	20400
3	SF	969	0	1940	968	1290	323	10000
4	SF	969	323	2580	9040	645	323	9370
5	SF	1290	646	1620	1610	968	645	2260
6	SF	2580	323	645	13600	5820	323	16100

7	SF	4200	1290	7110	24600	26500	1610	4520
1	HM	3230	0	1940	12000	6460	646	9690
2	HM	1940	323	2580	2910	2580	323	19700
3	HM	968	0	969	12400	6140	323	10300
4	HM	4200	0	1290	16500	2580	323	6140
5	HM	969	0	969	12900	2910	323	18100
6	HM	645	323	2260	3550	2580	645	18400
7	HM	15200	323	2260	52300	37800	323	29100
1	CF	1940	0	2260	646	6130	0	13900
2	CF	968	0	2260	323	6140	323	14900
3	CF	969	323	1290	968	323	0	9370
4	CF	646	0	323	4520	3550	323	7430
5	CF	969	323	1610	2260	9040	1290	10300
6	CF	969	0	1610	1940	6140	646	12600
7	CF	969	323	2260	1940	3550	646	21600
Sample	Phenotype	hydroxybutrylyCoA	glycerate	glyoxylate	SAH_neg	Ascorbic Acid		
1	SF	969	9320000	108000	4520	3600000		
2	SF	1290	18800000	138000	3870	4660000		
3	SF	323	17200000	113000	2910	838000		
4	SF	2580	14900000	165000	9040	11100000		
5	SF	1620	12800000	9370	2260	704000		
6	SF	5490	7050000	153000	6460	2850000		
7	SF	37800	2680000	131000	17400	3360000		
1	HM	4840	13300000	93700	11600	13700000		
2	HM	1290	10100000	153000	5170	10300000		
3	HM	1940	3030000	91400	13600	1420000		
4	HM	1290	2980000	66900	7110	2850000		
5	HM	1290	1720000	81400	8400	852000		
6	HM	646	4410000	219000	4840	6340000		
7	HM	14900	2640000	136000	1940	462000		
1	CF	1940	12600000	94000	5490	4660000		
2	CF	646	8710000	121000	5170	406000		
3	CF	323	6860000	80600	1610	57200		
4	CF	2260	8090000	48100	6780	336000		
5	CF	323	9930000	156000	5490	8580000		
6	CF	1620	4740000	79500	2260	5980000		
7	CF	969	8700000	242000	9370	6360000		