THE IMPACT OF VITAMIN A SUPPLEMENTATION IN THE EARLY POSTNATAL PERIOD ON METABOLIC REGULATION RELATED TO CHILDHOOD OBESITY RISK

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ABSTRACT

Background: Childhood obesity has been recognized as a serious growing medical concern since the 1990s and is associated with many preventable immediate and long-term health complications. Without appropriate intervention, obesity from infancy and childhood is likely to be carried to adolescence as well as adulthood. Nowadays, the concept of “fetal origins of adult disease” has been extensively studied and well accepted. However, research is scarce about the effects of early-life nutritional interventions and, with respect to vitamin A (VA), little is known regarding VA supplementation on the risk of obesity development later in life.

Objective: The purpose of this study was to investigate whether early postnatal VA and/or VARA (a retinoid combination of VA and 10% retinoic acid) supplementation alters metabolic functions related to risk of childhood obesity development, using a diet-induced obese rat model with VA-marginal status. Specifically, we examined the changes of expression of genes related to insulin sensitivity (Rbp4) and thermogenesis (Ucp) in various tissues at two ages, in neonatal age and in young adolescent rats.

Hypotheses: We hypothesized that VA and/or VARA supplementation, administered in the early postnatal period, is associated with altered expression of Rbp4 (in liver, kidney, and adipose tissues) and Ucp (Ucp1 in brown adipose tissue, BAT; and, Ucp3 in skeletal muscle) in the neonatal period, with carryover effects to young adolescent age. We also hypothesized that early postnatal VA and VARA supplementation is associated with decreased adiposity in prepubescent rats.
Methods: A purified high-fat VA-marginal (HFVAM) diet, which contains 45 kcal% fat and a marginal level of VA (0.35 µg of retinol as retinyl palmitate/g diet), was fed to Sprague Dawley dams from pregnancy until pups were euthanized at the end of study. Three oral doses of placebo, VA, or VARA (adjusted based on body weight) were administered to pups on postnatal day (P) 0/1, P4, and P10. Serum and tissues were collected either in the neonatal period (P12; n=57) or at young adolescent age (5 weeks; n=49). Body composition of young adolescent rats was measured by dual-energy x-ray absorptiometry (DEXA). A group of age- and sex-matched normal chow-fed non-obese young adolescent rats that received no treatment were included as references for the purpose of body composition comparison.

Results: Early postnatal VA and VARA supplementation significantly increased total retinol concentrations in liver, lung, kidney, BAT, white adipose tissue (WAT), and muscle in neonatal pups. Besides liver, lung, and WAT, the effects of early-life VA and VARA supplementation on significantly elevated retinoid storage in other extrahepatic tissues (kidney, BAT, and muscle) were transient in the neonatal period and were not carried over to young adolescent age. The serum total retinol levels remained homeostatically controlled within a normal physiological range, regardless of the increase in hepatic retinoid storage by early-life VA and VARA supplementation. Treatment effects of early-life VARA treatment on up-regulating Rbp4 and Ucp1 steady-state mRNA expression in BAT were observed at young adolescent age; however, the Rbp4 (in liver, kidney, and WAT) and Ucp3 (in muscle) mRNA transcripts were not affected by early postnatal VA or VARA supplementation at both developmental stages. The results suggest the early postnatal VARA treatment may have long-term impacts on functions
related to metabolic regulation in BAT. No significant differences in body weight change were observed between treatment groups over the course of the experimental period, indicating similar growth patterns as well as successful pup randomization across treatment groups. Additionally, early-life VA and VARA supplementation was not associated with decreased adiposity in prepubescent rats. Overall, no sex differences were observed in tissue retinoid levels, tissue gene expression, and body composition within treatment group at both developmental stages (neonatal period and young adolescent age).

**Conclusions:** The study showed that early postnatal VA and VARA supplementation not only is effective in promoting retinoid reserves in hepatic and extrahepatic tissues (lung and WAT), but also may play important roles in the long-term regulation of insulin sensitivity and thermogenesis in BAT. In prepubescent rats, no adverse effects on body composition were observed from the administration of early-life VA and VARA supplementation. Thus, based on these results, VA supplementation early in life may be considered safe as viewed from the perspective of obesity risk later in life.

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LIST OF ABBREVIATIONS

**ABCA1**: ATP-binding cassette subfamily A member 1

**AI**: adequate intake

**ANOVA**: analysis of variance

**apoB**: apolipoprotein B

**ARAT**: acyl-CoA:retinol acyltransferase

**BAT**: brown adipose tissue

**BCO1**: β-carotene-15,15’-oxygenase

**BCO2**: β-carotene-9’,10’-oxygenase

**BMC**: bone mineral content

**BMD**: bone mineral density

**BPD**: bronchopulmonary dysplasia

**CD36**: cluster of differentiation 36

**cDNA**: complementary DNA

**CRABP**: cellular retinoic acid-binding protein

**CRBP**: cellular retinol-binding protein

**CYP26**: cytochrome P450 family 26

**DEXA**: dual-energy x-ray absorptiometry

**DRA**: 3,4-didehydroretinyl acetate

**DRI**: dietary reference intake

**EAR**: estimated average requirement

**FFQs**: food frequency questionnaires

**FOAD**: fetal origins of adult disease
**GDP:** gross domestic product  
**GLUT4:** glucose transporter 4  
**h:** hour(s)  
**HFD:** high-fat diet(s)  
**HFVAM:** high-fat vitamin A-marginal  
**HSC:** hepatic stellate cell(s)  
**IACUC:** Institutional Animal Care and Use Committee  
**IFN:** interferon  
**IU:** international units  
**LPL:** lipoprotein lipase  
**LRAT:** lecithin:retinol acyltransferase  
**MAPK:** mitogen-activated protein kinase  
**MRDR:** modified-relative-dose-response  
**mRNA:** messenger RNA  
**NAFLD:** non-alcoholic fatty liver disease  
**NIH:** National Institutes of Health  
**NOAEL:** no-observed-adverse-effect level  
**NPC1L1:** Niemann-Pick C1-Like 1  
**P:** postnatal day  
**PDA:** photodiode array  
**PIC:** polyriboinosinic:polycytidylic acid  
**PPAR:** peroxisome proliferator-activated receptor  
**RA:** retinoic acid  
**RALDH:** retinal dehydrogenase(s)
RAR: retinoic acid receptor(s)
RARE: retinoic acid response elements
RBP4: retinol-binding protein 4
RBPR2: RBP4 receptor-2
RDA: recommended dietary allowance
RDH: retinol dehydrogenase(s)
RDR: relative-dose-response
RE: retinyl ester(s)
REH: retinyl ester hydrolases
ROH: retinol
RP: retinyl palmitate
RT-PCR: reverse transcription polymerase chain reaction
RXR: retinoid X receptor(s)
SD: Sprague Dawley
SEM: standard error of the mean
SR-BI: scavenger receptor class B type I
STRA6: stimulated by retinoic acid gene 6
TMMP-retinol: trimethylmethoxyphenyl-retinol
TR: thyroid hormone receptor(s)
TTR: transthyretin
UCP: uncoupling protein
UL: upper level
UPLC: ultra-performance liquid chromatography
VA: vitamin A
VAD: vitamin A deficiency
VARA: a retinoid combination of vitamin A and 10% retinoic acid
VDR: vitamin D receptor(s)
WAT: white adipose tissue
WHO: World Health Organization
WT: wild type
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CHAPTER 1

LITERATURE REVIEW

1.1 Vitamin A

1.1.1 Introduction to vitamin A

Vitamin A (VA) is a generic term that encompasses all-trans-retinol (ROH) and its metabolites with biological activities; the group includes retinal, retinoic acid (RA), and retinyl esters (RE) (1-4). VA is an essential fat-soluble micronutrient that cannot be produced by human body and must be consumed from diet or supplements. Dietary VA can be found either as provitamin A carotenoids (i.e., α-carotene, β-carotene, and β-cryptoxanthin) in yellow or orange fruits and dark-green leafy vegetables or as preformed VA (i.e., retinol and RE) in dairy products and animal origin, such as liver, meats, and eggs.

The characterization of VA was a long incremental process that started in the 1880s, so it is unfair to refer to a single study as the ‘discovery’ of VA (5). In 1881, Nicolai Ivanovich Lunin and his mentor, Gustav von Bunge, showed that mice cannot survive on diet consisting of proteins, fats, carbohydrates, salts, and water, but can live in good
conditions on milk, suggesting milk contained unknown indispensable substances that were essential to life (5). Between 1909 and 1911, Wilhelm Stepp and his mentor, Franz Hofmeister, concluded that the unknown essential substances provided in milk were not only fat-soluble, but also alcohol- and ether-soluble (5). In 1912, Frederick Gowland Hopkins termed the unknown essential substances, which were suggested by Lunin’s study in 1881 and confirmed by Hopkins’s own work, as the ‘accessory factors of the diet’ (6). Hopkins then received the Nobel Prize in Physiology or Medicine in 1929 for the work. The nature of the ‘accessory factors’ was revealed independently by Elmer McCollum and Marguerite Davis at the University of Wisconsin-Madison and Thomas Osborne and Lafayette Mendel at Yale University in 1913 (7, 8). These investigators found that rats fed a basal diet with added lard or olive oil extract died, but they survived when an ether extract of butter or egg yolk was added to the diet, indicating that the ‘accessory factors’ were presented in butterfat, but not lard (7, 8). The term ‘fat-soluble A’ was suggested by McCollum in 1918 and later ‘vitamin A’ was proposed by Jack Cecil Drummond in 1920 (9).
1.1.2 Functions and properties of vitamin A

Vitamin A is critical in the regulation of various biological processes, including vision, cell growth and differentiation, gene expression, immunity, reproduction, embryonic development, and bone metabolism (10). The cones and rods are photo- and light receptors in the retina specialized for visual function in bright light and dim/dark environment, respectively. Rhodopsin, a VA-containing pigment protein, is found in rods and is composed of 11-cis-retinal and opsin. When light hits the retina, rhodopsin is cleaved to release 11-cis-retinal, which can be converted to all-trans-retinal for the purpose of neural signaling to the brain. In order to regain vision in the dark, the all-trans-retinal must be converted back to 11-cis-retinal, which can then reattach to opsin for rhodopsin reformation. A delayed vision recovery from darkness might be expected in populations with inadequate VA intake or storage (10).

Cell differentiation refers to the process of immature cells developing into functional mature cells. Retinoic acid, the major bioactive metabolite of VA, is often required for cell differentiation, especially for epithelial cells. For example, the presence of RA is essential for the differentiation of keratinocytes into mature epidermal cells (10). An ex vivo study showed that, with adequate VA, RA favors the development of myeloid
progenitors into myeloid dendritic cells for immune system actions (11). Retinoic acid, especially all-\textit{trans}- and 9-\textit{cis}-isomers of RA, is involved in the regulation of gene expression. Retinoic acid is transported from the cytosol into the nucleus facilitated by cellular retinoic acid-binding protein (CRABP) (10). Within the nucleus, all-\textit{trans}- and 9-\textit{cis}-RA bind to retinoic acid receptors (RAR) and retinoid X receptors (RXR), respectively, to form VA-receptor complexes (1-3). The VA-receptor complexes can then form various homodimeric or heterodimeric complexes and subsequently attach to retinoic acid response elements (RARE) within the DNA (10) to regulate the transcription of more than 500 genes (12). Besides forming heterodimer with RAR, RXR also interacts with other ligand-activated nuclear receptors, including the vitamin D receptors (VDR), peroxisome proliferator-activated receptors (PPAR), and thyroid hormone receptors (TR) (1-3). Retinoic acid also appears to be crucial for male and female reproductive processes as well as embryonic development, although the mechanism of actions remain to be elucidated (13). Additionally, VA is involved in bone metabolism, acting to maintain the balance between osteoblasts and osteoclasts (10).
1.1.3 Vitamin A metabolism

Dietary retinoids and carotenoids are absorbed by the small intestine and packaged into chylomicrons to be delivered to the liver and subsequently to target organs. The metabolic process involves various enzymes, for retinoid conversion, and several specific binding proteins, for retinoid transportation. Detailed information about retinoid metabolic process, including intestinal digestion and absorption, hepatic uptake, plasma trafficking, extrahepatic tissue metabolism, and enzymes and specific binding proteins involved, will be reviewed and discussed in the following sections.

1.1.3.1 Intestinal digestion and absorption

The absorption of dietary provitamin A carotenoids and preformed VA begins with digestion in the intestinal lumen, followed by cellular uptake, and then incorporation into chylomicrons for plasma transport. The newly ingested RE must be hydrolyzed to liberate unesterified retinol and corresponding fatty acid by pancreatic triglyceride lipase and cholesteryl ester hydrolase, which are nonspecific pancreatic enzymes located within the intestinal lumen, or by phospholipase B, which is one of the retinyl ester hydrolases (REH) located at the brush-border membrane (3, 14, 15). In order to complete the hydrolysis, the RE must be mixed with bile salts into lipid micelles along with dietary
carotenoids for RE solubilization as well as activation of hydrolytic enzymes (1, 4). The free unesterified retinol and carotenoids are then delivered to the surface of mucosal absorptive cells (also known as the “enterocytes”) by micelles for intestinal uptake (16).

The intact carotenoids are absorbed into the enterocytes by apical membrane transporters, including SR-BI, CD36, and NPC1L1, whereas the free retinol is taken up by the enterocytes through unidentified apical retinol transporter at physiological concentrations, or passive diffusion at pharmacological concentrations (2, 14, 16). Approximately 75% to 100% and 3% to 90% of ingested retinol and β-carotene, respectively, can be absorbed across the microvilli of duodenum and jejunum (16), as long as more than 5 grams of dietary fat is consumed along with the meal (17).

Once in the enterocytes, the free retinol binds to the cellular retinol-binding protein, type II (CRBP-II) and is subsequently re-esterified to RE through the actions of lecithin:retinol acyltransferase (LRAT), with the fatty acyl group limited to palmitic, stearic, and oleic acids, derived from lecithin (2, 3). Although acyl-CoA:retinol acyltransferase (ARAT) is identified as another retinol-esterifying enzyme, it has been suggested that ARAT is ineffective in retinol esterification in the intestine, with the exception of large amounts of retinol intake (2, 4, 14, 18). The intact carotenoids can be
cleaved by carotenoid cleavage enzymes (i.e., β-carotene-15,15'-oxygenase (BCO1) and β-carotene-9’,10’-oxygenase (BCO2)) to eventually yield retinal (19-21). The retinal can then be reduced to retinol by retinal reductase and esterified to RE by LRAT, or oxidized to RA by retinal dehydrogenases (RALDH) (1).

The newly formed RE are incorporated into nascent chylomicrons, along with triglycerides, cholesterol, cholesteryl esters, phospholipids, apolipoprotein B (apoB), and a small proportion of uncleaved carotenoids (14, 22). The nascent chylomicrons are secreted into the lymphatic system and eventually enter the general circulation to be delivered to tissues for future use (1-4, 14). The chylomicron VA concentrations in the plasma peak around 2 to 6 h after VA-containing meals (1). The unesterified retinol can be secreted from the basolateral mucosal cell membrane into the portal circulation by an unidentified basolateral efflux transporter or ABCA1 (via the apoA-I pathway) (14, 16). The RA, which is generated from carotenoid cleavage and retinal oxidation, is absorbed into the portal blood and transported by serum albumin (1, 23). In addition, Wang et al. (24), using a ferret model, reported that the circulating RA level can be increased within 2 h after a dose of β-carotene.
1.1.3.2 Hepatic uptake

Once in the circulatory system, the nascent chylomicrons become chylomicron remnants after lipolysis of triglycerides through the actions of lipoprotein lipase (LPL). During the LPL reaction, a minor fraction of RE from nascent chylomicrons may be transferred into tissues or exchanged with plasma lipoproteins (1), yet a significant amount of the original RE remain in the chylomicron remnants; the remnants are then taken up shortly after their formation, into liver parenchymal cells by receptor-mediated endocytosis (4, 25). Approximately 75% of postprandial RE in the chylomicron remnants are cleared into the liver from the circulation, while the remainder are taken up by extrahepatic tissues, including adipose tissue, kidney, lung, skeletal muscle, and heart (26, 27).

Liver is not only considered as the main storage organ for retinoids, but also responsible for maintaining whole body retinoid homeostasis (1). In the hepatocytes (liver parenchymal cells), the RE transferred from chylomicron remnants are rapidly hydrolyzed to retinol by REH (i.e., carboxylesterase ES-10) (26, 28, 29). Most of the retinol is transported by an unknown mechanism to hepatic stellate cells (HSC), which are VA-storing cells located in the perisinusoidal space of the liver (2). Hepatic stellate
cells (also known as Ito cells, fat-storing cells, or lipocytes) contain cellular retinol-binding protein, type I (CRBP-I) and LRAT, which together result in the formation and storage of RE within the cytoplasmic lipid droplets (1, 2). Under VA-adequate conditions, HSC can store approximately 50-80% or even up to 90% of total body VA, mostly as retinyl palmitate (1, 2, 30). Vitamin A can also be stored throughout the body, as stellate cells exist in several extrahepatic tissues, including pancreas, lung, kidney, and intestine (30).

The hydrolysis of newly-endocytosed RE is insensitive to VA status; in contrast, the process of RE formation and storage in HSC and the activity of LRAT highly depend on body VA status. During a VA-deficient state, LRAT mRNA expression and activity are down-regulated and the stored RE are mobilized from HSC (31), resulting in increased apo-CRBP (1). The increased apo-CRBP then stimulates REH activity to hydrolyze RE into retinol (32), which is eventually secreted into the circulation. Liver LRAT mRNA expression and enzyme activity can be restored shortly after the administration of VA or RA (31). Ross and colleagues (31) showed that liver LRAT mRNA expression is regulated by dietary VA exposure in a dose-dependent manner.
1.1.3.3 Plasma trafficking

Plasma retinol-binding protein, also known as retinol-binding protein 4 (RBP4), is a specific carrier of retinol in the blood that delivers retinol to retinoid-dependent peripheral tissues for their physiologic needs. In the fasting state, retinol is bound to apo-RBP4 (retinol-free RBP4) for the formation of holo-RBP4 complex, which is then secreted into the circulation (1-3). Approximately >95% of VA is present as all-trans-retinol in the plasma (1-3). In the circulation, nearly all holo-RBP4 complex is bound to a second protein, transthyretin (TTR, also known as prealbumin), in a molar ratio of 1:1 to form the holo-RBP4-TTR protein complex (33). The holo-RBP4-TTR complex serves to increase the molecular weight and prevent the loss of RBP4 and retinol by glomerular filtration (33, 34). A pool of apo-RBP4 accumulates in the hepatocytes in the state of vitamin A deficiency (VAD), suggesting that the secretion, rather than synthesis, of RBP4 by the liver is affected by body VA status (35). The apo-RBP4 can be rapidly released into the circulation as holo-RBP4 complex when VA-deficient animals are replenished with VA (35).
1.1.3.4 Extrahepatic tissue metabolism

Retinol is taken up into the extrahepatic tissues by stimulated by retinoic acid gene 6 (STRA6), a specific cell-surface receptor for RBP4 that functions as a major physiological mediator of retinol uptake (36). STRA6 acts to remove retinol from the holo-RBP4 complex and to transport retinol across the cell membrane (36, 37). STRA6 is widely expressed in murine embryonic and in adult organ systems, including brain, lung, kidney, spleen, testis, and female reproductive tract; however, the expression of STRA6 in the liver is very low or absent (36, 37). In contrast, RBP4 receptor-2 (RBPR2) is a membrane protein that shares structural similarity with STRA6 (38), but is expressed in liver and intestine where the expression of STRA6 is absent (36, 38). Alapatt et al. (38) reported that RBPR2 mediates hepatic retinol uptake and is inversely regulated by liver retinol stores and RA in vivo, suggesting the potential role of RBPR2 in coordinating whole body retinol homeostasis. The expression of STRA6 and LRAT in the lungs of neonatal rats can be significantly induced 6 h after a treatment of VARA (a retinoid combination of VA and 10% RA), along with increased RE formation observed (39).

Once transported across the plasma membrane, retinol is either bound to CRBP and reesterified to RE by LRAT, or oxidized to retinal by retinol dehydrogenase (RDH) (1-3).
The RE is stored in cellular lipid droplets and can be hydrolyzed by REH to liberate retinol, depending on physiologic requirements (1-3). The retinal can then be irreversibly oxidized by RALDH to RA, which is then bound to CRABP for delivery into the nucleus (1-3). In the nucleus, RA acts to activate RAR and RXR, which can then form homodimers or heterodimers to regulate the transcription of downstream target genes (1-3). The intracellular concentration of RA can be maintained by the enzyme CYP26, which belongs to the cytochrome P450 gene superfamily, to further oxidize RA into polar metabolites, such as 4-hydroxy-RA and 4-oxo-RA (40). The CYP26 subfamily is comprised of three genes (CYP26A1, CYP26B1, and CYP26C1), which are expressed in a tissue-specific manner (40). For instance, CYP26A1 and CYP26B1 play significant roles in embryonic development and postnatal survival, respectively (40).

1.1.4 Vitamin A homeostasis

LRAT and CYP26 are two essential enzymes that play predominant roles in maintaining whole body VA homeostasis. LRAT is responsible for retinol esterification to yield RE for storage in stellate cells; CYP26 catalyzes the oxidation of RA into polar metabolites. In other words, LRAT controls the availability of retinol for retinoid metabolism, while CYP26 maintains the concentration of RA to prevent detrimental
effects. The important functions of LRAT and CYP26 activities are closely regulated by all-trans-RA in a tissue-specific manner (31, 41). Both LRAT and CYP26 are strongly regulated in liver and lung, with CYP26 shows additional tissue specificity in testis and intestine (31, 41, 42). In the case of VAD, the LRAT and CYP26 mRNA expression and enzyme activity are significantly down-regulated and almost virtually absent in liver and lung of mice and rats (31, 41-47). In contrast, the LRAT and CYP26 mRNA and activity can be rapidly induced upon VA repletion in a dose-dependent manner (VA deficient < VA marginal < VA adequate < VA supplemented < therapeutic dose of exogenous RA) (31, 41-47). The whole body retinoid homeostasis is mediated by the autoregulatory system of RA and enzymes (LRAT & CYP26) (46).

Although liver total retinol concentration is proportional to dietary VA exposure, the liver retinoid reserves and serum total retinol levels are not linearly correlated (48). The serum retinol concentration reaches a plateau around 20 µg/g (~70 nmol/g) of liver VA storage, and remains relatively constant up to 300 µg/g (~1047 nmol/g) of liver VA reserves (48). Serum VA value falls when liver retinoid storage is exhausted (<20 µg/g), and rises when liver stores are excessive (>300 µg/g) (48). In other words, the serum VA concentration is homeostatically controlled over a wide range of liver reserves and
dietary or supplementary ingestion of VA, indicating that serum VA concentration is not an ideal indicator for body VA status except under extreme conditions (VA deficiency and VA toxicity).

1.1.5 Biomarkers of vitamin A status

Currently there are several biomarkers of VA status available for various purposes of research studies, program evaluation, policy formation, and clinical application. Some of the most popular methods for body VA status assessment, such as dietary assessment, functional/physiological measurements, and biochemical indicators, will be briefly reviewed as follows. Different dietary assessment methodologies, including food records, 24-h dietary recall, food frequency questionnaires (FFQs), and diet history, have been extensively studied and well validated (49). However, there are four variables that need to be carefully evaluated when assessing population-based dietary patterns: (a) the season of the year; (b) the times of the week (weekdays vs. weekends); (c) food fortification in the country; and (d) individual usage of VA-containing supplements (22). Since provitamin A carotenoids are only provided by relatively few seasonal fruits and vegetables sources, it is crucial to consider the times of the year for data collection. Some people have distinctly different dietary habits between weekdays and weekends, so the
times of the week to complete dietary assessment need to be taken into account. Researchers also need to be aware of food fortification in the country and individual usage of VA-containing supplements, especially with preformed VA as a high intake of preformed VA from fortified foods or supplements can potentially lead to hypervitaminosis A (50).

Dark adaptation testing is the most commonly used bioindicator for functional/physiological measurement of body VA status. Vitamin A is known to play a critical role in the visual cycle. The rods are specialized for low-intensity light condition as well as colorless night vision. Individuals with inadequate body VA storage have insufficient rhodopsin in rods for vision recovery after a light flash. Therefore, a VA-deficient population might suffer from night blindness due to impaired rods function (10). The dark adaptation test, which is to accurately measure the individual’s ability to adapt under low light intensity, is being used to detect VAD and to help define dietary VA requirements. Several different techniques that are currently available for dark adaptation testing: (a) classical testing; (b) rapid dark adaptation testing; (c) electroretinography; and (d) pupillary threshold testing (22). However, the accuracy of dark adaptation testing results may be affected by several confounders, such as eye
diseases, aging of the subjects, and zinc or protein deficiency (22). The advantages and limitations of different dark adaptation testing methods are summarized in Table 1.
Table 1. Advantages and disadvantages of different dark adaptation testing techniques (22).

<table>
<thead>
<tr>
<th>Methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical testing</td>
<td>1. No surrogate biomarkers needed 1. Confounders</td>
<td>2. Equipment is cumbersome and expensive</td>
</tr>
<tr>
<td></td>
<td>2. Precise</td>
<td>3. Not suitable for children</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Requires trained technician</td>
</tr>
<tr>
<td>Rapid dark adaptation testing</td>
<td>1. Inexpensive equipment          1. Confounders</td>
<td>2. Requires trained technician</td>
</tr>
<tr>
<td></td>
<td>2. Suitable for children</td>
<td>3. Needs further validation</td>
</tr>
<tr>
<td></td>
<td>3. Fast</td>
<td></td>
</tr>
<tr>
<td>Electroretinography</td>
<td>1. Objective                      1. Confounders</td>
<td>2. Invasive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Not suitable for children</td>
</tr>
<tr>
<td>Pupillary threshold testing</td>
<td>1. Objective                      1. Confounders</td>
<td>2. Have not been widely used</td>
</tr>
<tr>
<td></td>
<td>3. Rapid and portable</td>
<td>4. Requires trained technician</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>
Plasma retinol and RBP4 concentrations are two of the most widely used biochemical indicators to assess VA status at the population level (51). Plasma retinol is bound to RBP4 as the holo-RBP4 complex, to be transported through the circulatory system to peripheral tissues. During acute infection or inflammation, the release of holo-RBP4 complex from the hepatocytes is disrupted, leading to transiently depressed plasma retinol and RBP4 concentrations (52). Although liver total retinol concentration is considered as the “gold standard” for body VA status assessment (53), obtaining human liver biopsy requires ethical considerations. Instead, the relative-dose-response (RDR) test and the modified-relative-dose-response (MRDR) test are used for body VA status determination (22, 53).

The release, rather than synthesis, of RBP4 from the hepatocytes is dependent upon retinol availability (35). In the case of VAD when liver retinoid storage is low, apo-RBP4 accumulates in the liver and can be rapidly released in response to new VA ingestion (35). In other words, the mobilization of apo-RBP4 in the VA-deficient liver is depending on VA intake (35, 54). For the RDR test, an oral dose of RE is administered with two blood draws (baseline pre-dosing and 5 h post-dosing); in contrast, for MRDR test, a challenge dose of 3,4-didehydroretinyl acetate (DRA) or vitamin A₂ acetate is administered with
one single blood draw between 4 and 7 h post-dosing (22, 53). For both RDR and MRDR tests, the newly ingested RE and DRA doses are absorbed into the enterocytes and then incorporated into chylomicrons to be delivered to the liver. Once the esters are taken up by the hepatocytes, they can be hydrolyzed and the retinol can bind to apo-RBP4 to be released into the circulation as holo-RBP4 complex. The values from RDR and MRDR tests are useful indicators for liver retinoid reserves as well as VA-deficient status. The clinical cutoffs for body VA status using liver reserves (µmol/g) and plasma total retinol levels (µmol/L) are listed in Table 2.

Another useful biomarker is plasma RE, which can be used as a measure of VA toxicity. Liver, the main VA storage organ, stores approximately 90% of body VA (1); however, the storage capacity is not infinite. In normal circumstances, liver clears the RE within chylomicron remnants in plasma after VA consumption in a fat-containing meal (25). The liver VA storage can become overloaded with acute or chronic VA over-exposure, especially with preformed VA from meals or supplements (55). When liver is not able to clear the RE in the circulatory system, the RE remain in plasma with some converted to free unbounded retinol, which is highly toxic to cell membranes (22); thus, the RE that are not sequestered by the liver and overflow in the circulation may
potentially lead to VA toxicity (hypervitaminosis A).

Blood drawn for plasma RE measurement must be taken in the fasting state as the increase of circulating RE in the chylomicrons typically lasts about 3-5 h after dietary or supplemental intakes (22). Plasma RE determination is the most commonly used bioindicator for VA intoxication (22); however, several confounding issues require attention: (a) protein malnutrition (56); (b) liver disease (57); (c) hypertriglyceridemia (58); and (d) age (59). In the case of hypertriglyceridemia, the plasma RE levels may be high despite normal body VA status (58). In addition, the liver function of RE clearance from the circulation may be delayed among older adults (59).
Table 2. Clinical cutoffs for vitamin A status using liver reserves and plasma total retinol levels (22, 53).

<table>
<thead>
<tr>
<th>Vitamin A status</th>
<th>Liver reserves (µmol/g)</th>
<th>Plasma retinol levels (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excessive</td>
<td>&gt;1.0</td>
<td>&gt;3.00</td>
</tr>
<tr>
<td>Adequate</td>
<td>0.1-1.0</td>
<td>1.05-3.00</td>
</tr>
<tr>
<td>Marginal</td>
<td>0.07-0.1</td>
<td>0.70-1.05</td>
</tr>
<tr>
<td>Deficient</td>
<td>&lt;0.07</td>
<td>&lt;0.70</td>
</tr>
</tbody>
</table>
1.1.5.1 Vitamin A deficiency

Vitamin A deficiency (VAD) is a global public health problem. It not only causes preventable childhood blindness, but also contributes to morbidity and mortality from infections among populations at risk, especially in low- and middle-income countries (60). Infants, children, and pregnant and lactating women are considered as populations-at-risk of VAD due to their high nutritional demand. Vitamin A deficiency is caused by chronic insufficient intake of VA that fails to reach physiologic requirements for normal metabolism as well as tissue growth (60). The ‘synergistic’ effect of long-term deficiency in VA consumption and severe infections (e.g., measles) may lead to depressed appetite, depleted body VA storage, increased risks for other complications, and eventually death (60).

Vitamin A deficiency is defined based on specific clinical indicators (e.g., night blindness, conjunctival xerosis, Bitot’s spots, and corneal ulceration) and subclinical assessment (e.g., serum retinol concentration) (1). Night blindness (poor vision in darkness), conjunctival xerosis (dryness of the conjunctiva), and Bitot’s spots (white foamy lesion on the temporal side of the eye) are considered as early symptoms of VAD and can be reversed by VA supplementation (2, 61). Corneal ulceration is more severe
than xerophthalmia (dryness of the conjunctiva and cornea) and is irreversible even with VA treatments and therefore results in blindness (1, 2). According to the World Health Organization (WHO), approximately 19 million pregnant women and 190 million preschool-age children globally were affected by VAD as defined by low serum retinol levels (<0.7 µmol/L or <20 µg/dl) during 1995-2005 (60). In fact, the WHO had estimated that 122 countries (out of 193 countries) were classified as having a moderate to severe VA-deficient status in preschool-age children (<5 years of age) between 1995 and 2005 (60).

Vitamin A deficiency has been suggested to be a cause of anemia, since VA is involved in several biological mechanisms, such as immune modulation and iron metabolism (62). According to nutritional surveys, populations with VAD usually have a high prevalence of anemia (62). Many programs regarding VA supplementation have been developed with the purpose of reducing the prevalence of VAD; however, the impact of improving VA status on anemia has not received enough attention. It is hypothesized that VAD affects immune function and modulates iron metabolism, and eventually causes anemia (62). In experimental VA-deficient rats, iron could not be effectively used for erythropoiesis by bone marrow because it was accumulated in the
liver and spleen (63). da Cunha et al. (64) reported that during VA-deficient state, renal erythropoietin (stimulates erythropoiesis) expression was down-regulated in the kidney, leading to increased phagocytosis of undifferentiated erythrocytes and, subsequently, increased liver and spleen heme concentrations. In contrast, repletion of VA stimulated iron mobilization and utilization and contributed to optimum erythropoiesis (65). Although VAD is associated with anemia, the complete pathophysiology of VA-induced anemia requires further characterization.

1.1.5.2 Vitamin A toxicity

Hypervitaminosis A, also known as VA toxicity, can be induced by acute and chronic excessive consumption of preformed sources of VA (e.g., fish liver oil and fortified foods) and/or pharmaceutical supplements (55). VA toxicity is very unlikely to be induced with a high intake of provitamin A carotenoids because the provitamin A carotenoids are absorbed less efficiently, as compared to preformed VA; and, additionally, the bioconversion of provitamin A carotenoids to retinol is tightly regulated by body VA status (55). In other words, the body VA status can either positively or negatively affect the bioavailability of provitamin A carotenoids (66); for instance, the provitamin A carotenoids are cleaved to yield retinal at a higher rate among VA-deficient individuals.
In addition, Ribaya-Mercado et al. (67) reported that the bioconversion of plant carotenoids to VA is inversely associated with VA status in children aged 7-13 years.

Persons with an intake of VA greater than 150,000 µg in a single dose, or short-term large doses, may experience acute VA toxicity with symptoms including nausea, vomiting, headache, blurred vision, muscular incoordination (17). Individuals who ingested more than 30,000 µg/day for months or years were reported to experience severe adverse effects associated with chronic VA toxicity (17). The adverse effects of hypervitaminosis A are typically dose-dependent (1). Chronic VA toxicity is characterized by reduced bone mineral density, teratogenicity, and liver abnormalities (17). The loss in bone mineral density may lead to increased risk of hip fracture and development of osteoporosis, especially in pre- and postmenopausal women (17). An increased risk of birth defects (teratogenesis) appears to be associated with excessive intakes of preformed VA during the first trimester of pregnancy (17). Because liver is considered the main organ for VA storage, a causal relationship between high VA consumption and liver abnormalities (e.g., reversible enzyme elevation, and irreversible fibrosis and cirrhosis) has been observed in both human and animal studies (17).

As illustrated in the previous section (1.1.4 Vitamin A homeostasis), serum retinol
concentration is not an ideal indicator for liver retinol reserves because serum retinol levels are homeostatically controlled between 20-300 µg/g of liver retinoid reserves (48). Therefore, assessing body VA status is complicated since serum VA level only increases, in the form of RE, when the liver retinoid storage exceeds 300 µg/g (48, 68). Persons with serum total retinol level of >3.00 µmol/L are considered to be in VA-excessive status (Table 2). The signs and symptoms of acute and chronic VA toxicity have now been well evaluated and studied (69); however, the subtoxicity without clinical signs and symptoms of VA toxicity is an underappreciated health issue that requires immediate attention (55). A case of chronic hypervitaminosis A was observed with normal serum retinol concentration in which leading to underestimation of liver retinoid reserves and delayed treatment for chronic VA intoxication (70). In the U.S., the 95th percentile of VA intake, as preformed VA in fortified foods and supplements, exceeded the tolerable upper level (UL) but was lower than the no-observed-adverse-effect level (NOAEL) among children 1-3 years of age (71). In fact, approximately 75% of children exceeded the UL as contributed by VA supplementation alone (71). Since the VA intake was below NOAEL, no evidence about the adverse health outcomes with high levels of VA intake (predominately from VA supplementation) was reported (71). Future studies are warranted to fully evaluate and address the issue of VA subtoxicity.
1.2 Vitamin A and neonates

Vitamin A is not only essential for many events during embryonic development (i.e., early nervous system, spinal cord, and eye development) (13, 72), but also important for infant and neonatal growth (i.e., immune system and lung development) (73-75). Vitamin A, especially in the form of RA, is required for the patterning as well as neural differentiation of nervous system. In the case of VAD, shortened hindbrain and severe eye abnormalities were observed in quail and rat embryos (76-78). Additionally, administration of exogenous RA completely restored the destroyed lung architecture and severe loss of alveolar surface area, which was caused by dexamethasone, in mice, illustrating the role of RA in postnatal alveolar development, maintenance as well as regeneration (75). Ross and colleagues (73) found that RA combined with polyriboinosinic:polyribocytidylic acid (PIC), an inducer of interferon (IFN), were able to modulate the maturation and/or differentiation of cells from both innate and adaptive immune systems (i.e., neonatal B cells, natural killer cells, natural killer T cells, and antigen-presenting cells), suggesting a potential nutritional-immunological intervention that could target VA-deficient children, who are more susceptible to infectious diseases.
1.2.1 Vitamin A status in neonates

Rapid growing infants are in high-demand for ingestion of VA since VA is crucial for cell growth and differentiation; however, neonates usually begin their lives with low VA reserves in the liver. Olson et al. (79) reported that approximately two-thirds of healthy newborns <3 months had liver VA concentration $\leq$20 µg/g and one-fourth had liver storage of $\leq$5 µg retinol/g in America, which is an industrialized country (GDP $\geq$US$15,000) where pregnant women are assumed to be free of VAD of public health significance (60). Such a value of $\leq$20 µg/g (~0.07 µmol/g) of liver retinol level is considered as VAD in adults (as illustrated in Table 2); further validation of the value is required for the neonatal population. Neonatal rats also had low VA liver reserves (~0.06 µmol/g) on postnatal day 4 (P4) and the hepatic retinol levels decreased slowly over time if not supplemented with VA (80).

The VA status of suckling pups is positively correlated with VA content in maternal milk as well as maternal VA status (81). In a human cross-sectional study conducted in India, cord serum VA levels are positively related with newborn birth weight as well as gestational age and maturity (82). Agarwal et al. (82) further reported that low neonatal VA status is associated with prematurity and intrauterine growth
retardation. In fact, the low VA status in premature (very-low-birth-weight) infants leads to higher risk of experiencing bronchopulmonary dysplasia (BPD), damage in respiratory system epithelial cells, and chronic lung disease, as compared to term infants (83, 84). Together, VA status is generally low in newborns, especially lower in preterm infants; therefore, VA supplementation in neonates is critical for the prevention of VAD-related morbidity and mortality.

1.2.2 Vitamin A supplementation and reduced risk in neonates and children

The efficacy of VA supplementation among children (>6 months and <59 months old) has been examined in a substantial number of studies in lower income countries (e.g., India, Ghana, Nepal, and Bangladesh). Overall, supplementation of VA among children at risk for VAD (aged 6-59 months) is associated with reduced all-cause mortality, diarrhea specific mortality, morbidity and mortality in measles, and reduction in clinic attendances and hospital admissions in developing countries (85-89). However, studies showed inconsistent results regarding the effects of VA supplementation on survival among newborns 0-6 months of age (90-98). Humphrey et al. (92) reported that an oral dose of VA (50,000 IU as retinyl palmitate), administered to newborns on the first day of life, reduced infant mortality in Indonesia. In contrast, Benn et al. (96-98) doubted the
beneficial contribution of VA supplementation for newborn infants to improve survival. In fact, Benn et al. (97, 98) observed that infant girls (but not boys) that received VA supplementation at birth (within 72 hours) had a higher mortality rate in the first year of life, indicating that VA supplementation may cause adverse effects in girls.

Between 2010 and 2013, the WHO coordinated three large randomized, double-blinded, placebo-control trials in India, Ghana, and Tanzania (Neovita trials) to examine the effects of early neonatal VA supplementation on infant mortality; the findings were inconclusive (93-95). The trial conducted in India suggested that VA supplementation (50,000 IU as retinyl palmitate), administered within the first three days of life, was able to provide modest beneficial effects and reduced risk of death (95). However, the other two trials conducted in Ghana and Tanzania did not support the strategy of newborn VA supplementation to increase children survival rates, as neither adverse results nor beneficial effects were observed (93, 94). Populations-at-risk for VAD may respond better to VA supplementation, as compared to those with VA-adequate status; in other words, the effectiveness of VA supplementation may be population dependent (22).
Based on results of population-based studies, the WHO currently recommends 100,000 IU (30 mg RE) once and 200,000 IU (60 mg RE) every 4-6 months for infants 6-11 months of age and children 12-59 months of age, respectively (99). Since infants are generally born with low body VA storage (79) and possibly weak resistance to infections, the purpose of neonatal VA supplementation is to reduce morbidity and mortality and improve survival rate for infants and children in VA-deficient areas (100). However, currently there is no recommendation for VA supplementation targeting at infants 1-5 months of age due to inconclusive study results noted above (101). Further research is warranted to determine the beneficial impact of early neonatal VA supplementation.

1.3 Fetal origins of adult disease

David Barker, who was one of the most influential epidemiologists, first popularized the concept of “fetal origins of adult disease (FOAD)” (also called the Barker hypothesis) in 1995 (102). The FOAD concept suggests that fetal programming may generate permanent changes on one’s health status or risk of disease development throughout the lifespan (102, 103). In other words, events during critical periods when an organism is sensitive to its environment may have a profound impact on its health outcomes later in life. The programming process may extend beyond the in utero period
to early childhood developmental stage (i.e., perinatal period) (103). For instance, babies with disproportionate growth (thin and short) *in utero*, at birth, and during infancy, had a higher risk of developing coronary heart disease and related disorders in later ages (102). Nowadays, the concept of FOAD has been well accepted and extensively studied in both animal and human studies. The FOAD hypothesis provides new insights into the possible pathogenesis of chronic disease as well as potential preventative treatments during critical periods of disease development. The Barker hypothesis has been considered as a new developmental model of chronic disease that benefits not only the policy makers but also healthcare professionals.

1.4 Childhood obesity

Obesity is defined as excessive fat accumulation, which is caused by energy imbalance and may eventually affect health status. According to the WHO, the global prevalence of obesity has tripled since 1975, and more than 650 million adults worldwide were considered obese in 2016. Obesity not only is related to many preventable immediate and long-term health consequences, but also causes a significant economic burden on the health care system in the U.S. In addition, childhood obesity has been recognized as a growing medical concern since the 1990s. The WHO estimated that the
number of overweight or obese infants and young children (<5 years old) had increased from 32 million in 1990 to 41 million in 2016 and possibly to 70 million by 2025 if the current trends continue. Among the 9 million overweight or obese infants and young children increased between 1990 and 2016, 5 million children were from the WHO African Region, indicating a large number of overweight or obese children live in lower income countries. A substantial number of epidemiological studies have shown that childhood obesity (<5 years old) is significantly associated with adult overweight or obesity (104). Childhood obesity is also linked to a variety of adverse health complications; for example, type 2 diabetes, metabolic syndrome, and hypertension (105); therefore, early childhood intervention should be promoted to reduce the risk of adult obesity as well as disease development.

1.5 Metabolic regulation

Insulin resistance, one of the obesity-initiated metabolic syndromes, is a pathological condition in which insulin is not properly utilized by cells, leading to high blood sugar as well as high blood insulin level (106). Insulin, a hormone secreted by pancreatic β-cells, functions to stimulate glucose uptake into cells and maintain the normal range of blood glucose after carbohydrate consumption. Insulin resistance is
closely related to glucose intolerance and a key risk factor for the development of type 2 diabetes (107).

Traditionally, adipose tissues have been classified into two subgroups, white adipose tissue (WAT), and brown adipose tissue (BAT) (108). WAT functions to store energy as triglycerides in lipid droplets, whereas BAT is specialized for non-shivering thermoregulation through the actions of uncoupling protein (UCP) (108). Upon activation, UCP dissipates energy stored in protons generated by the respiratory chain as heat instead of in the production of ATP (109). In the last two decades, the manipulation of the thermogenic characteristics in BAT has been highly suggested as a potential strategy to combat obesity as well as related metabolic syndromes (108-110).

1.5.1 Insulin sensitivity

Serum RBP4 plays a significant role in VA metabolism because it is responsible for transporting retinol as the holo-RBP4 complex in the circulation (1-3). RBP4 is synthesized primarily in liver and adipocytes, with a small portion synthesized elsewhere, for example, kidney and retinal pigment epithelium (111). Serum RBP4, which belongs to the lipocalin superfamily, is a fat-derived adipokine and a biomarker for insulin sensitivity (111). Kahn and colleagues (112) showed that the serum RBP4 levels were
increased in insulin-resistant adipose-specific *Glut4*−/− mice; conversely, treatment with rosiglitazone (an anti-diabetic agent) to the insulin-resistant adipose-specific *Glut4*−/− mice reduced their elevated *Rbp4* mRNA expression in adipose tissue and normalized elevated serum RBP4 levels. Transgenic mice that overexpressed human RBP4 were insulin resistant; in contrast, *Rbp4*−/− mice exhibited enhanced insulin sensitivity, as compared to wild type (WT) mice (112). Kahn and colleagues (113, 114) also reported that RBP4-overexpressing mice were insulin resistant, which was triggered by activation of antigen-presenting cells and infiltration of macrophages and CD4 T-cells in adipose tissue. When comparing to high-fat diet (HFD)-fed WT mice, HFD-fed *Rbp4*−/− mice had reduced adipose tissue inflammation and improved blood glucose and insulin levels, indicating that deletion of RBP4 improves insulin sensitivity (113, 114). Together, RBP4 is involved in the development of insulin resistance.

Several population-based studies have found that serum RBP4 levels are positively associated with body-mass index, impaired glucose tolerance, and type 2 diabetes (115, 116). Additionally, Graham *et al.* (115) reported that serum RBP4 levels are found to be inversely correlated with adipocyte insulin-regulated glucose transporter 4 (GLUT4) protein. Transgenic mice overexpressing human RBP4 in adipose tissue had hepatic
steatosis, which is the precursor for the non-alcoholic fatty liver disease (NAFLD) (117). In fact, numerous studies have provided evidence that elevated serum RBP4 concentrations were more often observed in patients with NAFLD than those in non-NAFLD controls (118, 119). Childhood obesity is the major risk factor for NAFLD, which is the most common form of liver disease during childhood (120). The prevalence of NAFLD has doubled in the past two decades, which follows the trend of increased childhood obesity prevalence (120).

Palou and colleagues (121) showed that RA treatment down-regulates Rbp4 mRNA expression in WAT adipocytes of NMRI mice and in cultured adipocytes (3T3-L1 mature adipocytes and mouse embryonic fibroblasts-derived adipocytes), indicating that VA may regulate adipokine production and eventually affect insulin sensitivity. Previously our laboratory has reported that early postnatal VARA treatments significantly decreased liver Rbp4 transcripts in neonatal rats, as compared to in oil-treated (placebo) pups (122). We further demonstrated that the liver Rbp4 mRNA expression was also significantly decreased in adult rats that received VARA supplementation as neonates, but not as adults (122), suggesting that early postnatal VARA supplementation has a long-term impact (carryover effect) on liver Rbp4 transcripts.
1.5.2 Thermogenesis

Two types of thermogenesis, shivering and non-shivering thermogenesis, are heat production processes in organisms. Shivering thermogenesis occurs to raise body temperature when exposed to cold environments, whereas non-shivering thermogenesis is stimulated either by cold or diet and activated by the sympathetic nervous supply of the tissue. BAT-specific non-shivering thermogenesis is mediated by uncoupling protein 1 (UCP1), which is an inner mitochondrial membrane protein that uncouples the proton gradient from the electron transport chain and thereby dissipates energy as heat (123). There are five UCP homologues expressed in mammals, UCP1-UCP5 (124). UCP1 is found exclusive to BAT, UCP2 is distributed in many tissues and cell types, and UCP3 is mainly expressed in BAT and skeletal muscle (124).

Research by Palou and colleagues (125-127) reported that treatment of RA triggers a dose-dependent increase in expression of genes involved in fatty-acid oxidation and thermogenesis (UCP2 in liver and UCP3 in muscle), leading to decreased liver and muscle lipid content in mice. The effects of RA treatment on increased oxidative metabolism and Ucp1 expression in brown adipocytes were not only observed *in vivo* but also *in vitro* (123, 128-130). Studies in cultured brown and white adipocytes suggested
that RA acts to induce *Ucp1* gene expression in a p38 mitogen-activated protein kinase (p38MAPK)-dependent fashion (130, 131). Furthermore, RA treatment significantly up-regulated mRNA levels of BAT-specific *Ucp1* and genes involved in lipid oxidation in WAT, suggesting that RA treatment favors the remodeling of WAT into BAT-like properties in mice (132). The effects of RA treatment on reducing body weight, decreasing adiposity, and elevating *Ucp1* expression in BAT were observed independently of diets and VA status in mice (133).

Previously in our laboratory, we reported that neonatal VA supplementation transiently increased retinol uptake (~400%) into BAT around 4 hours after oral dosing and the retinol concentrations remained elevated for several days in a kinetic study (80). We also illustrated that VARA treatments have a more potent effect on retinol uptake into extrahepatic tissues in neonatal rats, as compared to VA dose alone (134). Together, the RA treatment effects on inducing tissue-specific thermogenesis through UCP activation have been suggested as a potential method for the prevention of obesity and type 2 diabetes.
1.6 Sex differences

The variable “sex” has been long neglected before women were mandated for inclusion in National Institutes of Health (NIH)-funded biomedical studies in 1993 (135); however, the differences between sexes have often failed to be addressed (136). In fact, 75% of articles in journals with high impact factors did not indicate sexes of animals used (137). In addition, males still dominated rat and mouse studies in 2010, as researchers tend to avoid using female animals due to hormone concerns. To take full advantage from research, NIH has implemented a new policy to consider on sex as a biological variable in cell studies, animal models as well as human biomedical research (135).

In studies using C57BL/6 mice as a model of HFD-induced obesity, male mice are known to develop greater weight gain than female mice, with no sex differences observed in energy intake (138, 139). Additionally, the obese male mice are more vulnerable to metabolic alterations, including hyperglycemia, hyperinsulinemia, hypercholesterolemia, and hyperleptinemia, as compared to HFD-induced obese female mice (139). Palou and colleagues (140) reported that obese female rats exhibit higher serum ghrelin levels and gastric leptin mRNA expression than obese male rats, suggesting sex-associated differences in the regulation of food intake control system and energy balance under HFD
exposure. In sum, studies using obese mice and rats showed that sex factor is involved in the alterations of weight-control as well as hormone-control systems. In order to understand better about the treatment effects using HFD-induced obese models, it is important to interpret results in a sex-specific manner.
CHAPTER 2

RATIONALE, HYPOTHESIS, AND SPECIFIC AIMS

2.1 Rationale of the study

To date, although the FOAD concept has been well accepted and replicated in various human and animal studies, research is limited which have investigated the effects of early life micronutrient supplementation on risk of disease development later in life. A substantial number of studies have been focused on the association between breast versus formula feeding and growth patterns as well as later health outcomes in adolescence and adulthood. Here, we determined whether VA or VARA supplementation, administered in the early postnatal period, alters metabolic functions related to obesity risk in neonatal pups and young adolescent-age rats. To be more specific, we examined the changes in expression of genes that are associated with insulin sensitivity and thermogenesis, with attention to sex differences. In addition, we measured body composition, including body fat and lean body mass, to determine whether early-life VA or VARA supplementation is associated with decreased adiposity later at prepubescent age.

Neonatal VA supplementation has become a significant public health focus, yet little is known regarding the potential beneficial or adverse effects of VA
supplementation on central metabolism among the neonatal population. Numerous interventions have been developed to test the efficacy of early-life VA supplementation to reduce infant morbidity and mortality in areas of the world where a high prevalence of VAD occurs; currently, no firm conclusions have been reached regarding neonatal VA supplementation as an effective survival strategy (101).

All-trans-RA, a carboxylic form of VA, is not only the regulatory metabolite of VA, but also a ligand for nuclear transcription factors. Previously in our laboratory, we have demonstrated that VARA treatment synergistically increases the retinol uptake and RE formation in the lung of neonatal as well as young adult rats (141-144). We also reported that neonatal VA supplementation effectively and rapidly increases retinol concentrations in extrahepatic organs among neonatal rats (80); with a more potent effect observed with early-life VARA treatment compared to VA supplementation alone (134). Moreover, a number of strong in vitro and in vivo evidences suggest that all-trans-RA treatment not only enhances lipid oxidation in muscle and liver, favors remodeling of WAT, but also significantly affects metabolic processes related to insulin sensitivity and thermogenesis in muscle, liver, and adipocytes (121-123, 125-133). In particular, RA treatment is
associated with induced tissue-specific UCP expression and reduced RBP4 production; thus, RA treatment potentially leads to decreased adiposity (121-123, 125-133).

However, most in vivo studies were conducted using VA-sufficient mice; in the current study, we created a rat model with VA-marginal status to determine how VA status may impact treatment effects. Also, we utilized both VA and VARA (a retinoid combination of VA and 10% RA) treatments, to compare side-by-side, for the evaluation of metabolic profile related to VA metabolism; in contrast, most in vivo and in vitro studies, to our knowledge, used only the RA treatment to assess retinoid homeostatic mechanism. Previously in our laboratory, we observed that WAT was nearly absent at birth until P12 and the weight of WAT (% relative to body weight) did not significantly increase from P12 to P28 in neonatal rats fed a VAM diet (contains 15.8 kcal% fat) (80); here in the proposed study, we investigated whether the weight of WAT (%) remains constant throughout the course of experimental period in animals fed a HFD (contains 45 kcal% fat). In addition, previously we reported that BAT was present at birth and, despite continuous tissue growth, the weight of BAT (%) decreased steadily throughout the neonatal period in non-obese neonatal rats (80). To capture the early presence of BAT and to investigate the changes of tissue retinoid metabolism overtime, we used neonatal
pups (P12) and prepubescent-aged rats (5 weeks old) in both sexes; in contrast, most in vivo studies were conducted in adult male animals.

The purpose of the study was to understand the association between early-life VA and VARA supplementation and risk of childhood obesity for the promotion of a healthier metabolic profile later in life. The study is innovative in investigating the potential influence of early postnatal VA and VARA supplementation on energy metabolism in two different life stages (neonatal period and young adolescent period), using an obese animal model with marginal status of VA.

2.2 Hypothesis

We hypothesized that early postnatal VA and VARA supplementation (given at a dose resembling that used in public health programs) in rats alters the expression of genes related to insulin sensitivity (Rbp4 in liver, kidney, and adipose tissues) and thermogenesis (Ucp1 in BAT and Ucp3 in skeletal muscle) in the neonatal period, with carryover effects to prepubescent age. Also, early postnatal VA and VARA supplementation is associated with decreased adiposity in young adolescent rats. Based on these hypotheses, our three specific aims are listed as follows.
2.3 Specific aims

**Aim 1.** To understand whether early-life VA or VARA supplementation reduces *Rbp4* gene expression in liver, kidney, and adipose tissues, in neonatal pups and/or young adolescent rats.

The early postnatal VA and VARA supplementation treatments were administered orally three times, on P0/1 (less than 24 h after birth), P4, and P10, with canola oil used as placebo/control. Serum and tissues were collected at P12 (neonatal period) and 5 weeks of age (young adolescence). No further treatment was administered after neonatal age; therefore effects at young adolescent age (5 weeks old) would represent a long-term “carryover” effect, consistent with the central concept of the FOAD hypothesis. In order to create an HFD-induced obese rat model, a purified high-fat vitamin A-marginal (HFVAM) diet was fed to dams from pregnancy until the pups were euthanized at the end of study; those pups that were weaned were weaned onto the same diet. Additionally, to understand better about the effects of early-life VA supplementation, we correlated tissue retinoid concentrations with *Rbp4* steady-state mRNA expression in the liver, kidney, and adipose tissues (BAT and WAT). We also examined sex differences as well as treatment x sex interaction.
**Aim 2.** To determine whether early-life VA or VARA supplementation increases *Ucp1* and *Ucp3* gene expression in BAT and skeletal muscle, respectively, in neonatal pups and/or young adolescent rats.

The correlation between tissue retinoid concentrations and tissue-specific *Ucp* (*Ucp1* in BAT and *Ucp3* in skeletal muscle) steady-state mRNA expression were examined. We also examined differences between sexes as well as treatment x sex interaction.

**Aim 3.** To investigate whether early-life VA or VARA supplementation is associated with altered body composition (i.e., decreased adiposity) in young adolescent rats.

The body composition of young adolescent rats was measured by dual-energy x-ray absorptiometry (DEXA) technology. A group of age- and sex-matched normal chow-fed non-obese young adolescent rats that received no treatments was included as a reference. Data were analyzed with attention to sex differences as well as treatment x sex interaction.
CHAPTER 3

EXPERIMENTAL DESIGN AND METHODS

3.1 Experimental design

To understand better about the concept of FOAD, we designed the study to investigate whether early postnatal VA or VARA supplementation alters metabolic functions related to obesity risk in the neonatal period as well as prepubescent age, using a rat model. The experimental design is shown in Figure 1. Briefly, a defined HFVAM diet, which contains 45 kcal% fat and a marginal level of VA (0.35 µg of retinol as retinyl palmitate per gram diet), was fed to Sprague Dawley dams from the beginning of pregnancy until pups were euthanized at the end of study. To avoid litter effects, pups were randomly assigned to receive three oral doses of either placebo, VA, or VARA treatments, which were adjusted based on body weight, on P0/1 (<24 h after birth), P4, and P10. No further VA or VARA treatment was administered after P10. On P21, pups were weaned onto HFVAM diet.

Serum and tissue samples were collected at either neonatal period (P12; n=57) or prepubescent age (5 weeks of age; n=49). A group of age- and sex-matched normal chow-fed non-obese young adolescent (5 weeks of age) rats that received no treatment
was included in the study as a reference for the purpose of body composition comparison.

The dose administration and animal euthanasia schedule is shown in Table 3. The sample size of the treatment groups is presented in Table 4.
**Figure 1. Experimental design of study.** The placebo, VA, or VARA treatments were administered orally on P0/1, P4, and P10 to pups of dams fed a HFVAM diet. The pups were then euthanized at either P12 (neonatal period; \( n=57 \)) or 5 weeks of age (young adolescence; \( n=49 \)), still fed the HFVAM diet. A group of age- and sex-matched normal chow-fed non-obese young adolescent rats that received no treatment was included in the study as a reference.
Table 3. Dose administration and animal euthanasia schedule. The HFVAM diet-fed pups received three oral doses of placebo, VA, or VARA treatments on P0/1, P4, and P10. The HFVAM diet-fed pups were euthanized at either neonatal period (P12) or young adolescence (5 weeks old). A group of normal chow-fed non-obese young adolescent rats was included for age- and sex-matched reference.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Treatments</th>
<th>Dose administration</th>
<th>Animal Euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P0/1</td>
<td>P4</td>
</tr>
<tr>
<td>HFVAM¹</td>
<td>Placebo²</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>HFVAM</td>
<td>VA³</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>HFVAM</td>
<td>VARA⁴</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>HFVAM</td>
<td>Placebo</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>HFVAM</td>
<td>VA</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>HFVAM</td>
<td>VARA</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Normal Chow⁵</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹HFVAM: high-fat (45 kcal% fat) VA-marginal (0.35 µg retinol as retinyl palmitate/g diet) diet
²Placebo: canola oil was used as placebo for the purpose of control treatment
³VA: the mass of the VA dose was ~6mg retinol/kg of body weight
⁴VARA: a retinoid combination of VA and 10% RA
⁵Normal chow diet group: age- and sex-matched normal chow-fed non-obese young adolescent rats as references
Table 4. Sample size of the treatment groups. The study design included two different life stages (neonatal period and young adolescence) and three different treatments (placebo, VA, or VARA), with $n=5$-11 animals per age per sex per treatment group. The total sample size was $n=106$. A group of age- and sex-matched normal chow-fed non-obese young adolescent rats was included as a reference.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Treatment</th>
<th>Age (total $n=106$)</th>
<th>Neonate (P12; $n=57$)</th>
<th>Young adolescent (5 weeks; $n=49$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFVAM</td>
<td>Placebo</td>
<td>$n=18$ (8M, 10F)</td>
<td>$n=13$ (7M, 6F)</td>
<td></td>
</tr>
<tr>
<td>HFVAM</td>
<td>VA</td>
<td>$n=19$ (9M, 10F)</td>
<td>$n=12$ (6M, 6F)</td>
<td></td>
</tr>
<tr>
<td>HFVAM</td>
<td>VARA</td>
<td>$n=20$ (9M, 11F)</td>
<td>$n=12$ (5M, 7F)</td>
<td></td>
</tr>
<tr>
<td>Normal Chow</td>
<td>N/A</td>
<td>N/A</td>
<td>$n=12$ (5M, 7F)</td>
<td></td>
</tr>
</tbody>
</table>
3.2 Experimental methods

3.2.1 Ethical statement

The Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University approved the animal protocols and surgical procedures (IACUC #42706). According to the National Research Council’s Guide for the Care and Use of Laboratory Animals and the United States Animal Welfare Act, the temperature was maintained between 68-79°F with humidity controlled between 30-70% in the animal facility. Animals received daily care (365 days a year) by professional animal care technicians from the Animal Resource Program and were exposed to a 12-h light/dark cycle with ad libitum access to food and water. Animals were allowed to acclimate for at least seven days upon arrival prior to the start of experimental work. After arrival, animals were housed in an isolated rack with specific pathogen-free conditions in which the plastic cages are ventilated with sterilized air.

3.2.2 Animal model

Sprague Dawley (SD) rats, the animal model used in the study, were purchased from the Charles River Laboratories International, Inc. (Wilmington, MA). The strain was originated by Robert W. Dawley in 1925 and was transferred to Charles River
Laboratories in 1950 from Sprague Dawley, Inc. SD rats are an outbred general multipurpose model that is commonly used for diet-induced obesity and oncology, nutrition, and aging research. The SD rats were approximately 14 weeks of age at arrival. Male and female adult rats were co-housed for 12 days for mating process. Post-weaning pups were separated by sex and housed in groups of two to four. To ensure statistical power and to achieve a minimum of $n=5$ rats per age per sex per treatment group (total $n=106$), we completed five rounds of animal breeding; all three treatment groups (placebo, VA, and VARA) were included in each round of breeding.

### 3.2.3 Diets

Beginning from the breeding process until the end of the study when pups were euthanized at 5 weeks of age, animals were fed a customized HFVAM diet (Research Diets, Inc.; New Brunswick, NJ), modified from AIN-93G growing rodent diet formula (145) to contain high-fat (45 kcal% fat) and a marginal level of dietary VA (0.35 µg retinol as retinyl palmitate/gram diet) (146). The formula of the purified HFVAM diet used in the study (product #D16120606) is shown in Table 5. The detailed lists of ingredients in the mineral mix (S10022C) and vitamin A-free vitamin mix (V13002) are listed in Tables 6 and 7, respectively.
Previously, our laboratory showed that rats fed a defined VA-marginal diet were able to develop marginal status of VA, as confirmed by minimal liver retinoid storage, low plasma retinol concentrations, and a plasma total retinol:RBP4 molar ratio of <1, in young, middle-aged, and old rats (147). The marginal level of VA (0.35 µg retinol as retinyl palmitate/gram diet) contained in the diet was chosen to resemble the intake of VA among VA deficient populations. Such diets are seldom ever totally lacking in VA, and so a marginal level was selected instead of a VA-deficient diet.

The level of fat (45 kcal%) in the high-fat diet was chosen to resemble a typical “American” dietary pattern (148). A typical American diet consists of 35 kcal% fat, 50 kcal% carbohydrate, and 15 kcal% protein with the assumption of 2200 kcal of daily intake (148). Currently, two types of standard HFD, which consist of 45 kcal% fat (with 35 kcal% carbohydrate and 20 kcal% protein) and 60 kcal% fat (with 20 kcal% carbohydrate and 20 kcal% protein), are commonly used for research in diet-induced obese animal models. To mimic the typical American dietary pattern, we chose the former HFD formula (45 kcal% fat, 35 kcal% carbohydrate, and 20 kcal% protein).
Table 5. **Formula of the purified HFVAM diet.** The detailed composition of purified HFVAM diet used in the study. The HFVAM diet was modified based on AIN-93G growing rodent diet formula (145), to contain high-fat (45 kcal% fat) and a marginal level of dietary VA (0.35 µg retinol as retinyl palmitate/gram diet) (146).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm/kg</th>
<th>kcal/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>24.2</td>
<td>20.3</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>41.4</td>
<td>34.7</td>
</tr>
<tr>
<td>Fat</td>
<td>23.9</td>
<td>45.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>kcal/gm</td>
<td></td>
<td>4.78</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm/kg</th>
<th>kcal/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, lactic</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Corn starch</td>
<td>105</td>
<td>420</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>132</td>
<td>528</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70</td>
<td>630</td>
</tr>
<tr>
<td>Lard</td>
<td>130</td>
<td>1170</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.014</td>
<td>0</td>
</tr>
<tr>
<td>Mineral mix (S10022C)</td>
<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>19.5727</td>
<td>0</td>
</tr>
<tr>
<td>Potassium phosphate, monobasic</td>
<td>6.86</td>
<td>0</td>
</tr>
<tr>
<td>Potassium citrate, 1 H2O</td>
<td>2.4773</td>
<td>0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>2.59</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin A-free vitamin mix (V13002)</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Retinyl acetate, 500,000 USP/g</td>
<td>0.00275</td>
<td>0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>837.57</td>
<td>4000</td>
</tr>
</tbody>
</table>
Table 6. Detailed list of ingredients used in the mineral mix (S10022C) in the HFVAM formula. The ingredients of mineral mix used in HFVAM diet are identical to the AIN-93G mineral mix, as report by Reeves (145).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm</th>
<th>Amount in 3.5 gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium oxide (60.3% Mg)</td>
<td>24</td>
<td>Mg-0.5 gm</td>
</tr>
<tr>
<td>Potassium sulfate (44.9% K, 18.4% S)</td>
<td>46.6</td>
<td>K-0.73 gm</td>
</tr>
<tr>
<td>Potassium iodate (59.3% I)</td>
<td>0.01</td>
<td>I-0.2 mg</td>
</tr>
<tr>
<td>Ferric citrate (21.2% Fe)</td>
<td>6.06</td>
<td>Fe-45 mg</td>
</tr>
<tr>
<td>Chromium potassium sulfate, 12 H₂O (10.4% Cr)</td>
<td>0.275</td>
<td></td>
</tr>
<tr>
<td>Cupric carbonate (57.5% Cu)</td>
<td>0.3</td>
<td>Cu-6.0 mg</td>
</tr>
<tr>
<td>Manganous carbonate (47.8% Mn)</td>
<td>0.63</td>
<td>Mn-10.5 mg</td>
</tr>
<tr>
<td>Sodium selenate (41.8% Se)</td>
<td>0.01025</td>
<td>Se-0.2 mg</td>
</tr>
<tr>
<td>Zinc carbonate (52.1% Zn)</td>
<td>1.65</td>
<td>Zn-30 mg</td>
</tr>
<tr>
<td>Sodium fluoride (45.2% Fl)</td>
<td>0.0635</td>
<td></td>
</tr>
<tr>
<td>Sodium silicate, 9 H₂O</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>0.0174</td>
<td></td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.0815</td>
<td></td>
</tr>
<tr>
<td>Nickel carbonate</td>
<td>0.0318</td>
<td></td>
</tr>
<tr>
<td>Ammonium molybdate, 4 H₂O (54.3% Mo)</td>
<td>0.00795</td>
<td></td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>0.0795</td>
<td></td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>0.0174</td>
<td></td>
</tr>
<tr>
<td>Ammonium vanadate</td>
<td>0.0066</td>
<td></td>
</tr>
<tr>
<td>Ammonium molybdate (54.3% Mo)</td>
<td>0.00795</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>18.806</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
Table 7. Detailed list of ingredients used in the vitamin A-free vitamin mix (V13002) in the HFVAM formula. The ingredients of vitamin A-free vitamin mix used in HFVAM diet are identical to the AIN-93G vitamin mix, as report by Reeves (145), without added vitamin A (146).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm</th>
<th>Amount in 10 gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A palmitate, 500,000 IU/gm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin D₃, 100,000 IU/gm</td>
<td>1.0</td>
<td>1000 IU</td>
</tr>
<tr>
<td>Vitamin E acetate, 500 IU/gm</td>
<td>15.0</td>
<td>75 IU</td>
</tr>
<tr>
<td>Vitamin K as phylloquinone</td>
<td>0.075</td>
<td>0.75 mg</td>
</tr>
<tr>
<td>Biotin, 1.0%</td>
<td>2.0</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Cyancocobalamin, 0.1%</td>
<td>2.5</td>
<td>25 µg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.2</td>
<td>2 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>3.0</td>
<td>30 mg</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>1.6</td>
<td>16 mg</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>0.7</td>
<td>7 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.6</td>
<td>6 mg</td>
</tr>
<tr>
<td>Thiamin HCl</td>
<td>0.6</td>
<td>6 mg</td>
</tr>
<tr>
<td>Sucrose</td>
<td>972.725</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td></td>
</tr>
</tbody>
</table>
3.2.4 Dose preparation and administration

Since there is no current recommendation for VA supplementation in infants 1-5 months of age from the WHO (101), the VA dosage given to animals in this study was selected to resemble, per body weight, the amount of VA (50,000 International Units (IU) per 2.5 kg of infant body weight) previously given to human newborns to reduce the risk of infant mortality and morbidity, as shown in human clinical interventions (92, 95, 149). Two forms of VA, all-trans retinyl palmitate (RP) and all-trans RA (Sigma-Aldrich, Co., St. Louis, MO), were used for VA and VARA dose preparations. Pure canola oil (Crisco, Cincinnati, OH) was used as placebo and as the vehicle for VA and VARA doses. Treatments were freshly prepared and stored at -20°C in foil-wrapped vials to protect VA from photodegradation. The VA and VARA doses were prepared and administered as described previously (80, 141, 150).

The mass of VA dose was ~6 mg retinol/kg (or ~10.96 mg RP/kg) of body weight, using the conversion factor of 0.3 µg retinol/IU (or 0.56 µg RP/IU). The VA dosage was calculated to be ~210 nmol of retinol for a 10-g rat pup, with the retinol molecular weight of 286.44 g/mole applied. The final concentration of the prepared VA dose was 0.05 mmol/mL; thus, each animal received 0.4 µL VA dose/g of rat body weight, with an
additional 1 μL VA dose added as allowance for retention of the dose in the pipette tip. The VARA treatment is a mixture of the VA and RA at a molar ratio of 10:1; thus, the mass of VARA dose was 6 mg retinol/kg of body weight plus 0.6 mg RA/kg of body weight (80, 141, 150).

Pups were weighed immediately before each treatment for the purpose of dose adjustment based on body weight. All treatments were administered orally by using a MICROMAN® pipette with capillary pistons (Gilson, Inc., Middleton, WI), which is ideal for the delivery of viscous liquid (e.g., oil) into mouth of the pups.

### 3.2.5 Euthanasia and sample collection

Animals were weighed every 2-3 days until the end of study. Animals were euthanized at either neonatal age (P12) or young adolescent age (5 weeks), using carbon dioxide. Blood was withdrawn from the abdominal vena cava into heparinized syringes and stored temporarily on ice to be centrifuged for serum preparation. Tissues, including liver, lung, kidney, skeletal muscle, subscapular BAT, and inguinal WAT, were dissected and frozen immediately in liquid nitrogen to be stored at -80°C for later analysis. Animal sexes, weights, and tissue weights were recorded.
Olson et al. (151) reported that the coefficient of variation in retinoid analysis was lower when sampling from a specific lobe in human liver autopsy, as compared to random sampling from the whole liver. The main reason for the uneven distribution of VA gradients is because HSC (VA-storing cells) are irregularly located across liver lobes (151). The regional differences in liver VA storage were also found in other species, such as rat and mouse (151-153). A consistent plan was followed to minimize differences between regions of the liver. The schematic of liver portions used for retinoid analysis and gene expression analysis are shown in Figure 2.
Figure 2. **Schematic of liver portions used for different analyses.** The left median lobe, the right median lobe, and the right lateral lobe were used for retinoid analysis by UPLC. The left lateral lobe and the caudate lobe were used for gene expression analysis by reverse transcription polymerase chain reaction (RT-PCR).
3.2.6 Tissue analysis

Blood glucose was measured by a portable Contour® glucometer (Bayer Corporation, Whippany, NJ). For prepubertal rats (5 weeks old), the blood and liver were collected prior to body composition measurement to avoid blood clot and DNA degradation during DEXA scan. The body composition was measured using a Lunar PIXIImus Densitometer (GE Medical Systems, Inc., Waukesha, WI), which utilizes DEXA technology to estimate bone mineral content (BMC), bone mineral density (BMD), and the percentages of body fat and lean tissues.

Retinoid concentrations in the serum, liver, lung, kidney, muscle, BAT, and WAT were analyzed by ultra-performance liquid chromatography (UPLC) (Acquity UPLC system, Waters Corporation, Milford, MA) after saponification to convert any RE to retinol. Rbp4 mRNA transcript levels in liver, kidney, and adipose tissues (WAT and BAT) were determined by reverse transcription polymerase chain reaction (RT-PCR) using an Applied Biosystems® StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) after RNA isolation and reverse transcription. Ucp1 and Ucp3 mRNA transcripts levels in BAT and muscle were also measured by RT-PCR. The detailed steps of retinoid analysis and gene expression analysis are illustrated as follows.
3.2.6.1 Serum and tissue retinoid analysis

Total retinol concentrations in the serum and tissue samples were quantified by UPLC after homogenization, saponification, and lipid extraction. Briefly, portions of tissue samples (~0.027-0.190 g) were placed in pure ethanol (200 proof; Decon Laboratories, Inc., King of Prussia, PA), with three to six 2.8 mm ceramic beads (Omni International, Inc., Kennesaw, GA), to be homogenized using a Bead Ruptor 12 Homogenizer (Omni International, Inc., Kennesaw, GA). Serum aliquots (~30-100 µL) and tissue homogenates were then incubated in 2 mL of pure ethanol for at least 1 h after tissue homogenization. Next, for the purpose of complete hydrolysis of esterified lipids, 5% or 20% (only for WAT) (80) of potassium hydroxide (Sigma-Aldrich, Co., St. Louis, MO) and 1% of pyrogallol (Sigma-Aldrich, Co., St. Louis, MO) were added to each sample. The samples then went through saponification at 55°C in a hot water bath for 30 minutes. Lipids were extracted with 4 mL of hexanes (HPLC grade; Avantor Performance Materials, Inc., Center Valley, PA) containing 0.1% of butylated hydroxytoluene (BHT; Sigma-Aldrich, Co., St. Louis, MO) as antioxidant, and 2 mL of deionized water after saponification, to separate the organic and aqueous phases.
After centrifugation at 1600 rpm for 15 minutes, the upper organic phase of total lipid extract of each sample was transferred to a new 7 mL scintillation vial (VWR International, LLC., Radnor, PA) and a known amount of internal standard, trimethylmethoxyphenyl-retinol (TMMP-retinol; provided by M. Klaus, Hoffmann-La Roche, Basel, Switzerland), was added to the solvent. The lipid extraction with hexanes/BHT for WAT (lipid-rich tissue) was repeated for 3 times to achieve 95% extraction efficiency (80). The combined extracts for each sample were then evaporated under nitrogen in a 37°C water bath and each was immediately reconstituted in 100 µL of methanol (HPLC grade; EMD Millipore Corporation, Billerica, MA) for UPLC analysis. The retinoid analytical procedure was performed in the dark or under ultraviolet (UV) protection for the prevention of VA from photodegradation.

Approximately 7-10 µl of the reconstituted samples were injected onto a C-18 reversed-phase Acquity UPLC column (Waters Corporation, Milford, MA) and eluted isocratically with 92.5% methanol and 7.5% water at a flow rate of 0.6 mL/min for 1.2 minutes. The eluate was monitored with an Acquity UPLC photodiode array (PDA) detector (Waters Corporation, Milford, MA), and absorbance at 325 nm was extracted during data processing.
The total retinol mass (the sum of both unesterified and esterified retinol) was calculated based on the ratio of the peak area of TMMP-retinol (known amount) compared to the total retinol peak area of the same sample; the peak areas were analyzed by the Empower 2 software (Waters Corporation, Milford, MA). The calculated total retinol mass was then normalized to tissue weight or serum volume used for retinoid analysis to yield the reported total retinol concentrations (nmol/g or µmol/L). The reported total retinol concentrations in WAT were corrected for lipid extraction efficiency (95%) (80).

3.2.6.2 RNA isolation, reverse transcription, and qPCR analysis

The total RNA from each sample was isolated with TRIzol™ reagent (Invitrogen, Carlsbad, CA). Briefly, individual tissue sample (~0.1 g) was placed in 1 mL of TRIzol reagent to be homogenized, using a hand-held T10 basic ULTRA-TURRAX® homogenizer (IKA® Works, Inc., Wilmington, NC). Next, the tissue homogenates with TRIzol™ reagent underwent phase separation by adding 0.2 mL of chloroform (Sigma-Aldrich, Co., St. Louis, MO). The upper aqueous phase was then carefully removed after centrifugation at 12,000 g for 15 minutes at 4°C. The aqueous phase of each sample underwent RNA precipitation by adding 0.5 mL of isopropyl alcohol
(Honeywell International Inc., Muskegon, MI), followed by centrifugation at 12,000 g for 10 minutes at 4°C. The RNA pellet was washed with 75% ice-cold ethanol prior to resuspension in nuclease-free water. Each RNA sample was purified using silica-membrane RNeasy spin column by the RNeasy® Mini Kit (QIAGEN, Venlo, Netherlands) to yield high-quality total RNA.

A NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) was used to determine the purity and concentration of purified total RNA samples. The ratios of 260/280 absorbance ranged from 1.90 to 2.18 for all samples. The purified total RNA (2 µg for liver, BAT, WAT, and kidney; 1 µg for muscle) was then reverse transcribed into its complementary DNA (cDNA) with reverse transcriptase (200 U/µL) (Promega, Madison, WI). The diluted reaction product was used with PerfeCTa® SYBR® Green Supermix, ROX™ (BioSciences, Inc., Gaithersburg, MD) and designed primer pair (10 µM) in a final volume of 20 µl for real-time qPCR analysis.

The primers designed to detect mRNA expression were sent to Integrated DNA Technologies (IDT, Coralville, IA) for sequencing. The forward and reverse gene sequence of the primers, including Rbp4, Ucp1, Ucp3, and 18S, are listed in Table 8. 18S rRNA was chosen as the internal control for RNA integrity. To calculate the relative
mRNA expression of target genes, we applied the formula of comparative $C_T$ ($\Delta\Delta C_T$). $C_T$ represents the threshold cycle number, which refers to the minimum cycle number needed to detect the product. The formula is $2^{\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ (VA- or VARA-treated sample) – $\Delta C_T$ (placebo sample) and $\Delta C_T = C_T$ (target gene; $Rbp4$, $Ucp1$, $Ucp3$) – $C_T$ (housekeeping gene; 18S). Briefly, $\Delta C_T$ is the difference in $C_T$ values between the target genes and housekeeping genes and $\Delta\Delta C_T$ is the difference in $\Delta C_T$ values between VA- or VARA-treated and untreated (placebo) samples. In other words, the steady-state mRNA expression of each sample was first normalized to 18S rRNA in the same sample and then standardized to average value of the young adolescent (5 week) placebo group (set to 1.0), prior to statistical analysis.

3.3 Statistical analyses

Data are presented as group means ± standard error of the mean (SEM). Prior to statistical analyses, normality and equality of variances were tested; in cases of abnormal distribution and/or unequal variances, data were log10-transformed (as illustrated in figure legends). Data were analyzed by student’s $t$-test, one-way analysis of variance (ANOVA), and two-way ANOVA using Prism 7.0 (GraphPad Software Inc., La Jolla, CA). Student’s $t$-test was used for the comparison of animal body weights and postprandial blood
glucose between sexes. One-way ANOVA was used to compare animal body weights and gene expression between diet/treatment groups. Two-way ANOVA was used to determine how outcomes of interest were affected by two factors (sexes and treatment groups). Differences among sexes and treatment groups were determined by Tukey’s multiple comparison tests. A $p$-value of less than 0.05 was considered statistically significant.
### Table 8. Gene sequences of the primers designed for PCR amplification.

The forward and reverse gene sequence of the primers designed to detect steady-state mRNA transcript levels are listed as follows.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Accession No.</th>
<th>Gene sequences of the primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rbp4</strong></td>
<td>NM_013162.1</td>
<td>Forward: 5’-CTG TGG ACG AGA AGG GTC AT-3’&lt;br&gt;Reverse: 5’-GGA ATA CTG CAG AGC GAA GG-3’</td>
</tr>
<tr>
<td><strong>Ucp1</strong></td>
<td>NM_012682.2</td>
<td>Forward: 5’-GGC CAA GAC AGA AGG ATT GC-3’&lt;br&gt;Reverse: 5’-GAC AAG CTT TCT GTG GTG GC-3’</td>
</tr>
<tr>
<td><strong>Ucp3</strong></td>
<td>NM_013167.2</td>
<td>Forward: 5’-GTT GGA CTT CAG CCA TCA GAA-3’&lt;br&gt;Reverse: 5’-GTG GGT TGA GCA CAG GTC AC-3’</td>
</tr>
</tbody>
</table>
CHAPTER 4

RESULTS

4.1 Results for specific aim 1

*Liver retinoid storage in the neonatal period was significantly increased by early-life VA and VARA supplementation, with carryover effects to young adolescent age*

The comparisons of liver total retinol levels (nmol/g) between sexes and treatment groups in neonatal and young adolescent rats are shown in Figure 3. The pups that received VA and VARA supplementation treatments in early postnatal period had significantly higher liver total retinol concentrations in the neonatal period (P12), as compared to the placebo group (Figure 3A). Moreover, the VARA-supplemented pups (both male and female) exhibited even higher liver retinol levels than VA-treated male pups (Figure 3A). Since no further treatment was administered after P10 and a marginal level of VA was provided by the HFVAM diet, the liver retinoid storage in VA and VARA supplemented groups decreased over the course of the experimental period, yet the elevated effects of VA and VARA treatments on liver retinoid reserves remained significant at young adolescent age (5 weeks) (Figure 3B). Within treatment groups, there were no significant differences observed between sexes in liver total retinol levels.
Early postnatal VA and VARA supplementation significantly increased lung retinoid reserves, with carryover effects to young adolescence in female

The total retinol (nmol/g) analysis in lung of neonatal and young adolescent rats by sexes and treatment groups are shown in Figure 4. Early postnatal VA and VARA treatments significantly increased retinoid storage in lung of neonatal pups, as compared to the placebo control group (Figure 4A). When comparing to the pups that received VA supplementation, the lung total retinol levels were even higher in VARA-supplemented group (Figure 4A). Similar to hepatic retinoid storage, the lung retinoid reserves decreased from neonatal period (P12) to young adolescence (5 weeks), yet still remained significantly elevated in the VA- and VARA-treated female young adolescent rats (Figure 4B). Overall, no sex differences were observed within treatment groups at both developmental stages.

Serum retinol levels were homeostatically controlled regardless of early postnatal VA or VARA treatments

The serum total retinol concentrations (µmol/L) among neonatal and adolescent rats by sexes and treatment groups are shown in Figure 5. There were no significant differences observed in serum total retinol levels between treatment groups and sexes at
either neonatal period or young adolescent age, indicating that the relationship between
liver retinoid storage and serum retinol concentrations is not linear. Therefore, the serum
retinol levels remained to be homeostatically regulated regardless of the increase in liver
retinoid reserves by VA and VARA treatments in the early postnatal period.

*Early-life VA or VARA supplementation did not affect liver Rbp4 mRNA expression*

The steady-state *Rbp4* mRNA levels (display as relative fold change) in liver of
neonatal and young adolescent rats are shown in Figure 6. Data were normalized to
housekeeping gene in the same sample and standardized to average value of the young
adolescent placebo group (set to 1.0), prior to statistical analysis. Early-life VA or VARA
supplementation did not have any impacts on *Rbp4* mRNA transcript levels in the liver of
neonatal pups as well as young adolescent-age rats. No sex differences were observed
within treatment groups at both developmental stages.
Figure 3. Retinoid analysis in liver of neonatal and young adolescent rats. Total retinol concentrations (nmol/g) in liver of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. Different letters denote p<0.05 by two-way ANOVA and post-hoc test after log transformation (a>b>c). Early-life VA and VARA supplementation significantly increased liver total retinol reserves in the neonatal period, with elevated effects carried over to young adolescence, as compared to the placebo-treated group. No sex differences were observed within treatment groups at both developmental stages.
Figure 4. Retinoid analysis in lung of neonatal and young adolescent rats. Total retinol concentrations (nmol/g) in lung of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. In panel A, different letters denote p<0.05 by two-way ANOVA and post-hoc test after log transformation (a>b>c). In panel B, *p<0.05 and **p<0.01 by two-way ANOVA and post-hoc test. Early postnatal VA and VARA supplementation significantly increased lung total retinol concentrations in the neonatal period, with carryover effects to young adolescence in female (but not in male), as compared to the placebo group. Also, within treatment groups, no sex differences were observed at both developmental stages.
Figure 5. Retinoid analysis in serum of neonatal and young adolescent rats. Total retinol concentrations (μmol/L) in serum of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. No overall significant differences in serum total retinol levels were observed between sexes and treatment groups at both developmental stages. The relationship between liver retinoid storage and serum retinol concentrations is not linear.
Figure 6. Rbp4 mRNA expression in liver of neonatal and young adolescent rats. Steady-state Rbp4 mRNA transcript levels in liver of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. The Rbp4 mRNA expression level of each sample was normalized to 18S rRNA (housekeeping) in the same sample and standardized to average value of the young adolescent placebo group (set to 1.0), prior to statistical analysis. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. Overall, early postnatal VA or VARA supplementation did not affect liver Rbp4 mRNA transcripts at either neonatal period or young adolescence. Within treatment groups, no sex differences were observed at both developmental stages.
The kidney retinoid storage in the neonatal period were increased by early postnatal VA and VARA supplementation

Figure 7 shows the comparisons of kidney total retinol concentrations (nmol/g) between sexes and treatment groups in neonatal pups as well as young adolescent rats. The total retinol levels in kidney were significantly increased by early postnatal VA (in female) and VARA (both sexes) supplementation in the neonatal period, as compared to the control group (Figure 7A). Unlike other organs, the kidney retinoid reserves from all treatment groups increased over the course of the experimental period. Interestingly, at young adolescent age, the placebo group (both sexes) had significantly higher retinoid storage in kidney, compared to VA- and VARA-treated female rats (Figure 7B). This may be related to the function of VA recycling and disposal in kidney in which will be discussed later. Within treatment groups, there were no significant differences observed between sexes at both developmental stages.

Early-life VA or VARA supplementation did not alter kidney Rbp4 mRNA expression at both developmental stages

The steady-state Rbp4 mRNA transcript levels in kidney of neonatal and young adolescent rats are shown in Figure 8. Despite the elevated levels of kidney retinoid
storage, the kidney *Rbp4* mRNA transcript levels in neonatal pups and young adolescent rats were not altered by early postnatal VA or VARA supplementation treatments. Overall, at both developmental stages, no sex differences were observed within treatment groups.
Figure 7. Retinoid analysis in kidney of neonatal and young adolescent rats. Total retinol concentrations (nmol/g) in kidney of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. In panel A, the data were log-transformed prior to statistical analysis. Different letters denote p<0.05 by two-way ANOVA and post-hoc test (a>b). Early postnatal VA (in female) and VARA supplementation significantly increased total retinol levels in the kidney in the neonatal period, compared to the placebo control group. The retinoid reserves in the kidney increased over the course of the experimental period regardless of different treatments. At young adolescent age, the placebo-treated rats had significantly higher kidney total retinol concentrations than VA- and VARA-treated female rats. No sex differences were observed within treatment groups at both developmental stages.
Figure 8. *Rbp4* mRNA expression in kidney of neonatal and young adolescent rats. Steady-state *Rbp4* mRNA transcript levels in kidney of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. The *Rbp4* mRNA expression level of each sample was normalized to 18S rRNA (housekeeping) in the same sample and standardized to average value of the young adolescent placebo group (set to 1.0), prior to statistical analysis. Results are presented as mean ± SEM; *n*=5-11 per sex per treatment group. Early-life VA or VARA supplementation did not alter kidney *Rbp4* mRNA transcripts at either neonatal period or young adolescence. No sex differences were observed within treatment groups at both developmental stages.
The effects of early-life VA and VARA supplementation on increasing BAT retinoid storage were transient in the neonatal period

Total retinol levels (nmol/g) in BAT of neonatal and young adolescent rats by sexes and treatment groups are shown in Figure 9. Early postnatal VA and VARA supplementation transiently increased BAT total retinoid reserves in the neonatal period (Figure 9A). However, the elevated effects of early-life VA or VARA supplementation on BAT retinoid storage were not carried over to young adolescence (Figure 9B). At both developmental stages, no significant sex differences were observed within treatment groups.

Treatment effects of early postnatal VARA supplementation were observed on BAT Rbp4 mRNA expression at young adolescent age

The comparisons of steady-state Rbp4 mRNA expression in BAT of neonatal and young adolescent rats are shown in Figure 10. Overall, early postnatal treatment effects ($p=0.0256$) on up-regulating BAT Rbp4 mRNA transcript levels were observed in the young adolescence, but not in the neonatal period (Figure 10A & 10B). Since neither sex differences nor treatment x sex interactions were observed within treatment groups at young adolescent age, data from both sexes within treatment groups were combined for
one-way ANOVA analysis of treatment effects. The *Rbp4* expression levels in BAT were significantly increased by early-life VARA supplementation at young adolescent age (Figure 10C), suggesting that early postnatal VARA treatment may have long-term effects on inducing *Rbp4* expression in BAT.
Figure 9. Retinoid analysis in BAT of neonatal and young adolescent rats. Total retinol concentrations (nmol/g) in BAT of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. In panel A, different letters denote p<0.05 by two-way ANOVA and post-hoc test after log transformation (a>b). Early-life VA and VARA supplementation significantly increased BAT total retinol reserves in the neonatal period, yet the elevated effects were not carried over to young adolescent age. Overall, at both developmental stages, no sex differences were observed within treatment groups.
Figure 10. \(Rbp4\) mRNA expression in BAT of neonatal and young adolescent rats.
Steady-state *Rbp4* mRNA transcript levels in BAT of (A) neonatal (P12) and (B) young adolescent (5 weeks) rats by sexes and treatment groups, and (C) young adolescent (5 weeks) rats by treatment groups. The *Rbp4* mRNA expression level of each sample was normalized to 18S rRNA (housekeeping) in the same sample and standardized to average value of the young adolescent placebo group (set to 1.0), prior to statistical analysis. Results are presented as mean ± SEM; *n*=5-11 per sex per treatment group. In panel C, different letters denote *p*<0.05 by one-way ANOVA and post-hoc test after log transformation (*a>*b). Early postnatal VARA treatment effects on up-regulating BAT *Rbp4* expression were observed at young adolescent age but not in the neonatal period. Early postnatal VARA supplementation was associated with significantly increased *Rbp4* expression levels in BAT at young adolescent age. Within treatment groups, no sex differences were observed.
The total retinol levels in the WAT in the neonatal period were significantly increased by early postnatal VA and VARA supplementation.

The comparisons of retinoid analyses (nmol/g) by sexes and treatment groups in WAT of neonatal and young adolescent rats are shown in Figure 11. The WAT total retinol concentrations in the neonatal period were significantly elevated by early-life VA and VARA supplementation, as compared to the placebo-treated pups (Figure 11A). The elevated effects were not carried over to young adolescence except for the VA-treated female rats (Figure 11B). Consistent with other organs examined, no sex differences were observed within treatment groups at both developmental stages.

The Rbp4 mRNA expression in WAT was not affected by early-life VA or VARA supplementation.

The steady-state Rbp4 mRNA transcripts in the WAT of neonatal and young adolescent rats are shown in Figure 12. Similar to the liver and kidney, the Rbp4 mRNA transcript levels in the WAT of neonatal pups and young adolescent rats were not altered by early postnatal VA or VARA supplementation. Within treatment groups, no sex differences were observed at both developmental stages.
Figure 11. Retinoid analysis in WAT of neonatal and young adolescent rats. Total retinol concentrations (nmol/g) in WAT of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. In panel A, the data were log-transformed prior to statistical analysis. Different letters denote p<0.05 by two-way ANOVA and post-hoc test (a>b). The total retinol levels in WAT of neonatal pups were significantly increased by early postnatal VA and VARA supplementation. The retinoid reserves in WAT remained significantly elevated in VA-treated young female adolescent. No sex differences were observed within treatment groups at both developmental stages.
Figure 12. *Rbp4* mRNA expression in WAT of neonatal and young adolescent rats.

Steady-state *Rbp4* mRNA transcript levels in WAT of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. The *Rbp4* mRNA expression level of each sample was normalized to 18S rRNA (housekeeping) in the same sample and standardized to average value of the young adolescent placebo group (set to 1.0), prior to statistical analysis. Results are presented as mean ± SEM; *n*=5-11 per sex per treatment group. Early-life VA or VARA supplementation did not alter WAT *Rbp4* mRNA transcript levels at either neonatal period or young adolescent age. Within treatment groups, no sex differences were observed at both developmental stages.
4.2 Results for specific aim 2

Similar to Rbp4 expression in BAT, treatment effects of early postnatal VARA supplementation on up-regulating BAT Ucp1 mRNA transcript levels were observed at young adolescent age.

The comparisons of steady-state BAT Ucp1 mRNA transcripts at both developmental stages are shown in Figure 13. Similar to Rbp4 expression in BAT (Figure 10), treatment effects (p=0.0117) of early-life VARA supplementation on up-regulating BAT Ucp1 mRNA transcript levels were observed at young adolescent age, but not in the neonatal period (Figure 13A & 13B). Neither sex differences nor treatment x sex interactions were observed within treatment groups in the young adolescence, thus, data from both sexes within treatment groups were combined for one-way ANOVA analysis of treatment effects. When comparing to the placebo control group, the early postnatal VARA supplementation was associated with significantly increased Ucp1 expression in BAT in young adolescent rats (Figure 13C), indicating that early-life VARA treatment may have a potential long-term impact on induction of Ucp1 transcript levels at young adolescent age.
Figure 13. *Ucp1* mRNA expression in BAT of neonatal and young adolescent rats.
Steady-state Ucp1 mRNA transcript levels in BAT of (A) neonatal (P12) and (B) young adolescent (5 weeks) rats by sexes and treatment groups, and (C) young adolescent (5 weeks) rats by treatment groups. The Ucp1 mRNA expression level of each sample was normalized to 18S rRNA (housekeeping) in the same sample and standardized to average value of the young adolescent placebo group (set to 1.0), prior to statistical analysis. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. In panel C, different letters denote p<0.05 by one-way ANOVA and post-hoc test after log transformation (a>b). Early postnatal treatment effects on up-regulating BAT Ucp1 expression were observed at young adolescent age, but not in the neonatal period. The Ucp1 expression levels in BAT were significantly increased by early postnatal VARA supplementation at young adolescent age. Within treatment groups, no sex differences were observed.
Early postnatal VA and VARA supplementation transiently increased muscle retinoid concentrations in the neonatal period

The comparisons of muscle total retinol levels (nmol/g) by sexes and treatment groups in neonatal and young adolescent rats are shown in Figure 14. The muscle total retinol concentrations in the neonatal period were significantly increased in VA- and VARA-supplemented pups, as compared to the placebo-treated group (Figure 14A); however, the elevated effects of early-life VA or VARA supplementation on muscle retinoid levels were not observed at young adolescent age (Figure 14B). Overall, no sex differences were observed within treatment groups at both developmental stages.

Early postnatal VA or VARA supplementation were not associated with increased muscle Ucp3 mRNA expression in neonatal and young adolescent rats

The comparisons of steady-state muscle Ucp3 mRNA expression at both developmental stages are shown in Figure 15. Overall, early postnatal VA or VARA supplementation did not alter muscle Ucp3 mRNA expression at both developmental stages. Within treatment groups, no differences in sex were observed.
Figure 14. Retinoid analysis in muscle of neonatal and young adolescent rats. Total retinol concentrations (nmol/g) in muscle of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment group. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. In panel A, different letters denote p<0.05 by two-way ANOVA and post-hoc test after log transformation (a>b>c). Early-life VA and VARA supplementation significantly increased muscle total retinol reserves in neonatal pups, yet the elevated effects were not carried over to young adolescent age. No sex differences were observed within treatment groups.
Figure 15. *Ucp3* mRNA expression in muscle of neonatal and young adolescent rats. 
Steady-state *Ucp3* mRNA transcript levels in muscle of (A) neonatal (P12), and (B) young adolescent (5 weeks) rats by sexes and treatment groups. The *Ucp3* mRNA expression level of each sample was normalized to 18S rRNA (housekeeping) in the same sample and standardized to average value of the young adolescent placebo group (set to 1.0), prior to statistical analysis. Results are presented as mean ± SEM; *n*=5-11 per sex per treatment group. No overall significant differences in muscle *Ucp3* mRNA transcript levels were observed between sexes and treatment groups at both developmental stages.
4.3 Results for specific aim 3

*The body weight change did not differ by diets and treatment groups over the course of the experimental period*

The animal growth patterns and body weight comparisons between diets and treatment groups are shown in Figure 16. A group of age- and sex-matched normal chow-fed non-obese young adolescent rats was included as a reference for the comparison of animal growth pattern (total $n=106$). Overall, no significant differences in body weight change were observed between diets and treatment groups over the course of the experimental period, indicating similar animal growth patterns across diets and treatment groups as well as successful group randomization to avoid litter effects. The body weight change did not differ by sexes in HFVAM diet-fed rats; however, normal chow-fed male rats had significantly higher body weight starting from P24 until the end of study (5 weeks old), as compared to normal chow-fed female animals.
Figure 16. Animal growth patterns and body weight comparison. The animal growth patterns and body weight comparisons between diets and treatment groups. Results are presented as mean ± SEM. A group of age- and sex-matched normal chow-fed non-obese young adolescent rats was included as a reference for the purpose of animal growth comparison (total n=106). There were no significant differences observed in body weight change between diets and treatment groups over the course of the experimental period.
Although the body weight did not differ, the body composition was different between HFVAM diet-fed and normal chow-fed rats at young adolescent age.

The comparisons of body composition between diets and treatment groups in young adolescent rats are shown in Figure 17. The HFVAM diet-fed rats had significantly higher body fat and lower lean body mass in the young adolescence, as compared to rats fed a normal chow diet. The results suggest that although the body weight did not differ (as shown in Figure 16), the body composition was affected by different diets (HFD versus normal chow diet). Also, the percentage of body fat and lean body mass did not differ by sexes.
Figure 17. Body fat and lean body mass comparison in young adolescent rats. The comparisons of (A) body fat (%), and (B) lean body mass (%) between diets and treatment groups in young adolescent rats. Results are presented as mean ± SEM; \( n=5\) to 7 per sex per treatment group. Different letters denote \( p<0.05 \) by two-way ANOVA and post-hoc test (a>b). The rats fed a HFVAM diet had significantly higher body fat and lower lean body mass in the young adolescence, as compared to rats fed a normal chow diet. Body fat and lean body mass did not differ by sexes.
No significant differences were observed in postprandial blood glucose between treatment groups at both developmental stages.

Figure 18 shows the postprandial blood glucose (mg/dL) comparisons between treatment groups in neonatal and young adolescent rats. There were no significant differences observed in postprandial blood glucose between treatment groups at both developmental stages (Figure 18A & 18B). A sex effect was observed in postprandial blood glucose levels at young adolescent age. Since neither treatment effects nor treatment x sex interactions were observed, results from different diets/treatments were combined for analysis of sex differences. Overall, male rats exhibited significantly higher postprandial blood glucose levels than females at young adolescent age (Figure 18C).
Figure 18. Postprandial blood glucose comparison in neonatal and young adolescent rats.
The comparisons of postprandial blood glucose (mg/dL) in (A) neonatal (P12), and (B) & (C) young adolescent (5 weeks) rats by sexes, diets, and treatment groups. Results are presented as mean ± SEM; n=5-11 per sex per treatment group. In panel C, *p<0.05 by student’s t-test. At both developmental stages, no significant differences were observed in postprandial blood glucose between diets and treatment groups, yet a sex effect of postprandial blood glucose levels was observed at young adolescent age.
Vitamin A is involved in many critical events during neonatal growth and development, yet neonates are generally born with low hepatic retinoid storage, even in countries where pregnant women are assumed to be free of VAD of public health significance (79). Although treatment with all-trans-RA has been shown to alter gene expression related to metabolic processes in vivo and in vitro (121-123, 125-133), most previous studies were conducted in non-obese adult mice with VA-sufficient status or in cultured cells. In addition, the concept of FOAD has been widely accepted and applied in many studies. However, studies are limited about the effects of micronutrient supplementation, administered in early postnatal period, on later health outcomes. In the current study, we proposed to determine whether early-life VA or VARA supplementation alters metabolic functions related to risk of obesity development in the neonatal period as well as prepubescent age, using a diet-induced obese rat model with marginal VA status. In particular, we examined changes in expression of genes that are associated with insulin sensitivity and thermogenesis, with focus on the variable of sex differences. We hypothesized that early postnatal VA and VARA supplementation is
associated with altered Rbp4 mRNA transcript levels (in liver, kidney, BAT, and WAT) and Ucp mRNA expression (Ucp1 in BAT and Ucp3 in skeletal muscle) in the neonatal period, with carryover effects to young adolescent age. We also hypothesized that early postnatal VA and VARA supplementation is associated with decreased adiposity in prepubescent rats. This study utilized both VA and VARA supplementation, by which, to our knowledge, the research design provides novel information on the potential influence in metabolic profiles generated by two different types of early-life VA supplementation.

5.1 Tissue total retinol concentrations

As shown in Figures 3, early postnatal VA and VARA supplementation significantly increased hepatic retinoid storage in the neonatal period (P12; 2 days after last dose administration on P10), with the elevated effects carried over to young adolescent age (5 weeks; 23-25 days after last dose administration on P10) despite the overall reduced liver retinoid reserves; in contrast, serum total levels were not affected by early-life VA or VARA treatments at both developmental stages (Figure 5). Therefore, the relationship between liver retinoid reserves and serum total retinol concentration is not linear, in which makes the concentration of retinol in serum not an ideal indicator for assessment of VA status. Olson suggested that serum retinol value rises to a plateau
around 20 μg retinol/g liver (70 nmol retinol/g liver) and is homeostatically controlled up to liver retinoid reserves >300 μg/g (1047 nmol/g) (48). In the current study, the serum total retinol levels in the neonatal period and young adolescence were tightly regulated in a normal physiological range (0.50-2.12 μmol/L) regardless of early postnatal VA and VARA exposure (hepatic total retinol concentration ranged from 42-1233 nmol/g). Although the placebo-treated young adolescent rats had slightly lower liver retinoid reserves (average 25 ± 1.9 nmol/g), their serum retinol levels were still maintained normal (average 1.4 ± 0.09 μmol/L). This may be contributed by kidney homeostasis, which will be discussed later.

Besides liver, lipid droplet-containing stellate cells also exist in the lung (30), in which makes lung an important organ for retinoid storage in rats with VA-adequate as well as VA-excessive status (154). The lung VA stores in rats decrease significantly after gestational day 18 (the end of prenatal period) and continue into P21 (neonatal period) when lung alveolarization is completed (155, 156); in contrast, the liver retinoid reserves before and after birth are not affected by lung development (155). Thus, the cell growth and differentiation in the lung during early postnatal period is highly dependent on local VA stores but independent of hepatic VA reserves (155). Although our primary interest
was the liver, we also desired to understand if there are beneficial effects of early postnatal VA and VARA supplementation on retinoid homeostasis in lung. Previously, Ross and colleagues (141-144) have reported that the VA and RA combined in the VARA treatment act synergistically to increase retinol uptake and esterification in the lungs of neonatal and young adult rats, as compared to VA or RA treatments alone. Since most of the studies were designed to determine short-term response to VARA supplementation (study endpoint less than 24 h after VARA administration), the persistence of VARA treatment effects on elevating lung retinoid storage remains to be answered. Ross and colleagues (141) then reported that without further treatment introduced after the neonatal period (P7), a sustained increase in VARA-derived lung RE in neonatal rats was still observed at P16 (9 days after last VARA dose administration on P7); however, the lung RE retention induced by early postnatal VARA supplementation was not carried over to adulthood (2 months old) (122). In the current study, we observed that early postnatal VA and VARA supplementation significantly increased lung retinoid storage in the neonatal period (P12; 2 days after last VARA dose administration on P10); yet, the elevated levels of lung retinoid reserves were carried over to young adolescent age (5 weeks) in female rats only (as shown in Figure 4). The results suggested a potential therapeutic method to effectively enhance lung VA stores in which may be
beneficial for lung development during neonatal period. Future experiments are needed to determine the sustainability of increased lung retinoid reserves by VARA treatment.

Kidney plays multiple roles in VA metabolism, for instance, retinol recycling as well as retinoid storage. The glomerulus of the kidney functions to filtrate the circulating holo-RBP4 complex that is unbound to TTR, for retinol recycling or disposal (157). The recycled retinol is reabsorbed in the tubules by megalin, a surface receptor that mediates retinol reuptake in renal proximal tubule cells (158). Prior to irreversible degradation, retinol can be recycled 12 to 13 times through kidney, liver, plasma, and other organs in adult rats (159-161). Because of the expression of STRA6 (36, 37) and the existence of stellate cells (30), a small amount of VA can be taken up and stored in the kidneys.

As shown in Figure 7, early-life VA and VARA supplementation significantly increased kidney total retinol levels in the neonatal period (P12; 2 days after last VARA dose administration on P10). This finding agrees with a kinetic study previously conducted in our laboratory by Hodges et al. (80), which showed that the elevation of kidney retinol concentration lasted for 2 days after VA dose administration, possibly due to the increase of retinol recycling. VA supplementation results in a significantly higher recycling number of retinol prior to irreversible disposal, as compared to the control.
group (162). Hodges et al. (80) also reported that a gradual accumulation of kidney retinol was observed in both placebo-treated and VA-supplemented rats from P18 to P28 (VA dose administered on P4). In addition, although not statistically significant, the kidney total retinol concentrations were higher in the placebo control group on P28 (24 days after VA dose administration), as compared to the VA-treated group (80).

Consistent with results from the previous study, we also observed that the kidney retinoid concentration increased, instead of decreased, over the course of the experimental period from P12 to 5 weeks, especially in the placebo-treated young adolescent group. The placebo-treated young adolescent rats (both sexes) exhibited significantly higher kidney retinoid stores, as compared to VA- and VARA-supplemented female adolescent rats. Besides treatment effects ($p=0.0007$) of early postnatal VA supplementation, the treatment x sex interactions ($p=0.0494$) were also observed in kidney total retinol concentration of young adolescent rats.

A possible explanation of the particular phenomenon is that VA status, especially liver retinoid reserves, can influence plasma retinol kinetics (160). That is, when VA status is low or marginal, extrahepatic tissues are involved in maintaining VA homeostasis (159). Since no further treatment was administered after neonatal period and
a marginal level of VA was provided by the diet, the liver retinoid storage in all treatment groups decreased over the course of the experimental period from P12 to 5 weeks. To maintain normal plasma retinol levels, it is possible that the kidney accumulates pools of VA through retinol recycling in response to the decreased liver retinoid reserves. The significantly higher retinol concentration in the kidney of placebo-treated young adolescent rats was related to especially lower hepatic VA stores. Also, comparisons of VA kinetics between neonatal and adult rats show several similarities and differences in different life stages (159). For example, the retinol turnover rate was faster in neonatal rats (recycling number ~144) (163), as compared to adult rats (recycling number ~12-13) (161). Thus, the VA kinetics in kidney of young adolescent rats may be distinct from that of neonatal rats.

Similar to other tissues examined in this study (i.e., liver, lung, and kidney), early-life VA and VARA supplementation significantly increased total retinol levels in BAT, WAT, and muscle in the neonatal period (P12; 2 days after last VARA dose administration on P10). However, the effects of early-life VA and VARA treatments on increasing tissue retinoid stores were mostly transient. The elevated tissue retinoid levels were not carried over to young adolescent age except in WAT of VA-supplemented
female rats (as shown in Figures 9, 11 & 14). This observation was similar to findings reported by Hodges et al. (80) that the total retinol levels in BAT were significantly elevated for 4 days only after VA dose administration; whereas in WAT, the concentration of total retinol remained significantly increased for 18 days after VA dose administration. The transient elevation of retinol levels in BAT and muscle by early-life VA and VARA supplementation indicates that the retinol retention capacity is relatively low in these organs. The finding also suggests WAT as a possible long-term reservoir for VA storage.

5.2 Expression of genes related to insulin sensitivity and thermogenesis

Previously, Mourey et al. (164) reported that, using cultured hepatoma (HepG2) cells, RBP4-specific mRNA is positively regulated by treatment of retinoids (both retinol and RA) in a dose-dependent manner. In contrast, Palou and colleagues (121) suggested that RA treatment is associated with significantly decreased liver RBP4 protein content (but not Rbp4 mRNA expression) and increased serum RBP4 protein levels in NMRI mice. A possible explanation is that RA treatment triggers the release of RBP4 protein from liver into the circulation, to further prevent hepatic retinoid intoxication (121). In addition, since RA treatment has been shown to improve glucose tolerance and insulin
sensitivity, the increase of liver-derived RBP4 in the serum seems unlikely to cause systemic insulin resistance \textit{in vivo} (121, 165). Palou and colleagues (121) also illustrated that RA treatment significantly down-regulates \textit{Rbp4} mRNA expression in fat depots (epididymal and inguinal WAT) of NMRI mice as well as in cultured white adipocytes (3T3-L1 mature adipocytes and mouse embryonic fibroblasts-derived adipocytes); nonetheless, research about the effects of retinoid treatments on the regulation of adipose-derived RBP4 is scarce. Together, the correlation between reduced RBP4 expression in WAT and improved systemic insulin sensitivity after RA treatment demonstrates the potential role of retinoids in regulation of adipokine production.

Similar to the findings by Palou and colleagues, Owusu and Ross (122) showed that \textit{Rbp4} mRNA expression in the liver of neonatal rats was significant down-regulated by early postnatal VARA supplementation. Moreover, a significant reduction in liver \textit{Rbp4} transcripts was also observed in adult rats that received VARA supplementation in early postnatal period (up to P10, no further treatment ever since), along with increased hepatic retinoid reserves. Therefore, a “carryover” effect of early-life VARA supplementation on down-regulation of liver \textit{Rbp4} expression levels as well as elevation of hepatic retinoid storage in adult rats has been suggested. However, the endpoint of most animal studies
was set within 24 h after the last VA or RA dose administration; thus, short-term regulatory effects of retinoid on tissue Rbp4 mRNA were observed. Here in the current study, tissues were collected at P12 (neonatal period) and 5 weeks of age (young adolescence), which were 2 days and ~23-25 days, respectively, after the last VA and VARA dose administration on P10. Despite the significantly elevated retinoid levels, early postnatal VA or VARA supplementation did not alter steady-state Rb4 mRNA levels in the liver, kidney, and WAT in the neonatal period (as shown in Figures 6, 8 & 12), indicating that the treatment effects on tissue gene expression, if they exist, may be transient and were not captured by the design of the current study. Hence, the findings about Rbp4 transcripts in the liver, kidney and WAT from the current study did not support our original hypothesis.

Interestingly, the early postnatal VARA treatment effects on up-regulating Rbp4 mRNA transcript levels in BAT were only observed in young adolescent age rats, but not in the neonatal period (as shown in Figure 10). When comparing to the placebo control group, early postnatal VARA supplementation significantly increased BAT Rbp4 mRNA at young adolescent age, suggesting that VARA supplementation, administered at early postnatal period, may have a long-term impact on up-regulating Rbp4 expression in BAT.
The effects of RA treatment on regulation of gene related to thermogenesis in BAT and muscle have been tested in several in vivo and in vitro studies. Palou and colleagues (132, 133) reported that RA treatment significantly up-regulated the mRNA expression and protein levels of Ucp1 (brown-adipocyte specific gene) in inguinal WAT of NMRI mice, independent of dietary VA consumption. Together with increased numbers of multilocular adipocytes observed (132), studies suggested that RA treatment favors the browning of white adipocytes in vivo. Moreover, RA treatment not only increases UCP1 content in BAT of NMRI mice (129), but also up-regulates Ucp1 expression and UCP1 content in cultured mouse brown adipocytes (129-131) as well as white adipocytes (but not in human adipocytes) (130, 166). The Ucp1 transcripts in cultured cell models are induced RA treatment in a p38MAPK-dependent manner (130, 131). Palou and colleagues (125, 127) further reported that acute RA treatment was associated with up-regulation of Ucp3 mRNA expression in skeletal muscle of NMRI mice.

Results from the current study about Ucp expression partially agreed with our original hypothesis and review of literature. We found that the up-regulation of Ucp expression induced by early-life VARA supplementation only happened in BAT at young adolescent age, but not in muscle at both developmental stages, as compared to the
placebo group (as shown in Figure 13 & 15). Similar to the effect of VARA on elevating BAT Rbp4 mRNA expression, VARA supplementation is also suggested to have a long-term impact on induction of Ucp1 expression in BAT. We observed that BAT is a particular tissue of interest in that genes related to metabolic processes in BAT were affected by retinoid treatment. Further studies are warranted to understand the effects of RA treatment on lipid and energy metabolism in BAT.

5.3 Animal model and body composition

Since the development of laboratory rats is not proportional to the pace of human growth, the endpoints of the study were chosen carefully. On average, the weaning age is approximately 6 months (180 days) for humans and P21 for experimental rats (167). Based on the calculation (one human year = 42.4 rat days in this phase) (167), the first endpoint in the current study for pups was selected to be P12, which is roughly equivalent to neonatal humans ~3-4 months old. In general, humans do not reach puberty until ~11.5 years old, while laboratory rats reach sexual maturity at around P38 (167); according to the formula (one human year = 3.3 rat days in this phase) (167), the second endpoint in the current study was selected to be P33-P35, which is approximately equal to 10-11 years of age in young adolescent humans. The study was designed to gain comprehensive
insights on developmental changes in retinoid as well as energy metabolism after early-life VA and VARA supplementation, using a diet-induced obese rat model with VA-marginal status.

Rolls and Rowe (168) illustrated that diet-induced (10-20 weeks of HFD) maternal obesity is associated with poor growth pattern and higher mortality in pups, as compared to pups of non-obese normal chow-fed dams. Since obese dams lost significantly more weight post-partum, abnormal milk production and negative energy balance are the speculated reasons for impaired lactational performance (168). In other words, the surviving pups of obese dams are predisposed to a lower growth rate, which was confirmed by significantly lower pre-weaning body weights (pups of obese dams weighed 6.4 g less than pups of control dams) (168). Here in the currently study, the pup survival rate by dams fed a HFVAM diet (from the beginning of pregnancy) was only ~80%, whereas all of the pups of normal chow-fed dams survived. The finding further demonstrates that diet-induced maternal obesity is associated with a higher mortality rate in pups, regardless of feeding duration.

Previously, Hodges et al. (80) reported that no dissectible WAT was found until P12, and the weight of WAT (% relative to body weight) remained constant overtime in
neonatal rats whose dams were fed a VAM diet; in contrast, despite continuous tissue growth, the BAT weight (%) decreased over the course of the experimental period. Consistent with the observation by Hodges et al., we also found that the inguinal WAT weight (%) remained constant overtime, whereas the BAT weight (%) decreased steadily from neonatal period to young adolescent rats fed a HFVAM diet. As shown in Figures 16 & 17, although the body weight change did not differ by diet and treatment groups over the course of the experimental period, the body composition was significantly different between HFVAM diet (contains 45 kcal% fat)-fed and normal chow (contains ~13.4 kcal% fat)-fed young adolescent rats. The rats fed a HFVAM-diet had higher body fat (%) and lower lean body mass (%), as compared to the normal chow-fed group. Both inguinal WAT and BAT weight (%) in normal chow-fed young adolescent rats were significantly lower than HFVAM-diet fed groups, indicating that the differences in body composition was partially contributed by less subcutaneous fat (%) in normal chow-fed rats. It is worth to note that in normal chow-fed rats, male exhibited significantly higher body weight (after P24 to the end of study) and postprandial blood glucose (at young adolescent age), as compared to the females in normal chow group (as shown in Figure 18).
RA treatment has been shown to increase lipid oxidation and thermogenic capacity in liver, adipose tissues, and muscle of mice, leading to reduced total body and adipose tissue weights, adiposity, and lipogenesis, with no differences observed between pre- and post-treatment food intake (123, 125, 126, 129, 132, 133). Besides in vivo studies, the association between RA treatment and increased oxidative metabolism was also confirmed in a cultured white adipocyte model (128). However, results from the current study rejected our hypothesis that early-life VA and VARA supplementation is associated with decreased adiposity in young adolescent rats. In previous animal studies, mice were administered 10, 50, 100, and even up to 170 mg/kg of body weight of RA treatment for 4 consecutive days immediately before the day of euthanasia; and, a RA dose-dependent loss of body weight was observed. In contrast, here in the current study, rats received oral doses of VA (6 mg/kg of body weight) or VARA (6 mg retinol + 0.6 mg RA/kg of body weight) treatments on P1, P4, and P10 (total of 3 doses, no further treatment ever since); and, body composition was measured on P33-P35 (23-25 days after last VA and VARA dose administration). Together, the low dose of RA (0.6 mg RA/kg of body weight vs. >10 mg RA/kg of body weight) contained in the VARA treatment and an extended study endpoint (euthanasia ~23-25 days post-treatment vs. euthanasia within 24 h post-treatment) are likely reasons that a reduced adiposity in young adolescent rats with
early postnatal VARA treatment was not found. Although early-life supplementation of VA (as RP) has been shown to induce higher adiposity in rats fed a HFD than control rats (169), the diverge was observed only after 40 days of HFD introduction (rats aged P60). We did not observe increased adiposity associated with early life VA supplementation since our endpoint was set at P33-P35. Additionally, although low physiological concentration of RA dose has been suggested to promote the differentiation of adipocytes and the accumulation of lipid in vitro (170), future research is warranted on the investigation of RA treatment on changes in adiposity in vivo.

5.4 Future directions

There are several limitations in the current study that can be improved for future research in the field. First, the purpose of the study was to determine whether early postnatal VA supplementation is associated with decreased risk of developing childhood obesity. Based on this objective, we intended to generate a diet-induced obese young adolescent rat model with marginal status of VA using the customized HFVAM diet. Although the intervention group (HFVAM diet-fed rats) exhibited higher body fat (%) than the control group (normal chow-fed rats), there were no significant differences observed in body weight. Therefore, the length of HFD feeding (i.e., starting from
pregnancy to pups 5 weeks of age) might be slightly too short to create such an animal model that meets our expectation. Alternatively, using a HFD that contains 60 kcal% fat instead of 45 kcal% fat may be worth considering in future studies.

Second, we observed treatment effects of early-life VARA supplementation on inducing $Rbp4$ and $Ucp1$ mRNA transcript levels in BAT at young adolescent age, suggesting that early postnatal VARA supplementation may have long-term impacts on functions related to metabolic regulation in BAT. However, the specific mechanism of how early postnatal VARA supplementation regulates metabolic function related to risk of obesity development in BAT remains to be elucidated. Previous studies have shown that RA treatment acts to induce $Ucp1$ gene expression in a p38MAPK-dependent fashion \textit{in vitro} (130, 131). Future work includes examining profiles of cytokines and chemokines and/or potential signaling pathways related to increased $Rbp4$ and $Ucp1$ mRNA expression \textit{in vivo}, and exploring possible mitochondrial biomarkers and epigenetic changes in BAT associated with early-life VARA treatment.

Finally, in recent years, the association between gut microbiota and health status has become a great topic of interest in research. Human gut microbiomes differ across populations in a region- and age-specific manner (171). A link between early
development gut microbiota and risk of childhood obesity has been proposed in a number of studies (172-174). Moreover, Hibberd et al. (175) reported that micronutrient deficiencies, especially acute VA deficiency, have tremendous impacts on bacterial community in the gut. Yet, the gut microbiota in maternal as well as neonatal and young adolescent rats was not assessed in the current study. Future research may delve into the specific topic to determine if early-life VA and/or VARA supplementation is associated with altered gut microbiota related to risk of childhood obesity development. Identifying possible mechanism related to gut microbiota, if any, may help to generate relevant information on studying the effects of early-life VA supplementation.
CHAPTER 6

CONCLUSIONS AND PUBLIC HEALTH IMPLICATIONS

The current study is innovative as it is the first study to determine the effects of early postnatal VA and VARA supplementation, compared side-by-side, on changes related to energy metabolism in the neonatal period as well as young adolescent age. We observed that VA and VARA supplementation, administered in the early postnatal period, significantly increased total retinol stores in tissues examined, including liver, lung, kidney, BAT, WAT, and muscle, in neonatal pups. Besides liver, lung, and WAT, the significant elevation of retinoid reserves in kidney, BAT, and muscle were transient in the neonatal period and were not carried over to prepubescent age. Regardless of the increase in hepatic retinoid storage, the serum total retinol concentrations remained homeostatically regulated within a normal physiological range at both developmental stages. Treatment effects of early-life VARA supplementation on inducing Rbp4 and Ucp1 mRNA were observed in BAT. Both Rbp4 and Ucp1 steady-state mRNA expression were significantly up-regulated in VARA-treated young adolescent rats, as compared to the placebo control group; indicating that VARA supplementation in the early postnatal period may impact long-term regulation of metabolic functions related to
risk of obesity development. However, early-life VA and VARA supplementation did not alter \(Rbp4\) transcripts in liver, kidney, and adipose tissues, and \(Ucp\) expression in BAT and muscle in the neonatal period.

Body weight change did not differ across diets and treatment groups, suggesting similar growth patterns and successful group randomization to avoid litter effects; yet, the body composition was different between HFVAM diet-fed and normal chow-fed rats at young adolescent age. Although the HFVAM diet-fed prepubescent rats had significantly higher body fat and lower lean body mass, VA and VARA treatments in the early postnatal period were not associated with decreased adiposity in HFVAM diet-fed prepubescent rats. Overall, within each treatment group, no sex differences were observed in tissue retinoid stores, tissue gene expression, and body composition at both developmental stages (neonatal period and young adolescent age).

The current study is significant since it provides new scientific evidence related to impacts on long-term energy metabolism by early-life VARA supplementation, which may be translated into new research questions with a focus on the relationship between early-life VARA supplementation and prevention of childhood obesity. Currently, the WHO has not made a recommendation regarding VA supplementation to infants 1-5
months old. For dietary VA recommendation, an Adequate Intake (AI) is the only available dietary reference intake (DRI) for infants 0-12 months of age (17). The value of the AI is defined based on the amount of consumption for the specific nutrient in non-deficient population; thus, the amount of nutrient consumed may exceed optimal needs and no further information is provided. Generally, AI is only used when estimated average requirement (EAR) and recommended dietary allowance (RDA) is not available, and it is considered the least interpretable of all DRI values. Our study provides new insights into mechanisms on how VA supplementation in the early perinatal period could potentially alter energy metabolism later in life. The information could ultimately be helpful for policy-making and nutrition intervention designing. Although clinical trials and population-based observation are required to establish the dietary recommendation, animal trials are essential along the long gradual process. In sum, understanding the impacts of VA supplementation in the early postnatal period on later metabolic processes helps to fill important knowledge gaps and to benefit future public health decisions.
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APPENDIX

Total retinol concentrations in normal chow-fed young adolescent rats

<table>
<thead>
<tr>
<th>Organs</th>
<th>Total retinol concentrations (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (n=5)</td>
</tr>
<tr>
<td>Serum (µmol/L)</td>
<td>1.37 ± 0.19</td>
</tr>
<tr>
<td>Liver (nmol/g)</td>
<td>362.35 ± 36.08</td>
</tr>
<tr>
<td>Lung (nmol/g)</td>
<td>39.29 ± 12.35</td>
</tr>
<tr>
<td>BAT (nmol/g)</td>
<td>2.23 ± 0.68</td>
</tr>
<tr>
<td>WAT (nmol/g)</td>
<td>4.49 ± 0.84</td>
</tr>
<tr>
<td>Kidney (nmol/g)</td>
<td>13.34 ± 12.44</td>
</tr>
<tr>
<td>Muscle (nmol/g)</td>
<td>0.32 ± 0.09</td>
</tr>
</tbody>
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