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A STUDY ON THE EFFECTS OF VITAMIN A MARGINAL DIET ON THE ADULT EXPRESSION OF GENES, RETINOL CONTENT IN LUNGS, AND MORPHOLOGY OF LUNGS

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ABSTRACT

A clinically significant public health problem affecting children worldwide is vitamin A deficiency. Our knowledge of vitamin A (VA) effects on the ontogeny of the lung is limited despite the worldwide relevance of vitamin A deficiency. To better understand the impact of vitamin A deficiency on the lungs, this thesis aims to reveal how a diet of vitamin A adequate compared to vitamin A marginal status impacts the expression of retinoid-regulatory genes during ontogeny (early postnatal life), proliferation-associated genes, and surfactant proteins, and morphology of lung tissue. Rats were assigned a nutritionally adequate diet with marginal or adequate VA until adult age, 8 weeks old. Genetic expression of homeostatic and proliferation-associated genes was determined by (q)RT-PCR and retinol content was measured using UPLC. Histology was used to analyze the lung's morphologic characteristics. UPLC results indicated lower levels of ROH in the VA deficient lung. Expression of homeostatic and proliferation-associated genes did not vary significantly between the VA adequate and VA marginal rats. mRNA expression of EGFR was significantly lower in the VA deficient lungs, the only gene that was significantly different. Alveolar counts, a measure of lung development, were generally lower in the VA deficient rats and varied more throughout the lung of VA deficient rats, but there was no significant difference. Overall morphology did not vary drastically between the VA adequate lung and VA deficient lung. Vitamin A deficiency did not have a severe impact on the adult lung and most genes regulating VA metabolism and lung development were not affected. In conclusion, adults may have stronger resilience than neonates against the potential effects that vitamin A deficiency poses on the lungs, or adults may have a protective mechanism to compensate for the effects of low dietary VA.

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List of Abbreviations

VA = vitamin A
EGFR = epithelial growth factor receptor
VAD = vitamin A deficiency
WHO = World Health Organization
ROH = retinol
BPD = bronchopulmonary dysplasia
VADD = vitamin A deficiency-related disorders
RE = retinyl esters
RA = retinoic acid
CRBP = cellular retinol binding protein
LRAT = Lecithin retinol acyltransferase
RALDH 1 = Retinaldehyde dehydrogenase 1
RALDH 2 = Retinaldehyde dehydrogenase 2
RALDH 3 = Retinaldehyde dehydrogenase 3
RBP = retinol binding protein
CRABP = cellular retinoic acid-binding proteins
STRA6 = "Stimulated by retinoic acid 6" gene
CYP26B1 = Cytochrome P450, family 26, subfamily B, polypeptide 1
RAR = retinoic acid receptor
RXR = retinoid X receptor
VEGF = vascular endothelial growth factor
PDGFRα = plate-derived growth factor alpha
SP-A = surfactant protein A
SP-B = surfactant protein B
SP-C = surfactant protein C
VAA = vitamin A adequate
VAM = vitamin A marginal
(qt)-PCR = reverse-transcriptase polymerase chain reaction
UPLC = ultra performance liquid chromatography
H&E = Hematoxylin and Eosin

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Chapter 1: Introduction

Vitamin A deficiency (VAD) is a public health problem severely affecting infants in at least 60 developing countries throughout the world. Attention was first drawn towards VAD when ocular manifestations such as night blindness in humans and animals were detected.[1] Appropriate dietary interventions eradicated VAD and the illnesses associated with VAD in the 20th century, but VAD remained a prevalent health issue in developing countries. Today, VAD is one of the most common micronutrient deficiencies in the world.[1]

More than half of all the countries in the world are battling the public health problem of VAD.[1] VAD is most notably seen in developing countries located in Southeast Asia and Africa.[1] Within developing countries, high rates of poverty, poor health infrastructures, economic constraints, sociocultural limitations, high levels of infectious diseases, and food instability exist, conditions which render limited access to adequate amounts of food containing vitamins needed for healthy body functioning. In socioeconomic circumstances like these, inadequate intake of nutrients is unavoidable, and the onset of vitamin deficiencies are likely to follow in suit. Those most likely to develop VAD due to chronically low intakes of VA are populations who have high demands for larger quantities of vitamins including, infants, children, pregnant women and lactating women.[1]

Deficiency of VA is defined as the failure to meet physiological needs due to chronically insufficient levels of VA. According to the World Health Organization (WHO), the biochemical definition of VAD is a serum retinol (ROH) threshold of less than 0.70 μmol/L.[1] The primary

causes of VAD are chronically low dietary intake of VA that cannot meet the physiological needs of the body.[1]

Deficiency of VA is a large contributor of mortality and morbidity due to its leading roles in immune competence, maintenance of cell growth, red blood cell production, epithelial integrity, and normal functioning of the visual system. VAD is the leading cause of preventable blindness, or xerophthalmia, in children. WHO estimates, "approximately one third of the world's preschool-age population is estimated to be vitamin A deficient, with just less than 1% being night blind at a given time."[1] VAD in infants increases risks of developing bronchopulmonary dysplasia (BPD), the most chronic form of lung disease in infants.[2] Additionally, deficiency in VA increases the risk of developing diseases, most of which are easily preventable, and death from severe infection by weakening resistance to infection. Even prior to the onset of vitamin A deficiency-related disorders (VADD), the ramifications of infections and diseases, like measles, diarrhea and malaria, are heightened when there are inadequate stores of VA. Lack of VA in the body imparts a risk of developing easily preventable diseases, and hinders the body's recovery from infection. In fact, VAD is thought to be the leading cause of death due to infectious diseases worldwide. A myriad of research confirms the important role of VA for maintaining the immune system to function properly. Measles and infections causing diarrhea are commonly seen in individuals with a deficient VA intake. Infections associated with diarrhea and respiratory infections exacerbate the problem of low intake of VA by decreasing appetite and absorption and increasing excretion of vitamins. To this day, measles, along with several other diseases are managed by the use of VA supplementation, as suggested by WHO and UNICEF.[1]

One of the lesser researched areas of VAD is its effects on the lungs and development of lungs in adult rats, specifically how deficiency of VA affects the expression of retinoid-regulatory and proliferation-associated genes during ontogeny. Additionally, there is a lack of knowledge, particularly in regards to the effects of VAD on the metabolism of VA during lung development, expression of genes involved in VA metabolism, quantity of VA, and morphological characteristics of the lung. Implications of deficiency of VA on the lung of infants and adult requires more research in order to achieve a better understanding of the possible detrimental effects of VAD. Studying the ontogeny of lung development in animals that differ in VA status will expand our understanding of how important VA is involved in preventing chronic lung diseases and chronic illnesses associated with VAD. Exploring this missing link will help to better understand how VA functions in the body, while providing insight into effective methods of preventing and treating VAD in infants, children, and adults. Furthermore, insight gained from this study can be utilized to devise appropriate treatments of VAD-induced BPD. By comparing peer-reviewed literature articles of the relationship between VA and the growth and development of the lungs, the following literature review will show that further research in this field is warranted.

Chapter 2: Literature Review

What is Vitamin A?

VA is the general term used to define retinoids demonstrating biological activity of ROH. VA is an essential micronutrient necessary for normal reproduction, embryonic development, cell and tissue differentiation, vision, and immune function.[1, 3] The fat-soluble vitamin is naturally present in many foods. Preformed VA and provitamin A are two main forms of VA in the diet. Preformed VA, primarily ß-carotene, α-carotene, and ß-cryptoxanthin, is found in meat, poultry, fish, and dairy products. Provitamin A, primarily retinyl esters (RE) and retinol, is found in fruits, vegetables, and plant-based products. Another source of VA found in plants are carotenoids. A-catotene, ß-carotene, ß-cryptoxanthin, lycopene, lutein, and zeaxanthin, most notably are carotenoids providing sources of VA. ß-carotene exhibits the greatest amount of provitamin A activity. Preformed VA and provitamin A are important precursors for the biosynthesis of metabolites of VA.[3]

VA has many metabolic forms including ROH, RE, retinal, and retinoic acid (RA) (Figure 1). ROH is the active form of VA. RE is the major form of VA in the body and the form stored in tissues. 11-*cis*-retinal and all-*trans* RA (Figure 1) are essential components in the metabolic pathways of VA and are quintessential chemicals for normal cellular activity and bodily functions. 11-*cis* retinal is very important because this form is required for vision. All-*trans* RA, the most bioactive form, is required for cell differentiation and the regulation of gene transcription in many tissues.[4]

Metabolism of Vitamin A

VA needs to be digested to be absorbed. Upon ingestion, VA is bound to other food components. ROH is bound to fatty acids and RE and carotenoids are usually bound to proteins. Pepsin in the stomach hydrolyzes the carotenoid and RE from proteins. Fat globules form when RE and carotenoids coalesce. The fat globules move to the duodenum where enzymes continue to digest the lipids by hydrolases and esterases. Fat is required for digestion and absorption of VA. Bile emulsifies the fat globules to be broken down to smaller droplets. Micelles form and transport carotenoids and preformed VA across the brush border of the small intestine. Preformed VA is absorbed more efficiently than carotenoids.[3, 4 ,5]

VA metabolism begins in the enterocyte. In the enterocyte, carotenoids are cleaved to yield 2 retinal molecules by 12,15'-monooxygenase. Retinal is reduced to ROH or oxidized to RA as shown Figure 2. RA exits the enterocyte and is transported through the blood by albumin to the liver. Retinal and ROH are bound to cellular retinol binding protein (CRBP) II once in the cell. Retinal reductase converts retinal to ROH. CRBPII-bound retinol is esterified with a fatty acid by enzyme lecithin: retinol acyl transferase (LRAT). Retinyl palmitate bound to CRBP II is the most common product of esterification. RE's are incorporated into chylomicrons for transport to other areas of the body. Chylomicrons leave the enterocyte and enter the lymph. Chylomicrons deliver VA to several organs of the body including bone marrow, blood cells, spleen and adipose tissue. Chylomicron remnants, composed of RE and carotenoids, are shuttled to the liver. Within the liver, carotenoids may be cleaved to produce ROH, incorporated into very low density lipoproteins or other lipoproteins, or are stored in the liver.[3, 4, 5]

RE are released into the hepatocyte from a chylomicron. RE hydrolase converts RA to ROH. ROH binds to CRBP and LRAT or ARAT esterifies CRBP-retinol forming RE. The RE

may be stored in hepatic stellate cells. Besides conversion to RE, ROH has several fates. CRBP-retinol is converted to CRBP-retinal by NADPH-dependent ROH dehydrogenase. CRBP-retinal is then converted to RA by retinal dehydrogenase (RALDH). The family of RALDH consists of RALDH 1, RALDH 2, RALDH 3, and RALDH 4. Conversion of ROH to retinal to RA is depicted in Figure 2. Retinal conversion to RA is irreversibly catalyzed by RALDH. ROH may bind with retinol binding protein (RBP); once bound to RBP, ROH may exit the hepatocyte and travel through the blood with the help of transthyretin, also known as pre-albumin. RBP synthesis is dependent on ROH status; if there is low ROH, RBP synthesis increases. The last fate of ROH is conjugation with glucuronic acid to form retinyl ß-glucuronide, a VA derivative excreted in the bile, also shown in Figure 2. The RA produced from ROH binds with cellular RA binding proteins (CRABP). CRABP-retinoic acid can be conjugated to glucuronic acid and excreted into the bile, similar to ROH, or moved to the nucleus to regulate gene expression controlling cellular differentiation and other bodily functions.[3, 4, 5]

ROH bound to transthyretin is transported to other cells through the blood. Transmembrane proteins take up ROH complex via endocytosis. These transmembrane proteins are encoded by STRA6. STRA6 is an integral transmembrane cell-surface receptor protein for ROH-RBP. ROH-RBP is the form by which ROH is taken up to the cell. LRAT, STRA6, RALDH 1, 2, and 3, and CYP26B1 are classified as homeostatic or retinoid-regulatory genes for their role in regulating VA levels throughout the body.[3] Once ROH enters the cell, LRAT, reversibly esterifies all-*trans* ROH to all-*trans* RE or reversibly converted to retinal by ROH dehydrogenase. Likewise, RE can be mobilized back to ROH.[3,4,5] Following treatment of adult rats with VARA, which is VA combined with its metabolite RA, the expression of STRA6 increases further confirming the role of STRA6 in the uptake of ROH into the cells and lung.[6]

LRAT is very important in VA metabolism due to its responsibility to esterify all-*trans* ROH needed for the proper functioning of the visual system.[7] RE are stored mainly in liver and several other tissue including the lung and kidney, and in adipose which contains both RE and ROH. Upon a deficiency of dietary VA, the rat mobilizes RE from these storage tissue.[6, 8, 9] RE and carotenoids, the storage form of VA, are stored in fat tissue. Lipoproteins transport RE and carotenoids throughout the body. Another enzyme called Cytochrome P450, family 26, subfamily B, polypeptide 1 (CYP26B1), is a key regulator for all-*trans* RA. CYP26B1 inactivates all-*trans* RA to hydroxylated forms. Expression of CYP26B1 in adult rats increased following treatment of VARA, demonstrating its involvement in the processing of VA in the body.[6]

Functions of Vitamin A

VA is an essential micronutrient responsible for proper immune function, and normal vision, development, differentiation, survival and death of epithelial cells, normal reproduction, and embryonic development.[1, 3, 4]

VA functions as an antioxidant; carotenoids, more specifically, are classified as a lipid-soluble antioxidant because their structures contain conjugated double bonds that can quench free radicals. Immune function is another very important role that VA maintains. Phagocytic activity and cytokine production increased in the presence of VA. T-cells need VA for proper functioning and B cells for the antibody response to infectious microbes or viruses. The protective capabilities of VA help the body fight off infections. In fact, animals and humans are at risk for increased infections if they are VA-deficient because humoral response to parasitic,

bacterial or viral infections, immunity (cell-mediated and mucosal), and T-cell activity are diminished from VAD.[1, 3, 4]

Proper functioning of the vision cycle requires VA. ROH produced is transported into the retina by the holo-RBP-transthyretin complex. ROH is then transported into the endothelium of the rod cell. LRAT esterifies ROH to give all-*trans* RE, and all-*trans* RE is converted to 11-*cis* ROH. 11-*cis* ROH is converted to 11-*cis* retinal, which attaches to opsin to form rhodopsin. Rhodopsin is found in the rods of the retina and are needed for healthy vision function. Rhodopsin is cleaved when light hits the molecules, and the 11-*cis* retinal component of rhodopsin converts to *trans*-retinal. Following this event, the brain receives signals involved in eyesight. If an individual persists with suboptimal VA levels, rhodopsin will be absent and rods will not function properly. Therefore, it is not uncommon for VA deficient individuals to lose the ability to see at night, a condition known as night blindness.[3, 4]

Another function of VA, and highly relevant to this study, is its role in assisting the differentiation of various cells, including epithelial cells on our skin and lining the respiratory tract, gastrointestinal tract, and urogenital tract and helping to maintain the structure and function of these cells. VA's role in cellular differentiation and proliferation is essential to maintain healthy vision and lung function because epithelial cells need VA to maintain their normal structure and function. One example where RA controls differentiation of cells is the differentiation of immature skin cells, called keratinocytes, into mature epidermal cells. VA functions as a signal to turn on genes encoding keratin and needs to be present for transcribing and translating keratin molecules. Squamous epithelial keratinizing cells differentiate into mucus-secreting cells with the help of VA. A lack of VA halts the differentiation of keratinizing cells into goblet cells. With too few goblet cells, whose function is to produce mucus, and too

many epithelial keratinizing cells, xerosis of the eye results, as well as complications in the respiratory and urinary tract, or any area where mucus is needed for proper functioning.[4, 10] The mucus aids in providing antimicrobial defenses to the tissue. But these defenses decrease when there is less VA available in the body. Additionally, there is a lack of regeneration and differentiation of cells lining protective tissue. As a result, the tissues will flatten and keratinize.[11] Therefore, the problem is two-fold; due to the lack of mucus produced by goblet cells, complications where mucus is needed for normal function, such as the lungs, arise and susceptibility to infection increases. The differentiation of myeloid precursors into myeloid dendritic cells, cells that present antigens to T-cells increasing immune response from the body, is also regulated by VA. This is another way in which lack of VA lowers the body's immune response to infection.[4]

VA impacts gene expression of proteins through its role as a nuclear regulator. All-*trans* RA or 9-*cis* RA moves into the nucleus using CRBP, and attaches to nuclear retinoid receptors. 11-*trans* RA binds to retinoic acid receptor (RAR) and 9-*cis* RA binds to retinoid X receptor (RXR). RAR and RXR bind to the RA response elements located on the promoter region of DNA. Binding of RXR and RAR promotes transcription of the DNA, thus initiating changes in protein synthesis.[3, 4]

VA has a role in growth, specifically growth in epithelial cells. Gap junctions, or channels between cells involved in cellular communication that occurs during growth in epithelial cell, are maintained through adequate VA status.[4] Overall, VA is an essential nutrient responsible for proper immune function, normal vision function, differentiation of cells, survival and death of epithelial cells, and development and growth. The following two sections of the

literature review will expand on how these functions are of particular importance for the normal development and function of the lungs.

What are the effects of vitamin A deficiency?

Due to the high demand of nutrients during critical periods of growth, neonates need to receive adequate amounts of VA to set the stage for normal development throughout infancy and childhood. Moreover, the late fetal and postnatal periods are of significant importance for VA to promote lung growth. Deficiency of VA during the neonatal critical time period, aged from the time of birth to 28 days old, can have irreversible consequences on the body.[6, 12] Therefore, adequate delivery of VA to neonates is fundamental for healthy development of the newborn and to prevent the onset of VADD throughout childhood and adulthood.

The WHO[1] emphasizes that there is a plethora of effects infants experience from lack of VA in their diet, further stressing that research in this field is invaluable and a hot topic for improving public health. Infants with insufficient levels of VA are at risk for mortality and likely to develop VADD during childhood and adulthood. VADDs include xerophthalmia, anemia, weakened resistance of infections, and poor growth.[1]

The most notable ramification of VAD are the effects on the eyes. Keratinization on the eye causes xerophthalmia, which is characterized by drying and thickening of the cornea. Xerophthalmia, is the most preventable form of blindness in children and encompasses all the vision abnormalities induced by VAD including the following: night blindness, conjunctival xerosis, Bitot's spot, corneal xerosis, corneal ulceration/keratomalacia, corneal scarring, and xerophthalmic fundus.[1, 4] Xerophthalmia results in various pathologies including Bitot spots,

drying and thickening of the cornea, corneal scarring and night blindness. Blindness at night results from impaired production of rhodopsin in cells of the retina, specifically rods. VA is needed for the epithelial cells of the respiratory tract and urinary tract as well. In the eye specifically, epithelial cells change from columnar epithelial cells to stratified epithelial cells. This is accompanied by loss of goblet cells (mucus-secreting cells), formation of a granular cell layer, and keratinization of the surface.[4, 13] Additionally, other areas of the body including the respiratory tract and urinary tract will accumulate keratin, leading to abnormal development and functioning of the tissues. For example, VAD is associated with inadequate lung development in children, and contributes to BPD.[12, 14]

Low VA concentration also decreases the body's antioxidant protection from free radicals, factors contributing to development of chronic diseases, and make infants more susceptible to other illnesses that accompany VAD.[15]

VAD during infancy and continuation throughout childhood may facilitate these effects later, during the stages of childhood and adulthood. Therefore, effective supplementation with VA during the neonatal stages of lung development may be critical to prevent the onset of these disorders explained by WHO[1] and documented by Jobe.[16] In light of these findings and the continued incidence of VAD and its associated diseases, and mortality, it is imperative to answer what are the effects of VAD, from birth to adult age, on lung status in adults, an area of research currently lacking attention. It is important for several reasons to understand how the lungs of adult rats may change as a result of low dietary VA intakes. One reason is that we can better understand the future health outcomes later in life of children who are VA deficient.

What is vitamin A's role in lung development?

VA has a crucial role in proper lung development.[1] The expression of genes for elastin, vascular endothelial growth factor (VEGF), platelet-derived growth factor receptor (PDGFRα), epidermal growth factor receptor (EGFR), and α-smooth actin (α-SMA), considered structural and proliferation-associated genes involved in lung development, may be altered due to lack of VA. Elastin is a critical structural, fibrous, protein responsible in moderating elasticity and resilience of lung tissue. Elastin fibers allow the lungs to stretch during inhalation. Normal lung function requires elastin, and when the lung is damaged, elastin is needed to repair lung lesions. Other tissues where elastin plays an integral role in these functional characteristics include the skin, large arteries, tendons, ligaments, and elastic cartilage.[17] VEGF is the protein that regulates the formation of red blood cells from pre-existing blood cells, also known as angiogenesis, and has a major function on endothelial cell function.[18] VEGF is highly expressed in the lung due to its vital physiological role in the development of the lung and maintenance in the adult lung. Evidence further suggests severe acute and chronic lung diseases are influenced by alterations in VEGF.[19] PDGFRα plays a role in the alveogenesis. α-SMA is in fact induced by PDGFRα expressing cells during alveolar development.[20] EGFR controls lung morphogenesis including epithelial branching and alveolarization by regulating matrix metalloproteinases-mediated activation of gelatinase.[21] α-SMA is the predominant protein in smooth muscles. Specifically in the lungs, α-SMA plays a major role in the development of airways and pulmonary vessels during branching morphogenesis and it is necessary for structure and integrity of cells. α-SMA is also involved in the myofibril-like cells during lung repair.[22] Restoring normal lung function and structure following an injury requires many of these proliferation-associated genes acting in concert.

Surfactant proteins A, B, and C (SA-A, SA-B, SA-C) are proteins involved with the regulation of specific lung properties such as pulmonary surfactant, a critical "biological detergent". By lowering the surface tension of the alveoli air sacs, surfactant prevents the alveoli from collapsing during exhalation and adhering together. A lack of surfactant renders the lungs dysfunctional, as surfactant contributes toward normal physiological functioning of the lungs, and makes the lungs prone to infection.[11, 23] Diseases of this nature are usually fatal, as seen in the condition congenital alveolar proteinosis.[24]

Due to these properties, the genes that code the enzymes and proteins involved in lung development and proper lung function will be analyzed in VA deficient and VA adequate rats. There is a lack of research confirming whether the expression of these genes are altered from VAD. One of the questions this study will seek to answer is if low dietary intake of VA changes expression of the genes, and if morphological changes occur in the lung following chronic low dietary VA intake, lend reasoning to why these changes occur.

In addition to examining expression of homeostatic, proliferation-associated genes, and surfactant proteins, this study aims to examine differences in morphology of the lung between rats fed a vitamin A adequate (VAA) diet and rats fed a vitamin A marginal (VAM) diet. Research does suggest lung development in neonates abnormalities spawn from deficiencies in VA, as shown in the research of Jobe.[16] Jobe[16] reviewed BPD, a chronic lung condition induced by hyperoxia and often found in premature infants characterized by inflammation and scarring of the lungs.[16] Hyperoxia is known to induce inhibition of lung septation and impair alveolar development.[25] Additionally, those infants diagnosed with and treated for respiratory distress syndrome, a breathing disorder characterized by immature development of the lungs and inability to produce adequate surfactant, are likely to develop BPD. Infants with BPD often have

low VA concentrations.[26] In general, BPD is one of the most common causes of long-term disability in infants born prematurely. Lung immaturity and lack of proper lung development, particularly at the saccular to alveolar stages of lung development, are linked to BPD. Exposure of the premature lungs to oxygen causes an array of detrimental effects that allow the disease to persist. Collagen, elastin, and proteoglycan deposition change, causing a loss of surface area of the lung. Emphysematous changes result in impaired septal formation and reduced alveolar formation. However, administration of VA has been shown to have a reparative role and to decrease the severity of BPD. Supplementation using RA induced septal formation and alveolar formation.[16] Additionally, asthma is another condition that may be exacerbated by VAD due VA's intricate role in lung development. Suboptimal levels of VA in animal models demonstrated adverse effects on lung development and researchers believe lack of VA heightens the risk of developing asthma.[15]

Research confirms VA has a role in lung structure development in neonates, in addition to its role in preventing abnormal health conditions of the lung. According to Esteban-Pretel et al.[27], during the neonatal period, VA in the lungs is vital to the formation and maintenance of alveoli, as well as their function post-development. Gene expression is regulated by RA through RA's interactions with receptors on the nucleus. The nuclear receptors are ligand-activated transcription factors that influence the transcription of DNA to mRNA in the production of proteins, ultimately affecting normal function of the cells comprising the lungs. Due to the pivotal role that VA plays in the development of alveoli, the building block of the lungs, a lack of VA can have huge ramifications on the function of the lungs. In fact, it may explain why VA inadequacies are associated with neonatal lung disorders. The alterations of gene expression, caused by the lack of RA, change the formation of tissue and facilitate the malfunction and

damage of the lungs.[27] VAD exerts a multitude of effects at both the macroscopic level and the molecular level, resulting in improper and immature lung development and development of disorders including BPD.[10]

Esteban-Pretel et al.[27] further affirmed that VAD alters the process of normal formation, architecture, and composition of the basement membranes by affecting the gene expressions of proteins collagen and laminin that control these processes. Composition of collagen IV and laminin, components of the basement membrane, were decreased in abundance under VA deficient conditions. Furthermore, the application of RA in VA deficient rats subsequently increased collagen IV chains and laminin β-γ chains. Collagen and laminin are also found to regulate gene expression and promote cell differentiation by mediating the binding of integrins to the specific types of collagen and laminin. Their ability to modulate these affects translates to the ability to alter lung development. Therefore a lack of RA could change composition of collagen and laminin, possibly inducing improper lung tissue formation and function.[27]

Lung maturation of rats was also affected by marginal VAD. Alveolar counts, total alveolar surface, and elastic fibers decreased in the VA deficient rat, while septa wall thickness and collagen fibers were increased in VA deficient rats of infant and adult age.[10] Normal lung structure was observed to be restored if rats were supplemented with VA during early post-natal stages. Therefore dietary intervention at birth restored damage inflicted on the lung due to lack of VA.[28, 29, 30]

Surfactant protein levels decreased in fetal lungs during neonatal development when rats were VA deficient.[31] Therefore VAD may be responsible for delayed lung maturation. The reduction of plasma ROH correlated with reduction of lung surfactant phospholipids, major surfactant phospholipids, and de-saturated phosphatidylcholine and a decrease in expression of

fatty acid synthase, the controller of synthesis of lipid precursors. The work of Chailley-Heu et al.[31] and Esteban-Pretel et al.[27] similarly showed how VA controls lung development by changing the expression of fatty acid synthase. In addition to the effects on expression of genes and amount of surfactant, the cells that produce surfactant itself, inadequately matured in VA deficient rats. The maturation of the type II alveolar cells was limited, causing a drop in surfactant production.

Antipastis[32] conducted a study examining rat pups born to VA deficient mothers. Overall, rats exhibited signs of VAD, including retarded lung maturation. Elastin mRNA expression decreased in the lung of fetuses and neonatal rats born to VA deficient mothers. Lungs of the fetus had a significant decrease in elastin mRNA and growth arrest-specific gene 6 (gas 6) expression in the fetus. However, the decreased mRNA expression of elastin in neonates was not significant. gas6 is involved in adhesion of cells necessary for producing the surface size where gas exchange occurs. The change in gene expression based on low VA status helps explain the biochemical basis for why changes in lung development are seen in VAD.[32] One study indicated VA is responsible for changes in VA metabolism through the action of CRBP.[33] Low VA intake may reduce the mRNA gene expression and diminish synthesis of proteins involved in lung development. This may perpetuate abnormal morphology due to decreased expression of genes responsible for the maturation and normal morphology of the cells.[32, 34] For example, VA binding to RAR is needed to induce lumen formation in alveoli, glandular glands and ducts.[35]

The evidence from several studies implies the importance of adequate amounts of VA for proper lung development. There is a consensus between Jobe[17], Esteban-Pretel et al.[27], Chailley-Heu et al.[31], and Antipastis[32] that a lack of VA in the diet results in major physiological issues in

lung development by directly affecting some gene expression, development of alveoli, composition of cells, and alveoli formation.[27, 31, 34] Even though these studies examined different qualities of lung development, the research similarly implied that levels of VA alter gene expression, which consequently controls the development of the lungs. However, there has been no aspect of these studies that have measured levels of expression of homeostatic or proliferation-associated genes that may be affected by suboptimal VA status. In addition, previous studies have not confirmed the presence of VAD by measuring VA content in the lung. VAD in animal studies is typically observed as differences in lung morphology between VA adequate and VA deficient rats; however, measuring ROH content will expand our knowledge of the lung's access to VA and its storage, which may help to explain why development of the lung is negatively affected by a suboptimal VA dietary intake. Additionally, there is a lack of research confirming the effects of VAD on adult lungs. Overall, these studies confirm VAD hinders normal lung development. Therefore, VA is beneficial and crucial for lung development of neonates.

Problem Statement/Hypothesis

There is little literature concerning how the morphology of the adult lungs is affected by a VAM diet during ontogeny, from infancy to the time of adulthood. This study aims to provide insight into how the body, specifically the lungs, are affected by a lack of VA by examining the effects of VAD diets on lung ontogeny, the expression of retinoid-regulatory and proliferation-associated genes, and the morphology of the lungs of rats fed VAM diet compared to VAA diet. The impact of the study will be to provide knowledge on how VAD affects the lung throughout

infancy and up until adulthood. Based on the review of literature, we have several hypotheses on how the VAM diet will affect lung morphology and lung development, as investigated in adult rats that were fed VAD diet from neonatal age.

1. VA deficient rats will experience a decrease in homeostatic, proliferation-associated, and surfactant protein gene expression compared to VA adequate rats.
2. ROH concentrations in VA deficient rats will be less than that of VA adequate rats.
3. Lungs of VA deficient rats will have abnormal morphological characteristics compared to VA adequate rats, i.e., alveolar counts in VA deficient rats will be diminished compared to alveolar counts in VA adequate rats.

Chapter 3: Material and Methods

Dr. A. Catharine Ross, Sarah Owusu, and I designed the experiments. Sarah and I maintained the VA dietary status of rats, euthanized the rats, and dissected the lungs for histology and reverse transcriptase polymerase chain reaction ((q)RT-PCR analysis). I measured the gene expression using (q)RT-PCR and measured the ROH content in the lungs using UPLC. Sarah fixed and paraffin embedded lung tissues for sectioning. In order to study lung morphology of rats, I sectioned the lung tissues using a microtome. I also stained the lung sections and captured digital images of sections in order to study characteristics of the lung using a light microscope. For analysis of alveoli count, I used programs iLastik and ImageJ.

Animal Experiment

All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University, and all animals were handled in accordance with institutional guidelines. A Sprague-Dawley dam was fed a VAM diet from the time of mating. VAM diet contained 0.35 µg retinol/g diet. The dam gave birth to pups on January 14, 2013. Pups received VAM diet indirectly through the maternal transfer of VA via breast milk. Once the rats (n=6) were of adult age (21 days old), they were weaned and fed a VAA or VAM semi-purified diet. VAA diet contained 4.0 µg retinol/g diet. VAM diet contained 0.35 µg retinol/g diet. The levels of VA in the diet are those found in reference 36.[36] VAM diet contained

approximately ten times less ROH than the VAA diet. At 8 weeks of age, each rat received a dosage of oil (the placebo for another treatment, but not included in this project). Treatment dose volumes were calculated using the following equation based on the weight of rats: (0.4 µL x weight (g)) + residual (1µL). Rats were euthanized using CO_2 inhalation 8 weeks after birth. The chest was opened, the left lung was inflated with formaldehyde to be used for histology, then excised and placed in formaldehyde for fixation. The left lung of rats 12, 13, 14, and 15 were used for histology. The right lungs of rats 10, 11, 12, 13, 14, and 15 were rapidly frozen in liquid nitrogen to be used for RNA and UPLC analysis.

Age: Adult				
Rat #	Treatment (Tx)	Weight (g)	Diet (Adequate or Marginal)	Tail Color
10	Oil	262	Marginal	Red
11	Oil	264	Adequate	Green
12	Oil	329	Adequate	Blue
13	Oil	359	Adequate	Black
14	Oil	264	Marginal	Brown
15	Oil	264	Marginal	No color

Table 1: Animal Subjects and Experimental Design. Experiment subjects, treatment, weight prior to euthanasia and diet given between 21 days and 8 weeks old.

Polymerase Chain Reaction

To address how VAA and VAM diet affects ontogenic gene expression, several retinoid homeostatic and proliferation-associated genes and proteins were profiled. Total RNA was extracted from samples using the TRIzol ® (Life Technologies) method. cDNA was prepared using reverse transcriptase. cDNA equivalent to 0.05 µg RNA was used for (q)RT-PCR analysis. mRNA expression in collected lung tissue was measured using (q)RT-PCR. mRNA expression of LRAT, STRA6, CYP26B1, RALDH 1, RALDH 2, and RALDH 3, PDGFRα,

VEGF, α-SMA, EGFR, GAPDH, SP-A, SP-B, and SP-C were measured on samples from rats 10, 11, 12, 13, 14, and 15.

RNA Isolation

RNA was isolated from lung tissue using the TRIzol method. First, lung tissue (50-100 mg) was homogenized in 1 mL of TRIzol reagent or 1 minute at high speed. Tissue volume did not exceed 10% of the volume of TRIzol used for homogenization. Phases were separated after homogenization. Samples were incubated for 5 minutes at room temperature to allow for complete dissociation of nucleoprotein complexes. Two hundred µL of chloroform was added per 1 mL of TRIzol reagent used in the homogenization step, and the tube of sample and reagent was shaken by hand for 15 seconds. The samples and reagent were incubated for 2-3 minutes at room temperature. Following incubation, samples were centrifuged at 12,000-16,000 x g for 15 minutes at 4°C to separate the mixture into phases. The upper colorless aqueous phase containing RNA was removed and placed in a new centrifugation tube. The remaining contents including the lower red phenol-chloroform phase and the interphase were discarded.

The third step for the RNA isolation procedure precipitated the RNA from the collected upper phase. RNA was precipitated by adding 500 µL of 100% isopropyl alcohol per 1 mL TRIzol reagent. Tubes were inverted 2-3 times, and a cloudy precipitation began to form. RNA precipitate and reagents were incubated for 10 minutes at room temperature, centrifuged at 12,000 x g for 10 minutes at 4^0C. The supernatant from the tube was removed, leaving the RNA pellet behind. In order to carry out the fourth step, which is the RNA wash step, 1 mL of 75% EtOH in DEPC ddH_2O per 1 mL of TRIzol Reagent was added to the RNA pellet. The samples

were briefly vortexed, and then centrifuged at 7,000 x g for 5 minutes at 4°C. The wash was discarded. The pellet was dried for 5-10 minutes under the ventilation hood. The fifth step of RNA isolation was re-dissolving RNA. RNA was dissolved in DEPC ddH_2O (50 µL for 50 mg of initial tissue). Samples were stored in -80°C fridge for long-term storage. A Nanodrop instrument was used to determined RNA concentration and quality. A 260/280 value for all the tissues ranged from 1.92-2.00 and were considered of good quality for further use.

cDNA Preparation

It was imperative to have kept the reagents on ice, due to their sensitivity to temperature. The mastermix (MM1) was first prepared using 0.2 µL of Oligo dT per sample, 2 µL of dNTP/sample, and 7.8 µL of DEPC/RNA free ddH_2O per sample. dNTP was added last to MM1. The master mix was vortex and centrifuged. 10 µL of MM1 was added to 5 µL of each sample. Therefore, total volume per tube was 15 µL. Tubes were incubated in the GeneAmp PCR System 9700 thermal cycler at 70°C for 5 minutes. Tubes were immediately placed in ice after 5 minutes, and cooled for 5-10 minutes. Tubes were gently vortexed and centrifuged briefly to ensure all liquid was at the bottom of the tube. A second master mix, MM2, was prepared which included 4 µL of 5X buffer per sample, 0.5 µL of RNAsin per sample, 1 µL of RT per sample, and 4.8 µL of ddH_2O per sample. MM2 was vortexed and centrifuged. 10 µL of MM2 was added to the sample and MM1 mixture, yielding a total volume of 25 µL per tube. Tubes were vortexed gently and centrifuged briefly to ensure all the liquid was at the bottom of the tube. The samples were placed in the GeneAmp PCR System 9700 thermal cycler and run using the Promega protocol. The Promega protocol is as follows: 42^0C for 90 minutes, 95^0C for

5 minutes, and 4^0C for 90 minutes. Each cDNA sample was diluted 5 times by adding 125 µL of DEPC/RNA free ddH_2O before PCR analysis.

(q)RT-PCR

cDNA, prepared using reverse transcriptase, was used for (q)RT-PCR. cDNA, equivalent to 0.05 µg RNA, Bio-Rad reagents and real-time PCR instrumentation, specifically DNA Engine Opticon 2 Continuous Fluorescence Detector, was used to run samples for real-time PCR analysis. A master mix was prepared which included 10 µL of SYBR green per sample, 4 µL of H_2O per sample, and 1 µL of primer per sample to yield a volume of 15 µL of master mix. Once the SYBR green, H_2O and primers were pipetted into the plate wells, 5 µL of cDNA for each sample was added to each well and. Master mix was prepared on ice. The PCR program that the samples were run on is as follows in this order: 94^0C for 10 minutes (activation of SYBR green), 94^0C for 15 seconds (denaturing), 60^0C for 1 minute repeated 40 times (annealing), 75^0C for 30 seconds (extension), 75^0C for 1 second (extension), 72^0C for 10 minutes, and 4^0C upon completion. This protocol was repeated for each gene or protein used in this study.

PCR Statistical Analysis

mRNA-to-GAPDH RNA ratio for relative gene expression was calculated to determine the gene expression. A series of calculations were completed to obtain the ratio mRNA-to-GAPDH RNA ratio. The average of all the C(t) values obtained by (q)RT-PCR for each sample using the primer, for example STRA6, was calculated. The difference (delta C(t)) between the C(t) value and the average C(t) was calculated for each sample. 2^-deltaC(t) was then

determined for each sample. The ratio of 2^-deltaC(t) of STRA6 and 2^-deltaC(t) of GAPDH was computed, followed by the average of the ratio. Values were normalized using the average ratio. Because each sample was analyzed twice, an average of the normalized ratio was obtained. The average of the latter values were taken to determine the mRNA fold change. The fold change of the group of samples from the VA adequate rats was set at 1.00 prior to statistical analysis. Averages of the normalized ratio of of 2^-deltaC(t) of STRA6 and 2^-deltaC(t) were graphed using PRISM. Statistical analysis was conducted using PRISM 6.0. The statistical test performed was a two tailed unpaired t-test with a 95% confidence interval and determined statistically significant or non-significant differences between the mRNA expression of each gene or protein for rats fed a VAA diet versus rats fed a VAM diet.

Ultra Performance Liquid Chromatography

While working with the lung tissues, all lights were turned off and window shades were closed so as not to diminish the content of VA extracted from the tissue and skew results. About 0.5 g of lung tissue was weighed from each sample. 1.5 mL of ethanol was added to the sample. Lung tissue was homogenized for 5-10 minutes with a glass rod, which was rinsed with 0.25 mL of EtOH twice so that the final volume was 2.0 mL. Samples stood at room temperature for 1 hour. After standing for 1 hour, 100% potassium hydroxide (1 g KOH/mL H_2O (Milli-Q water)) was added and samples were vortexed on the Vortex Genie 2 for five seconds; thereafter, 20% pyrogallol (0.2 g pyrogallol in 1 ml EtOH) was added to each sample and samples were shaken for five seconds. Samples were incubated at 55°C in a water bath shaker for 30 minutes. After 30 minutes, samples were taken out of the water bath and cooled down to room temperature

under a circulating air hood. Following cooling down, 4 mL of hexanes was added to each sample and vortexed to mix. 2 mL of double distilled H_2O were subsequently added to each sample and vortexed to mix. Samples were stored in the cold room overnight. The next day, samples were taken out of the cold room and centrifuged at 1600 revolutions per minute at room temperature for 10 minutes. Upper hexanes phase of each sample were transferred into a 7 mL vial. Volume of the upper hexanes phase was about 3.5 mL. The amount of TMMP added to each sample was calculated. The concentration of TMMP per sample (50 pmol) divided by the concentration of TMMP (6.84 pmol/µL) yielded 7.31 µL of TMMP to be added to each sample. The 3.5 mL hexanes extraction and TMMP were dried using nitrogen in a 37^0C water bath for 20 minutes. Vials were rinsed with 1 mL of hexanes and dried for approximately 5 minutes after. Once again, vials were rinsed with 0.5 mL of hexanes and dried for approximately 5 minutes. The purpose of rinsing the vials with hexanes was to bring ROH attached to the side of the vial to the bottom. It was important to make sure no hexanes were left over, as this would decrease the quality of the UPLC calculation of retinol content. Samples were cooled down to room temperature for 2-3 minutes. After the samples were cooled, 150 µL of methanol was added to each vial for the purposes of reconstituting the ROH. Reconstituted ROH was transferred into an insert placed in a mini vial for preparation of UPLC. Vials were centrifuged at 1600 rpm for 2 minutes at room temperature. Samples were loaded into the Acquity™ UPLC instrument and processed.

A series of calculations were completed to determine the ROH (µg/g) in each lung tissue sample. Using the known TMMP concentration (pmol) and area of TMMP and ROH determined by UPLC, the ROH concentration (pmol) was computed using the following ratio:

TMMP concentration/TMMP area = ROH concentration/ROH area

ROH pmol per gram (ROH pmol/g) of tissue was calculated using the computed ROH concentration and the weights of tissue of each lung used. Finally, ROH concentration as µg/g was calculated. The averages of ROH concentration (µg/g) for rats fed the VAA diet and VAM diet were computed and graphed.

UPLC Statistical Analysis

Statistical analysis was conducted using PRISM 6.0. The statistical test performed was a two tailed unpaired t-test with a 95% confidence interval and determined statistically significant or non-significant differences between the ROH measured in the lungs of rats fed a VAA diet versus rats fed a VAM diet.

Histology

Histology Sample Preparation

Four standard steps were followed to prepare the samples for microscopy including fixation, processing, embedding, and sectioning. The lung tissue was preserved using formaldehyde. Lung samples were fixed using a method called perfusion fixation, where the lungs were inflated via infusion with formaldehyde prior to excision and stored in formaldehyde until processing. Lung samples were dehydrated and paraffin wax, with which the samples were infiltrated, was prepared. Embedding is the third step completed to orient the tissue that can be easily used for sectioning. Tissues infiltrated with paraffin and cooled were placed in a cassette and placed in the paraffin wax. The cassette was then removed from the wax to be cooled on a

cooling surface of the Microtome machine. Once cooled, the cassette was trimmed of excess wax. Once embedding was complete, samples underwent sectioning to produce very thin sections of tissue. Lung tissues was sectioned using a microtome. All sections were 5 µM thick. Sections were placed on a microscope slide to be stained.

Tissue and Sectioning of Paraffin Embedded Tissue Specimens

A Leica EG 1150 C Microtome was used to section the paraffin embedded lung tissue. Paraffin embedded lung tissue was first trimmed prior to sectioning. A standard thickness of 10 µm was set on the microtome for trimming. The knife was placed in beneath the clamp. Using the course feed, the block was brought towards the knife. Rotating the hand wheel clockwise, the block was slowly advanced to the knife until the knife barely trimmed the block. The block was adjusted from side to side and up and down to achieve good sections. Clean sections were trimmed off using the handwheel to advance the specimen. Once the specimen in the paraffin block appeared to have a smooth surface to the knife, the handwheel brake was applied to prep for sectioning. To begin sectioning, "Section" was selected from the left control pad. A thickness of 5 µM was selected as the thickness for the sections. Additionally, a clean area of the knife was moved to the block using the clamp lever to achieve smooth, well-cut sections. The clamping lever was locked once the knife was properly adjusted. To begin sectioning, the brake was released and the handwheel was rotated clockwise. To achieve the best sections, "ribbons" of paraffin embedded tissue sections were produced. To collect the ribbons of tissue, the break was engaged, and using a fine brush, the sections were removed from the clamp plate and separated from the plate. Good sections were transferred to a water bath and placed on to a

microscope slide. Microscope slides were placed on a Fisher Scientific Slide Warmer to dry for a couple of hours. Once dried, a coverslip was placed over the tissue using xylene substitute mountant. The microscope slides were placed under the hood to dry. Slides were not stained for at least 24 hours after gluing down the coverslip.

Tissue Processing

The automatic tissue processor Leica TP1020 was used for processing the tissues. The specimens in Histosolve (Harleco® Neo-Clear®) were transferred to 70% ethanol and soaked for 30 minutes. Specimens were moved to 80% ethanol for 40 minutes, followed by 85% ethanol for 40 minutes and two 40 minutes rounds in 95% ethanol. Specimens were moved to 100% ethanol for 40 minutes, followed by 100% ethanol for 40 minutes. After, tissues were moved to Histosolve 40 minutes, and moved to another Histosolve for 40 minutes. Lastly, tissues were transferred to paraffin and soaked for 45 minutes. Tissues were transferred to paraffin once for 45 minutes and soaked for another 45 minutes. The total run time was 7 hours and 40 minutes.

Hematoxylin and Eosin (H&E) Staining

A Shandon Varistain Gemini ES Automatic Stainer was used to perform H&E staining on the microscope slides of lung tissue. Microscope slides of tissues were placed into a slide rack and reagents were poured into their designated containers. Samples were first deparaffinized in Xylene in three rounds, each lasting 2:30 minutes. Using ethanol 100% and 95% ethanol, tissue was rehydrated. Slides were bathed in 100% ethanol, followed by 95% ethanol for 1:30 minutes each. Tissues were rinsed in running water for 1 minute and then

stained with hematoxylin 560 for 4:30 minutes. Following hemotoxylin staining, tissues were rinsed in running water for 1 minute. Tissues were then immersed in Define for 1 second. Following immersion in Define, tissues were rinsed again with running water for 1 minute. Tissues were stained with Bluing Buffer 8 for 30 seconds, followed by a rinse in running water for 1:30 minutes. Tissues were rinsed in 95% ethanol for 1:00 minute. After the alcohol rinse, tissues were counterstained with Alcoholic Eosin Y 515 for 20 seconds. Tissues were then dehydrated using 100% and 95% ethanol in the following order: 95% ethanol for 30 seconds, 95% ethanol for 1:00 minute, 100% ethanol for 1:30 minutes, and 100% ethanol for 1:30 minutes. Lastly, tissues were rinsed in Xylene substitute for 1:30 minutes, followed by 2:30 minutes.

Microscopy

Three stained tissue sections were randomly selected from samples 12, 13, 14, and 15 for microscopy analysis. Olympus BX51 was used to take pictures of the slides. Four pictures of each section were taken at 20x magnification and 40x magnification. A total of 24 pictures were taken for each tissue sample, 12 pictures at 20x magnification and 12 pictures at 40x magnification. Pictures were taken of the same region for each section and labeled as a, b, c, and d. This was done for samples that underwent H&E staining and elastin staining. Basic Bright Field was the application utilized, and Köhler Illumination was performed to achieve best imaging results. All images were saved as Tiff to achieve the best imaging results.

Alveolar Counting

Images of tissue sections stained using H&E were used to count alveoli. Ilastik, a computer program used for image classification, segmentation, and analysis, was used to prepare images or alveolar counting. Four images were chosen to be the images whose analysis was the "standard" from which the program was set to analyze all of the other images. Multiple images where used to produce the standard from which others were rendered to increase specificity. Four images were selected and classified using selected features. Based on the selected features, images were rendered using a live prediction. Images were further edited by hand to ensure accuracy in identifying which areas were tissue or air. Once the images were rendered, a batch containing the rest of the images to be analyzed were uploaded and rendered using the rendering results from the four original images. Once images were rendered using Ilastik, Image J was used to count the alveoli of each image. Instructions for counting alveoli were adopted from Christine Lebno of University of Chicago's Integrated Light Microscopy Core Facility.[37] Images were converted to 16-bit. Next, threshold was adjusted to highlight all the structures to count. Threshold of 119/255 was used for the majority of images. A few images were set to higher thresholds to be sure areas of tissue would be included in the counting of alveoli. Once the threshold was appropriately set, a binary version of the image was produced with two pixel intensities (black = 0 and white = 255). To analyze the particles, in this case the alveoli, of each image, a pixel size was determined using trial and error method. Pixel ranges of 0-∞ are less exclusive and account for a lot of noise. Therefore, each image was first set to a pixel range of 100-∞ and appropriately adjusted. For example, to exclude noise from being counted as alveoli, the lower end of the pixel range was raised. To number alveoli being counted, each were "outlined" and numbered. Results of images, including total alveoli, were saved as an Excel file.

The average alveoli count was taken of the four images (a, b, c, and d) for each section of tissue. The three averages for each section were averaged to result in one average alveolar count for each sample. Alveolar counts of rat 12 (VAA diet) and rat 13 (VAA diet) were compared to alveolar count of rat 14 (VAM diet) and rat 15 (VAM diet).

Statistical Analysis of Alveolar Count

PRISM 6.0 was used to analyze results. The statistical test performed was a two tailed unpaired t-test with a 95% confidence interval that determined statistically significant or non-significant differences between the alveolar counts measured in the lungs of rats fed a VAA diet versus rats fed a VAM diet.

Chapter 4: Results

Gene Expression

Expression of genes in lung tissues was quantified by mRNA in terms of fold change. The expression of retinoid homeostatic genes LRAT (Figure 3), STRA6 (Figure 4), CYP26B1 (Figure 5), RALDH 1 (Figure 6), RALDH 2 (Figure 7), and RALDH 3 (Figure 8) in the lung was not significantly different between rats fed a VAA diet versus VAM diet. However, there were some differences in mRNA expression of retinoid homeostatic genes between the lungs of rats fed the two diets that are still important to note. LRAT expression in the lungs of rats fed the VAM diet was on average lower than in the lungs of rats fed the VAA diet. STRA6 and CYP26B1 expression in the lungs of rats fed the VAM diet was on average higher than in the lungs of rats fed the VAA diet. The expression of RALDH 1, 2, and 3 was slightly lower in lungs of rats fed a VAM diet compared to the lungs of rats fed a VAA diet, but the difference was not significant, possibly due to the small number of animals. The range of RALDH expression was much larger in the lungs of rats fed a VAA diet.

The expression of proliferation-associated genes including elastin (Figure 9), PDGFRα (Figure 10), VEGF (Figure 11), and α-SMA (Figure 12) in the lung were not significantly different in rats fed a VAA diet versus VAM diet. On average the expression of PDGFRα, VEGF, and α-SMA was lower in the lungs of rats fed the VAM diet than in the lungs of rats fed the VAA diet. Gene expression of the proliferation-associated gene EGFR (Figure 13) was

significantly lower in the lungs of rats fed a VAM diet versus lungs of rats fed a VAA diet (P=0.0067).

Gene expression for surfactant proteins A (Figure 14), B (Figure 15), and C (Figure 16) in the lung were not significantly different between rats fed a VAA diet versus VAM diet. The mean mRNA value for SP-A, SP-B, and SP-C in both groups was around 1 and was relatively consistent in value and range for all three surfactant proteins.

Ultra Performance Liquid Chromatography

Figure 17 shows the UPLC quantification of ROH in lungs of adult rats fed a VAA diet verses adult rats fed a VAM diet. ROH content in lungs of rats fed the VAM diet fed rats was significantly less than in rats fed the VAA diet fed rats (n=6, P = 0.0212). Rat 11 used for calculating ROH (µg/g) was taken from a previous UPLC run due to a discrepancy in the ROH value obtained from 11 on a second trial.

Alveolar Count

Comparison between the alveoli count in lungs of adult rats fed a VAA diet verses lungs of adult rats fed a VAM diet demonstrated the effect of VA intake on morphological features of the adult lungs. Rats 12, 13, 14, and 15 were assessed for alveoli counts. VAA rats 12 and 13 were averaged; VAM rats 14 and 15 were averaged. Although not statistically significant, Figure 18 shows that rats fed a VAM diet had an overall decreased number of alveoli counts in comparison to VAA counterparts. The decreased alveoli count in the lungs from a rat fed a VAM diet was not significantly different from the alveoli count of lungs from the rats fed a VAA

diet. Four pictures best depicting alveoli count and distribution are shown in Figures 19, 20, 21, and 22.

Lung Morphology

Walls of the alveoli in rats (Figures 23 and 24) fed a VAA diet appeared to be slightly more organized than the alveoli in rats (Figures 25 and 26) fed a VAM diet. Differences are slight and not significant, but suggestive. Septal crests (identified in Figure 23) were present in lungs of both treatments. There did not appear to be a difference in the abundance of red blood cells and capillaries in the lungs from the two groups. Blood cells and capillaries (red oval) and alveolar walls (purple) are identified in Figure 24.

Chapter 5: Discussion

ROH content in the lung, mRNA expression of genes and proteins involved in VA metabolism and alveolar count were assessed to quantify the effects of VAD in the lungs of rats fed a VAA diet or VAM diet. Rats fed a VAM diet received low dietary intake of VA to induce deficiency. Rats fed the VAA diet received normal amounts of VA to sustain normal physiological functions.

UPLC was used to measure ROH content in both cohorts. The ROH content of the lungs of rats fed a VAM diet was statistically significantly lower than in rats fed a VAA diet. The data is consistent with what was expected. Rats who were fed a diet of VAM status received less dietary VA. Concentrations of VA in tissues, including lung, liver, and kidney, as well as blood, where VA is processed, stored, or needed for normal physiological function of cells are consistent with dietary intake of VA.[38] Therefore low dietary VA decreases the amount of VA contained in the lung. Less than optimal levels of ROH in the lung manifests in abnormal functioning of the lung and abnormal morphological characteristics, and these manifestations differ depending on the age or stage of lung development.[39, 40]

The gene expression levels of retinoid-regulatory genes, also known as retinoid homeostatic genes, proliferation-associated genes and surfactant proteins, in adult rats were measured in lungs to determine whether gene expression is differentially regulated in response to VA nutritional status. LRAT mRNA in the lungs of rats fed a VAM diet was less than in the lungs of rats fed a VAA diet, although the difference was not statistically significant. LRAT functions to esterify ROH to RE and expression is found to be regulated by dietary VA.[41] RE are

packed into chylomicrons which travel in the blood to the liver and other extrahepatic tissues, including the lung. If there is an overall decrease in dietary VA intake, less ROH would be available to be esterified to RE. Therefore, the expression of the enzyme LRAT needed to carry out esterification would be diminished in the rats with less VA dietary intake.

As opposed to LRAT, mRNA expression of STRA6 increased, although not significantly, in the rats fed the VAM diet. One explanation for the increase in mRNA expression of STRA6, which is involved in the uptake of ROH into the cell, is the attempt for the lung to compensate for the low VA availability. Increasing the mRNA expression of STRA6 may increase the trafficking of ROH into the lung cells, to maintain adequate VA homeostasis. Similarly, expression of CYP26B1, which is directly involved in the terminal oxidation of RA in the lungs and neonate liver, increases in the lungs of rats fed a VAM diet. The increase in CYP26B1 expression was not statistically significant.

The pattern of expression of RALDH 1, 2, and 3 were very similar in both cohorts. RALDH 1, 2, and 3 are involved in RA production. Despite the dietary intake of VA, the body may attempt to keep RA levels at a healthy level, because RA is the most active metabolite of RA. As a consequence, the mRNA expression of the RALDH enzyme that gives RA would stay similar no matter the dietary intake. RA is responsible for more functions in the body than the other metabolites of VA. Growth and development and nuclear regulation via the binding of RA to RXR and RAR to promote transcription of genes of proteins necessary for normal physiological functions are the major roles executed by RA.[3, 4] Because RA has these potent characteristics, RA production is tightly regulated. In fact, research has shown that oral treatment of RA results in reduction of RALDH mRNA and protein expression.[42] If RA levels

were low, a myriad of problems would manifest, due to involvement of RA in these functions. This is a possible mechanism performed to maintain nontoxic RA levels in the body.

Elastin, a gene associated with structure and function, was examined to see whether gene expression changed upon VA intake. Elastin mRNA expression was only slightly higher in the lungs of rats fed a VAM diet, and the difference was not statistically significant. Elastin mRNA expression content is expected to decrease in the lung of fetus and neonate rats birthed from VA deficient mothers, resulting in retarded lung maturation.[32] It is not known whether adults have greater protective mechanisms against the repercussions of VAD than neonates. However, these results may suggest that adults have a protective or compensatory mechanism that may prevent infliction of damaging morphological changes in the lungs. Analysis of gene expression of elastin may be showing that adults do have a protective mechanism that helps prevent mRNA levels of necessary building blocks in the lungs from diminishing so severely as to inflict large morphological changes in the lungs.

Proliferation-associated genes such as PDGFRα, VEGF, EGFR, and α-SMA were examined to see whether gene expression is differentially regulated in response to nutritional status of VA. PDGFRα exhibited no difference in lung expression of rats fed a VAA diet versus rats fed a VAM diet. PDGFRα plays a role in the formation of the alveoli that carry out gas exchange.[20] The results of alveolar counts of the lung sections is not consistent with PDGFRα expression. Based on the similar mRNA expression of PDGFRα, alveolar counts should be similar in the lungs of both dietary groups. In contrast to what was expected based on the expression of the gene controlling formation of alveoli, alveoli counts were less in the lungs of rats fed the VAM diet. One reason to explain the contrasting results is that another gene or

protein may be aiding in the formation of alveoli in addition to PDGFRα, offsetting the morphological effects expected by PDGFRα.

mRNA expression of VEGF and EGFR were slightly lower, but not statistically different in the lungs of rats fed the VAA diet compared to the mRNA expression in the lungs of rats fed the VAM diet. VEGF is highly involved in the development of the lung and maintenance of the adult lung by maintaining endothelial cell function.[19] VA may affect VEGF expression based on the results. VA is needed for cell differentiation and functioning.[4] The results may be revealing that VA affects not only the differentiation of endothelial cells, but also the enzymes needed for maintain normal function of these endothelial cells in the adult lung. Repeating the experiment with a larger sample size would uncover a more accurate depiction of VEGF.

EGFR regulates epithelial branching and alveolarization.[21] The decrease in EGFR expression is consistent with the decrease in alveolar count in the lungs of the rats fed the VAM diet and may be a possible explanation of why the alveolar count diminished. Therefore, EGFR protein may be influenced by VA, such that low intake of VA decreases the expression of EGFR which manifests itself in producing abnormal morphological characteristics in the lung, like that of low alveolar count, characteristics commonly seen due to low VA status. There is no research to date explaining this potential theory. Further research would need to be completed to validate the possible explanation or reveal other ideas to support this opinion.

α-SMA is an important protein needed for proper development of the lungs. More specifically, α-SMA plays a role in development of airways and pulmonary vessels and branching morphogenesis. This protein is necessary for overall structure and integrity of cells. mRNA expression of α-SMA was slightly less in the lungs of rats fed a VAM diet compared to the lungs of rats fed a VAA diet. Although the difference is not statistically different, the

difference may lend reasoning towards the morphological differences seen in the tissues stained with H&E staining. VA may be affecting the expression of α-SMA, such that low intake of VA decreases the expression of α-SMA.[43]

Surfactant proteins A, B, and C are needed for proper functioning and structure maintenance of the alveoli. Based on my results, surfactant gene expression was relatively similar in the lungs of rats fed a VAA diet and lungs of rats fed a VAM diet. This was consistent with results found by Grummer et al.[44] who concluded that fetal lung surfactant proteins are not affected by VAD; however, other studies concluded the opposite effect.[26, 27, 31] VAD has been shown to decrease the amounts of surfactant present in the lungs during neonatal development, resulting in delayed lung maturation of neonates.[31] No studies to date have provided an explanation for the expression of surfactant proteins in the adult rat lung based on dietary VA intake. But, a similar principle that explains the results from the expression of surfactant proteins in the fetal lung can be extrapolated to explain the results in this experiment.

Based on this present study, the majority of the differences in mRNA expression of genes and proteins involved in VA metabolism and lung development were statistically non-significant. An improvement to be made in future experiments could be to increase the sample size. Larger sample sizes more accurately reflect the population and therefore increase the chances of producing statistically significant results by enhancing the statistical power of a test. The fact that many of the differences in mRNA expression of genes were not significant may shed light on the possibility that adult rats are not as severely affected by a suboptimal VA status as neonates. Knowledge of a physiological model of VA in neonates is limited. Based on the little information we know about some physiological and morphological effects that VAD has on neonatal lungs, neonates are more severely affected by VAD.[45] This assumption is based on the

knowledge from this study, demonstrating mRNA expression and alveolar count to be different, but not statistically significant, between the two dietary groups. If these results were repeated in further experimentation using larger sample sizes, there is a possibility that adult rat lungs may not be affected by VAD. A possible explanation is that the adult rats can offset/compensate for the adverse effects of VAD on physiological function and morphological characteristics of the lung.

Alveolar counts of the lungs were lower in the lungs of rats fed a VAM diet but results were not statistically significant. A possible explanation, formerly alluded to, for the lower number of alveoli is the potential effect that VA has on EGFR, the protein vital for controlling lung morphogenesis including epithelial branching and alveolarization.[20] If low VA decreases expression of EGFR, less EGFR would be available to form alveoli, resulting in an overall diminished yield of the rat with low dietary VA intake. A larger sample size could produce more promising and reliable results to confidently conclude whether low dietary intake affects the alveolar counts in the lung, and thus physiological functioning of the lung.

Morphology of the lungs did not vary widely in rats fed a VAA diet compared to rats fed a VAA diet. Lungs of VA deficient rats had slightly more organized walls. Several limitations made it difficult to interpret the tissues prepared via histology. The tissue, especially of VAM status, was very dry making it difficult to compare the lung to the VA adequate lung. Blood cells and capillaries appear to be no different in the VA adequate lung and VA deficient lung, and septal crests are developed in both VA deficient lungs and VA adequate lungs.

There are several comments to note regarding the results obtained by UPLC. Total lipid extraction and tissue retinol analysis by UPLC was performed twice. The data used for the analysis was from the second trial; however, the ROH measurement in rat 11 was an outlier and

not consistent with the ROH measurements of the two other VA adequate samples. To resolve this discrepancy, the ROH content for rat 11 used for analysis was taken from the first trial. The UPLC instrument is very sensitive to other solvents in the vial. The drying of hexanes step could especially be problematic; if the hexanes are not dried completely, even the slightest remnants left over will be detected by the HPLC and skew the results. Another factor to be taken into account that could explain the difference in rat 11's results between the two trials is the problem associated with extracting ROH from adult lung tissue. Adult lung tissue tends to be extremely tough. As compared to neonatal lung tissue, adult lung tissue requires stronger grinding actions to homogenize the tissue. Homogenization is important to mechanically break up the tissue to release ROH. The toughness of the tissue presents limitations of how efficiently tissue can be homogenized, and this is directly reflected upon the amount of ROH that will be extracted from the tissue.

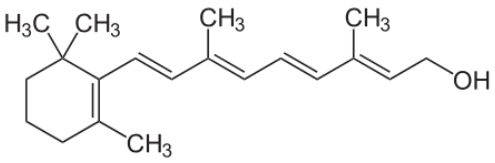
As previously stated, one way to gain more confident results would be to increase the sample size. PCR requires extremely precise measurements; slight errors in measurement have the potential to produce inaccurate results, which possibly explains the presence of outliers seen in the collected data. Additionally, acquiring good sections of lung tissue was limited by the dry nature of the lung tissue. Adult lung tissue is made up of 80% air, posing an extreme problem for obtaining histological good sections. While sectioning, the paraffin embedded tissue had to be constantly hydrated with water to moisten the tissue to acquire good sections of tissue. Much of the tissue was dry, despite these efforts. By limiting the areas of which images could be taken due to dryness, the samples from which morphologically characteristics are studied may not accurately represent the whole tissue. For future experimentation, methods for keeping tissue hydrated prior to section should be researched and carried out. There were several strengths in

this study that should be noted. Several facets of data were collected in this study including mRNA expression of 15 genes, content of ROH in the lungs, alveolar count, and characterization of the morphology of lung tissue to create a thorough understanding of what is occurring in the lung from a lack of VA. Investigating several perspectives lends a more comprehensive understanding of the microscopic and macroscopic changes occurring in the lung.

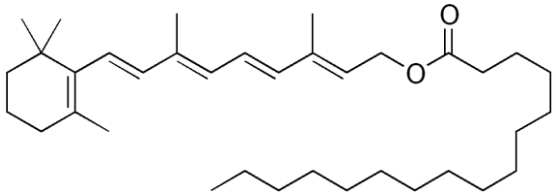
In summary, this study showed that VAD results in low tissue VA storage, even in the lung, and this could affect the expression of genes in the adult lung and the development and morphology of the lungs; however, no statistically significant results were attained, except for EGFR, ($P=0.0067$) to definitely conclude adult rats lungs are negatively affected by lack of VA. These results provided insight as to whether VA dietary status throughout development causes later effects in adults. A larger sample size for future experimentation will be necessary to more confidently decipher whether the changes seen in this experiment could be significant. Overall, there were no significant differences in the morphology of the lungs based on nutritional status. We can infer the possibility of protective mechanisms in adult rats, which neonates lack, to offset the effects of VAD; or adult rats are more resilient to changes in nutrition and therefore more capable of withstanding the effects of a nutritional deficiency of VA. The significant difference in the ROH content in the lungs demonstrates there was a definitive reduction in ROH stores and availability in the lungs of VA deficient rats; however, these did not manifest to significant morphological changes.

Appendix

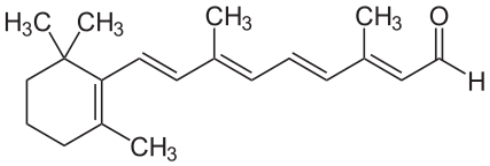
Retinol



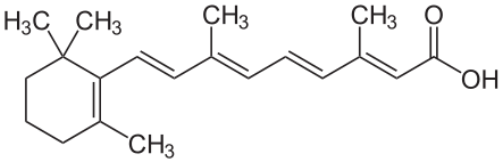
Retinyl Ester



Retinal



All-*trans* retinoic acid



11-*cis* retinal

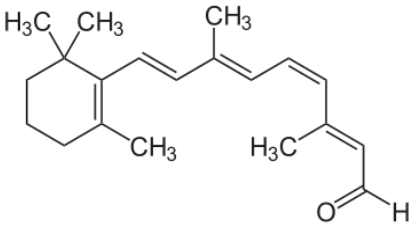


Figure 1: Chemical Structures of ROH, RE, Retinal, 11-*trans* RA, and 11-*cis* Retinal

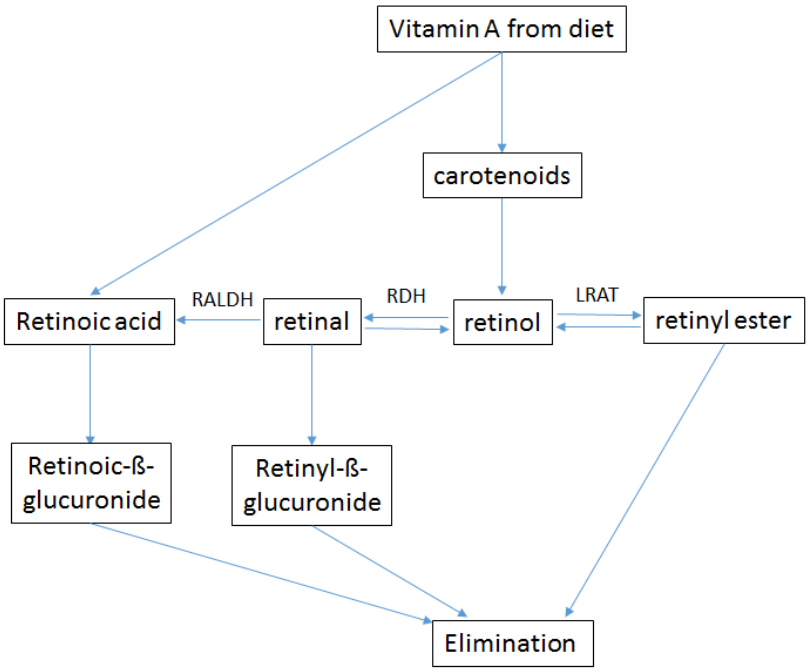


Figure 2: The Schematic of Vitamin A Metabolism

Homeostatic Genes

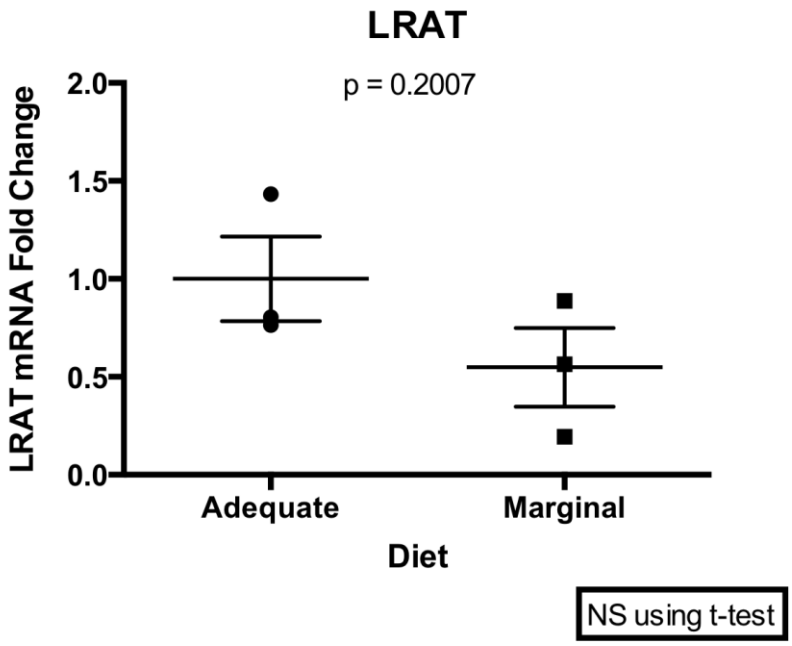


Figure 3: Gene Expression of LRAT in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. (p = 0.2007) There is no significant difference in LRAT mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

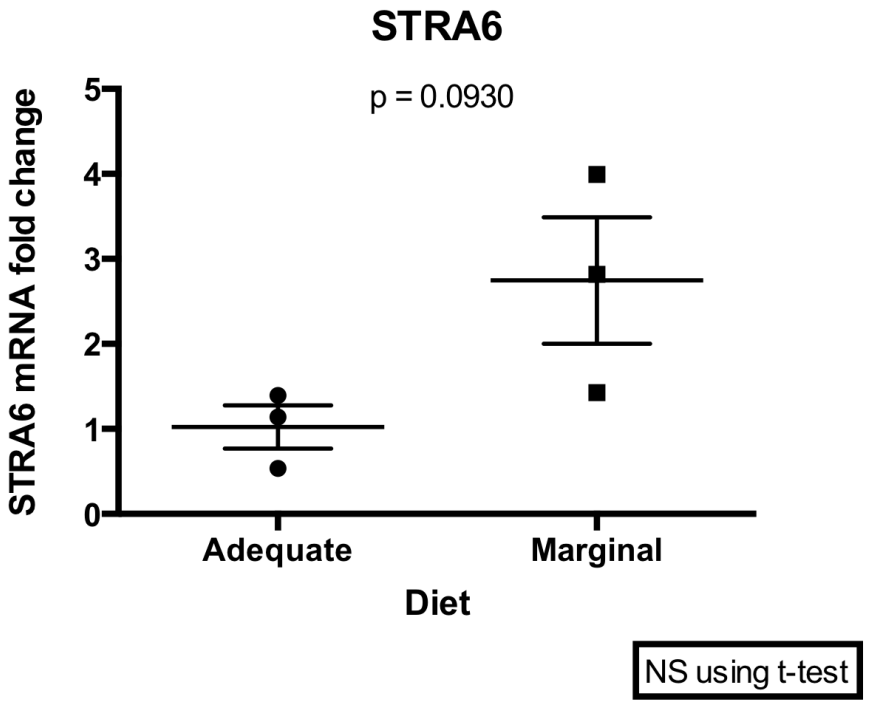


Figure 4: Gene Expression of STRA6 in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. (p = 0.0930) There is no significant difference in STRA6 mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

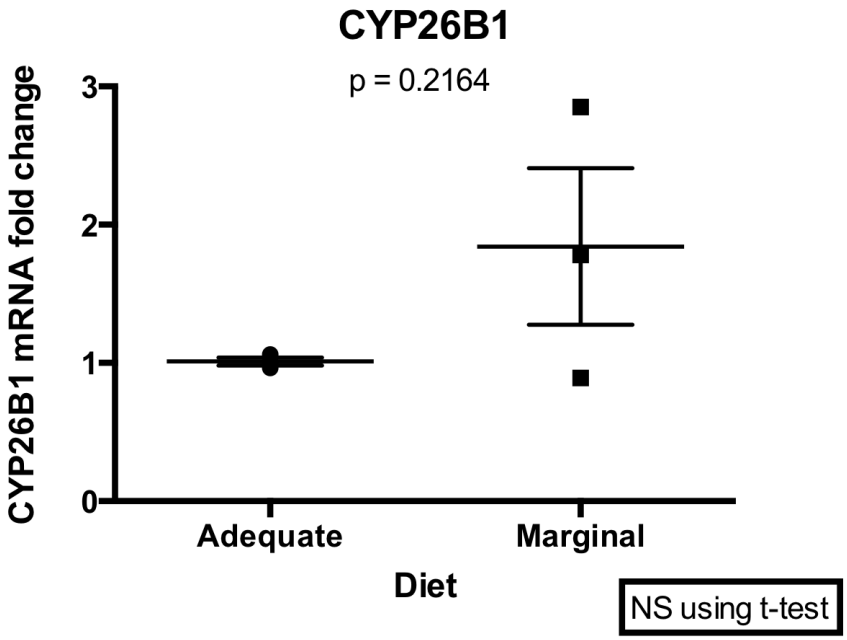


Figure 5: Gene Expression of CYP26B1 in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. (p = 0.2164) There is no significant difference in CYP26B1 mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

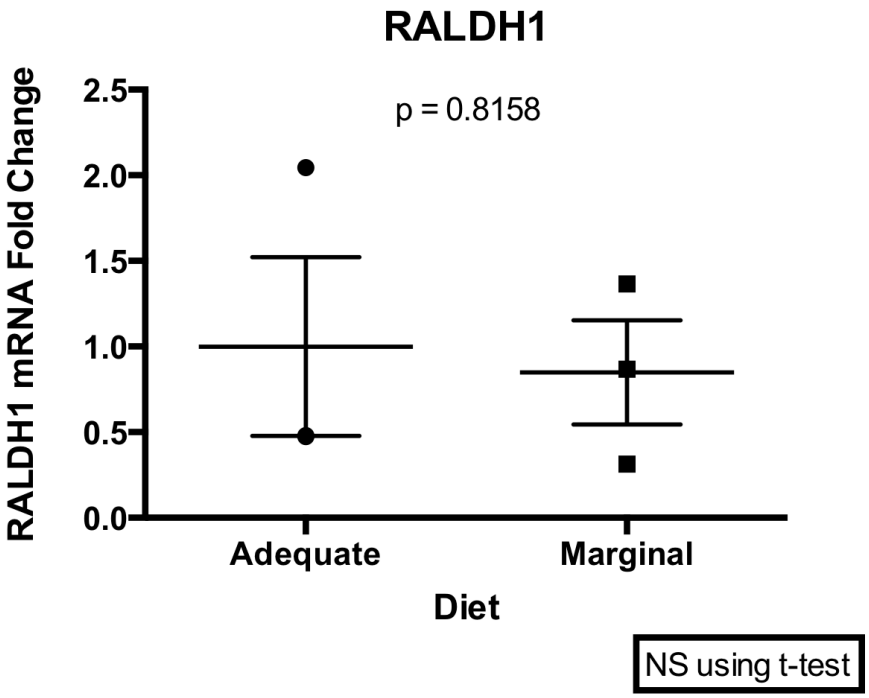


Figure 6: Gene Expression of RALDH 1 in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted (p = 0.8185) There is no significant difference in RALDH 1 mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

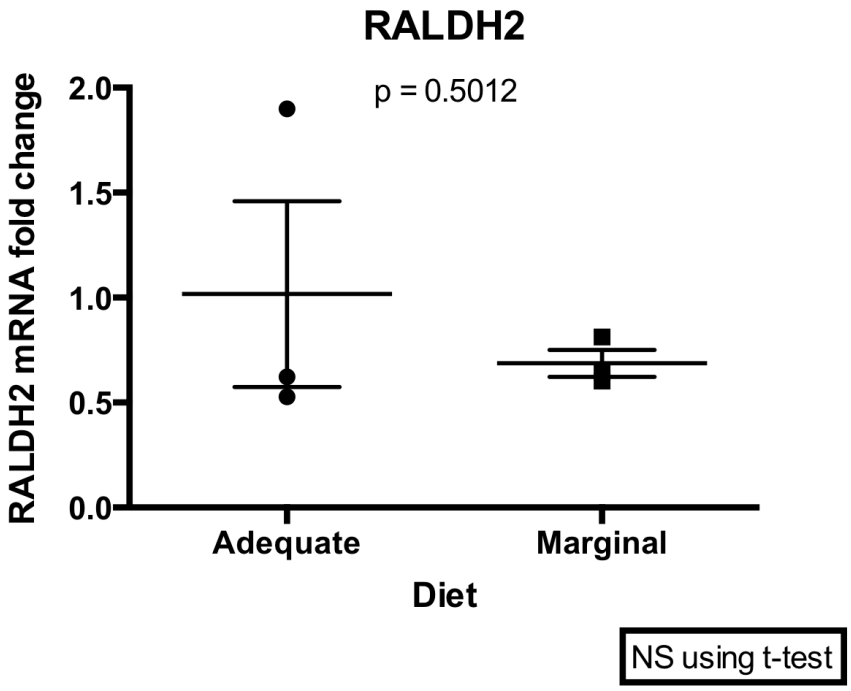


Figure 7: Gene Expression of RALDH 2 in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. (p = 0.5012) There is no significant difference in RALDH 2 mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

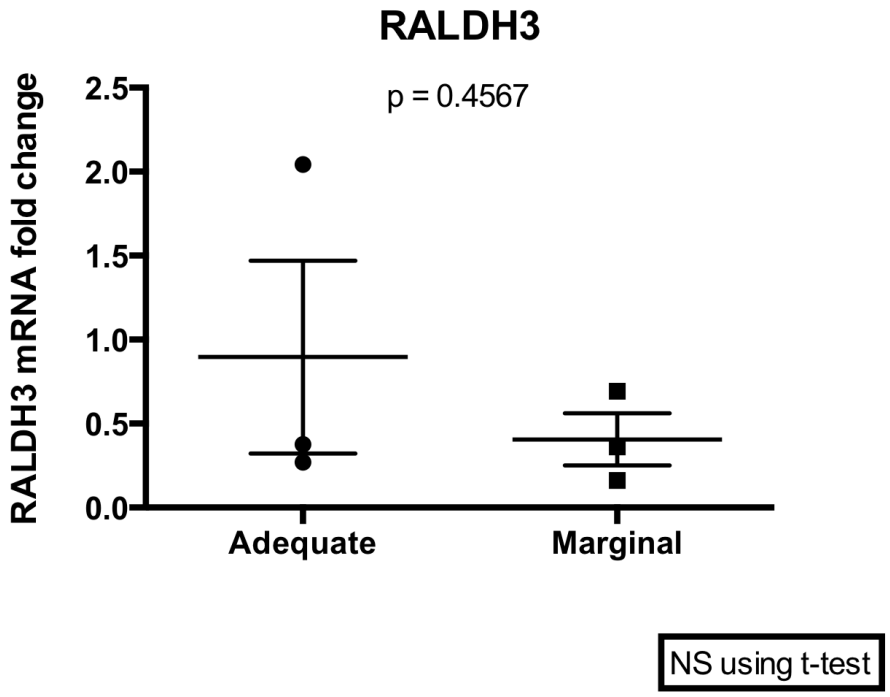


Figure 8: Gene Expression of RALDH 3 in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. (p = 0.4567) There is no significant difference in RALDH 3 mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

Proliferation-Associated Genes

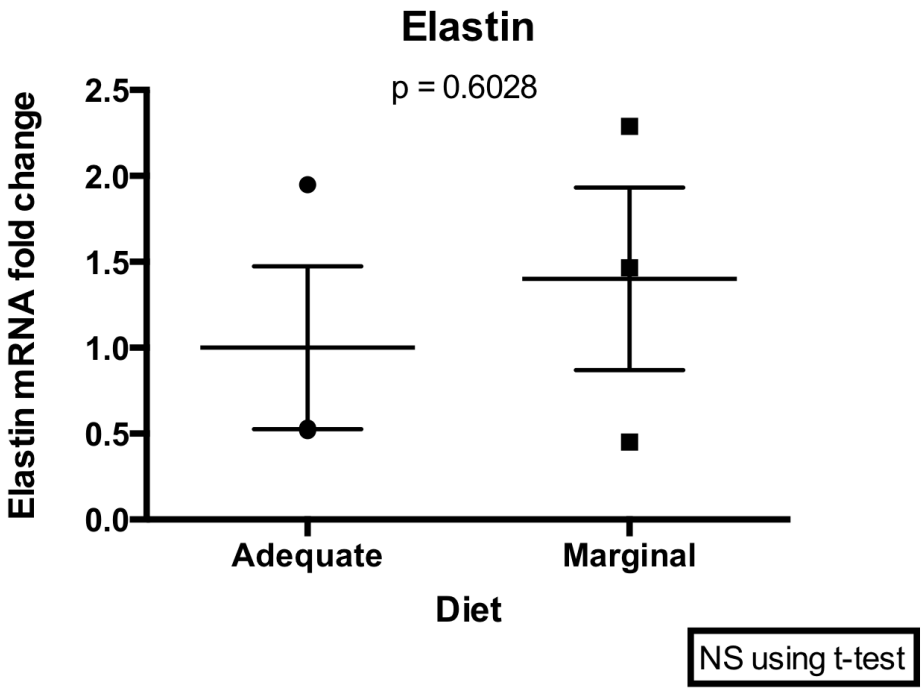


Figure 9: Gene Expression of Elastin in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. ($p = 0.6028$) There is no significant difference in Elastin mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

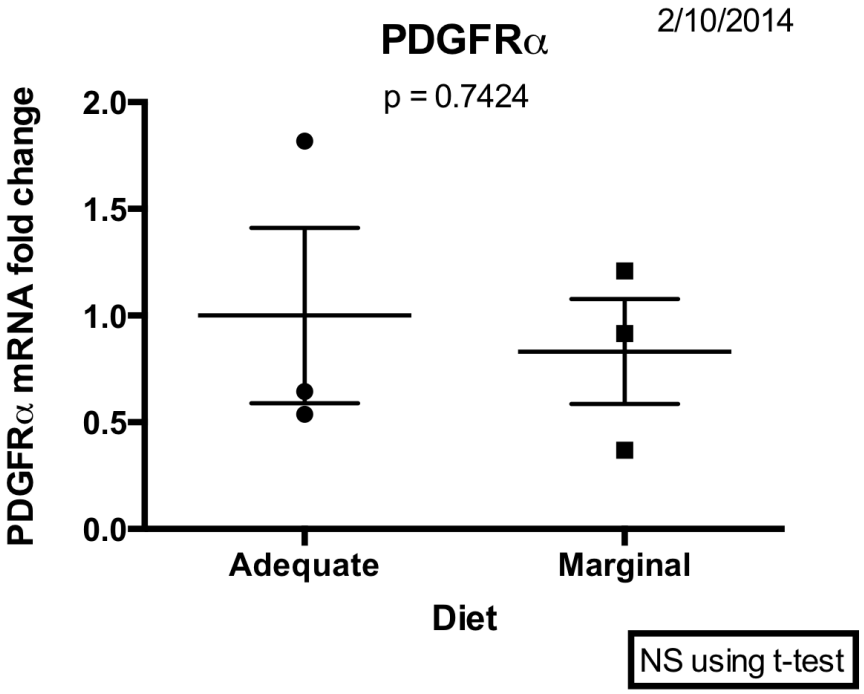


Figure 10: Gene Expression of PDGFRα in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. ($p = 0.7424$) There is no significant difference in PDGFRα mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

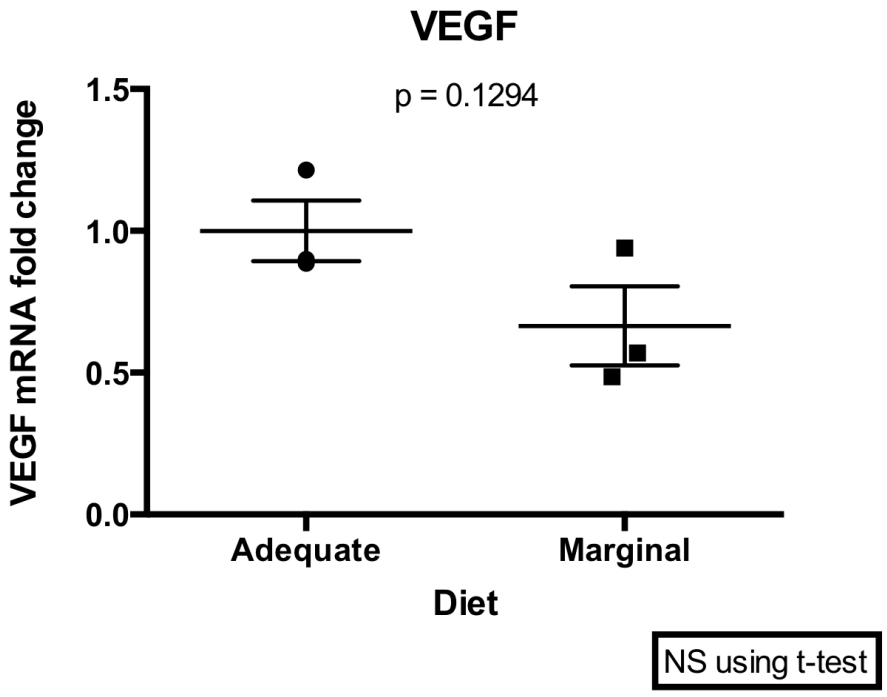


Figure 11: Gene Expression of VEGF in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. (p = 0.1294) There is no significant difference in VEGF mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

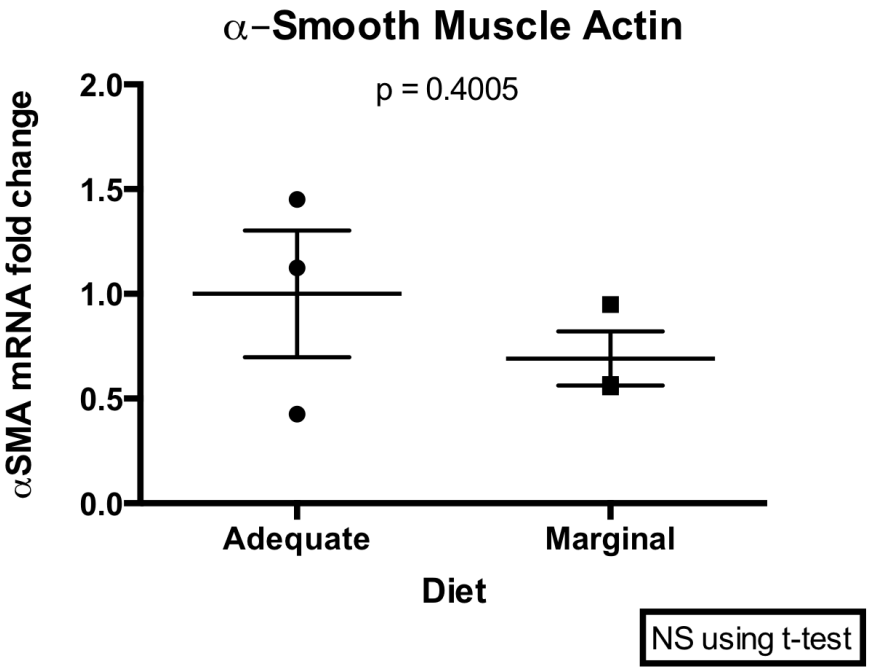


Figure 12: Gene Expression of α Smooth Muscle Actin in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. (p = 0.04005)There is no significant difference in α-smooth muscle actin mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

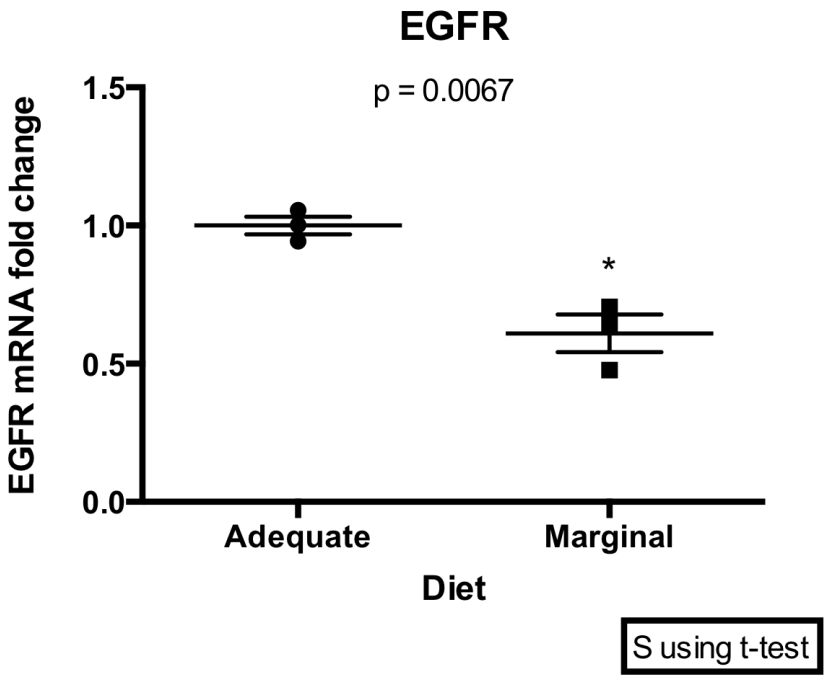


Figure 13: Gene Expression of EGFR in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. (p = 0.0067) There is a significant difference in EGFR mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

Surfactant Proteins

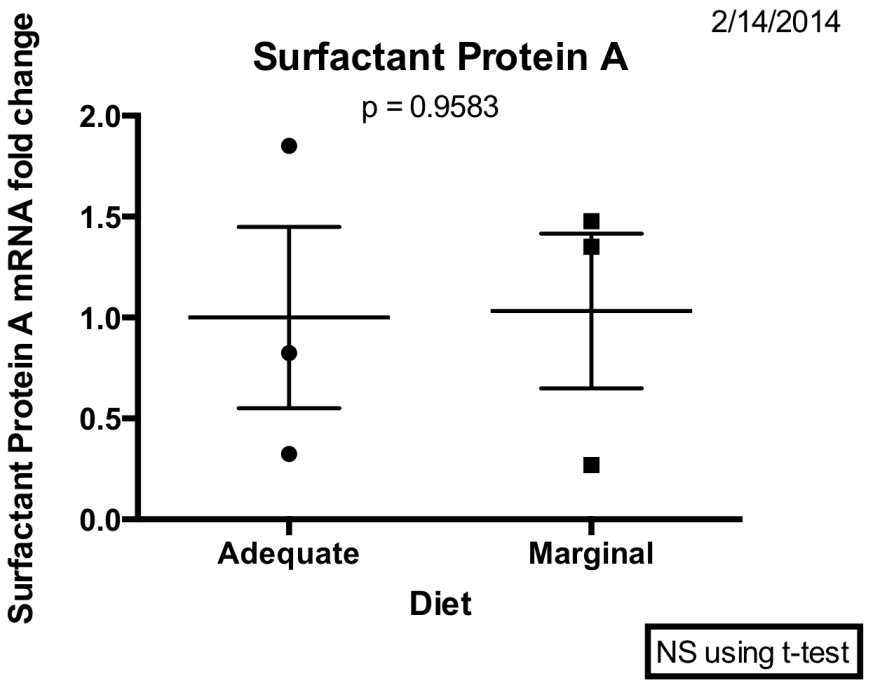


Figure 14: Gene Expression of Surfactant Protein A in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted (p = 0.9583) There is no significant difference in SP-A mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

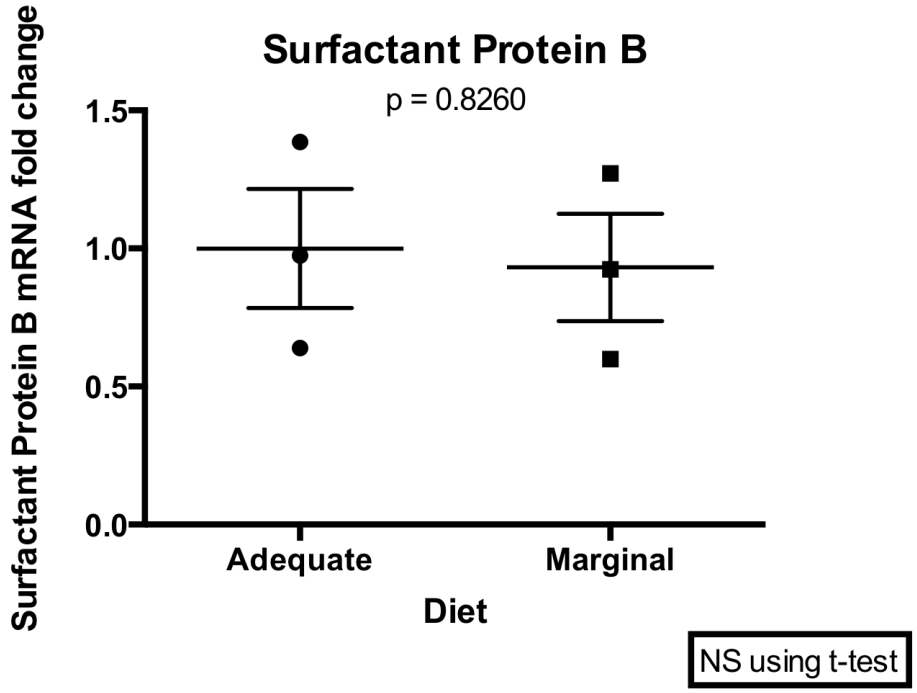


Figure 15: Gene Expression of Surfactant Protein B in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. ($p = 0.8260$) There is no significant difference in SP-B mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

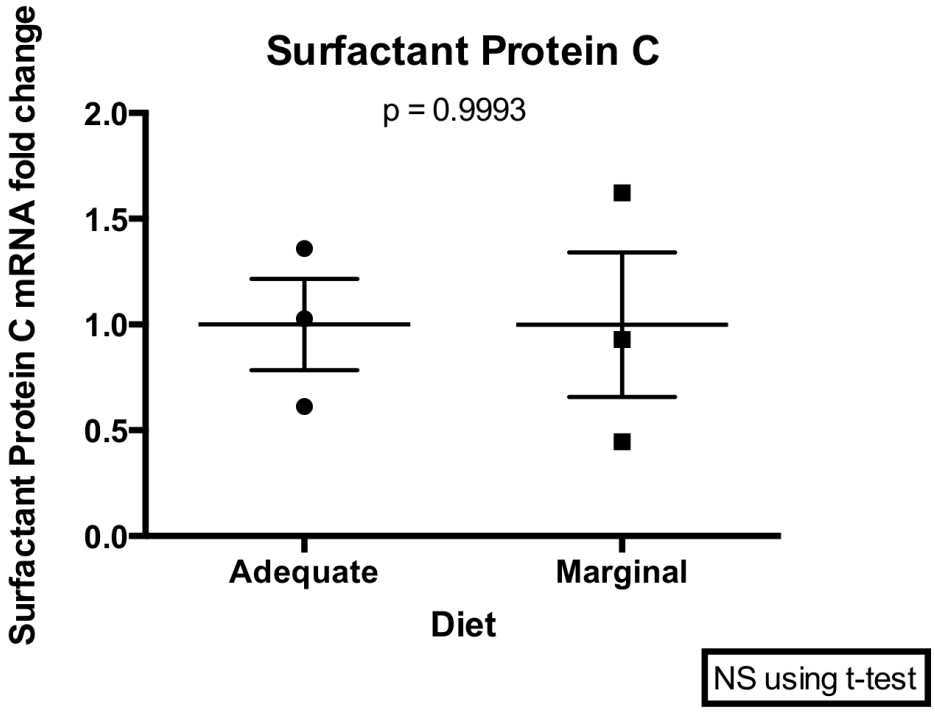


Figure 16: Gene Expression of Surfactant Protein C in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval was conducted. (p = 0.9993) There is no significant difference in SP-C mRNA fold change in lung tissue of VAA diet fed rats versus VAM diet fed rats.

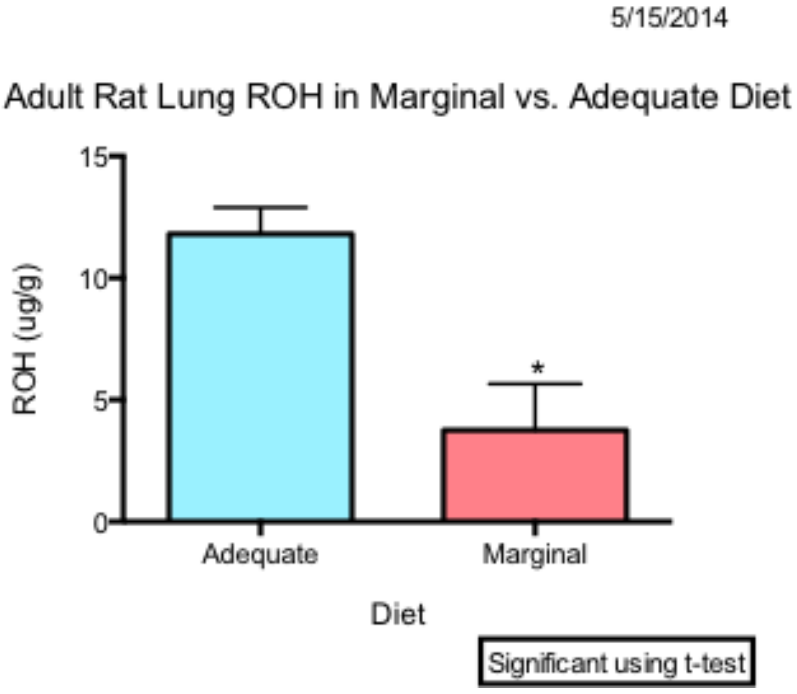


Figure 17: ROH (ug/g) in VAA versus VAM Diet fed Rats. A two tailed unpaired t-test with a 95% confidence interval showed a significant difference between the ROH content in lung tissue of VAA diet fed rats and VAM diet fed rats ($p = 0.0212$)

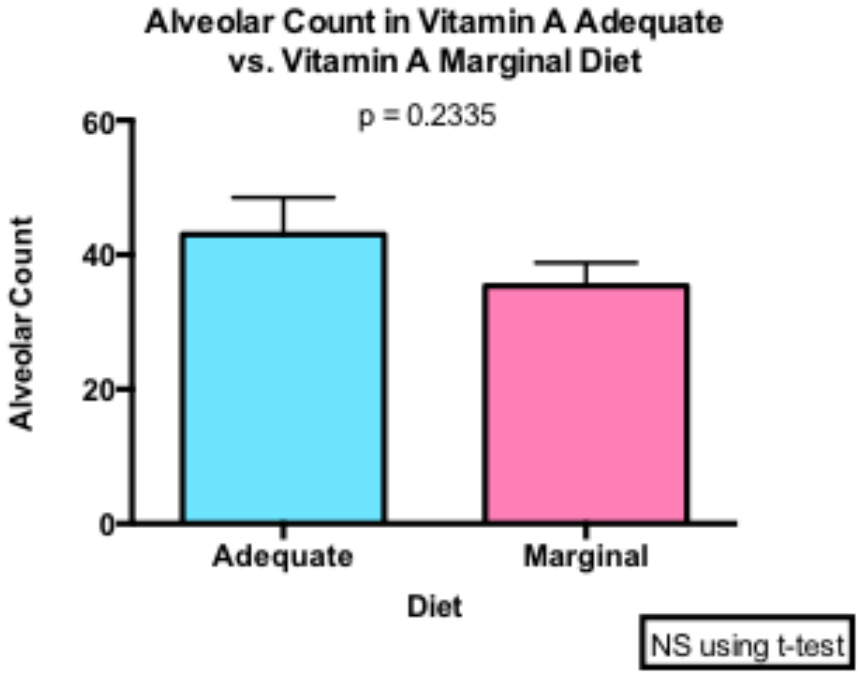


Figure 18: Alveolar Count of Adult Rat Lung fed VAA Diet versus VAM Diet. Alveolar counts of lung tissue section for VAA and VAM diet were averaged. A two tailed unpaired t-test with a 95% confidence interval showed no significant difference between the alveolar counts of the two diets. (p=0.2335)

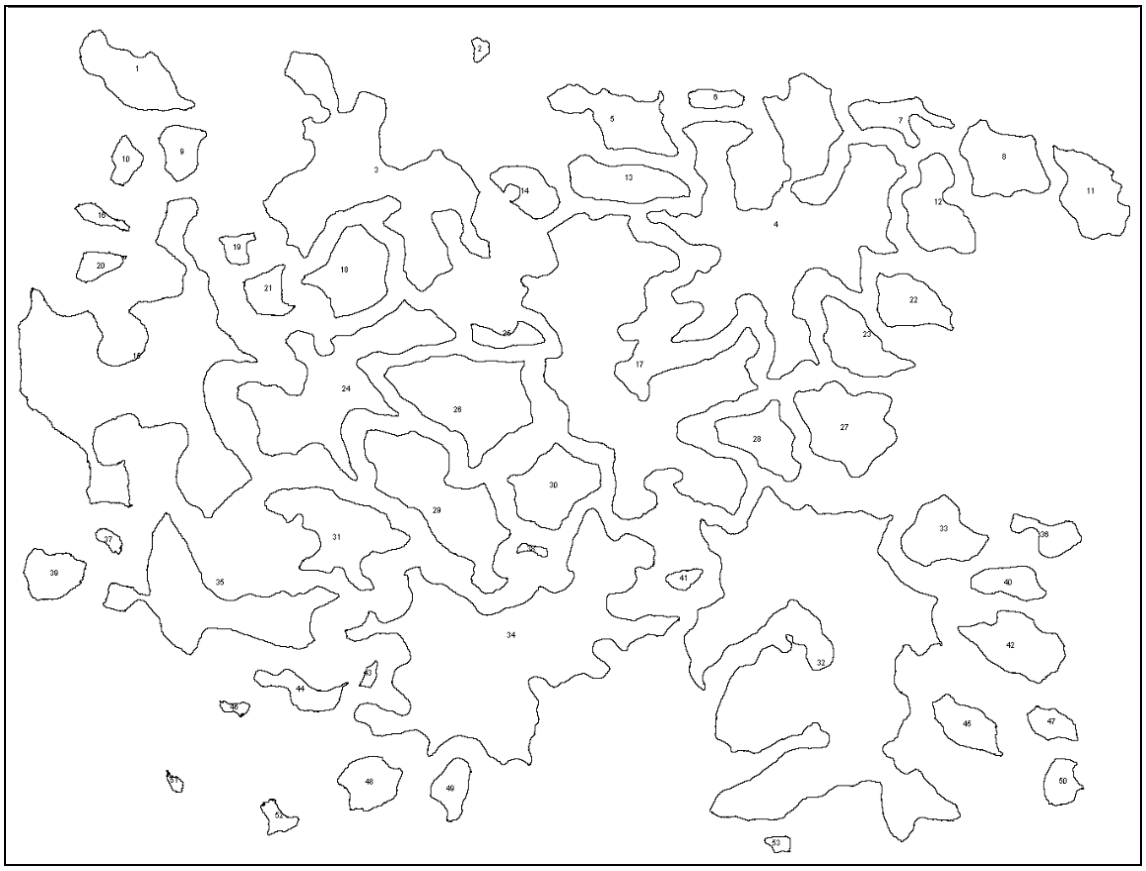


Figure 19: Alveoli count of lung from rat 12. Rat 12 was fed a VAA diet. Alveoli count was 53 alveoli. The image was taken from tissue sections stained with H&E, and analyzed using iLastik and ImageJ. Each alveoli is labeled with a number.

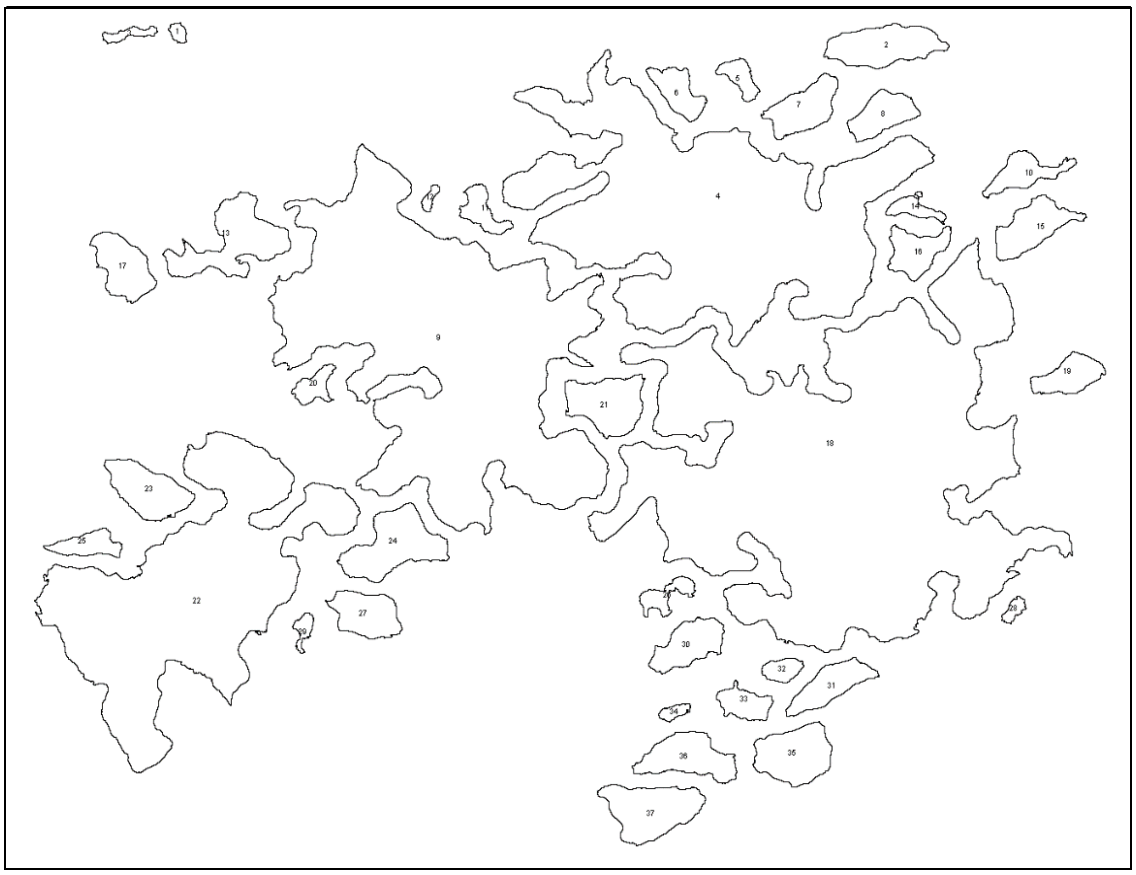


Figure 20: Alveoli count of lung from rat 13. Rat was fed a VAA diet. Alveoli count was 37 alveoli. The image was taken from tissue sections stained with H&E, and analyzed using iLastik and ImageJ. Each alveoli is labeled with a number.

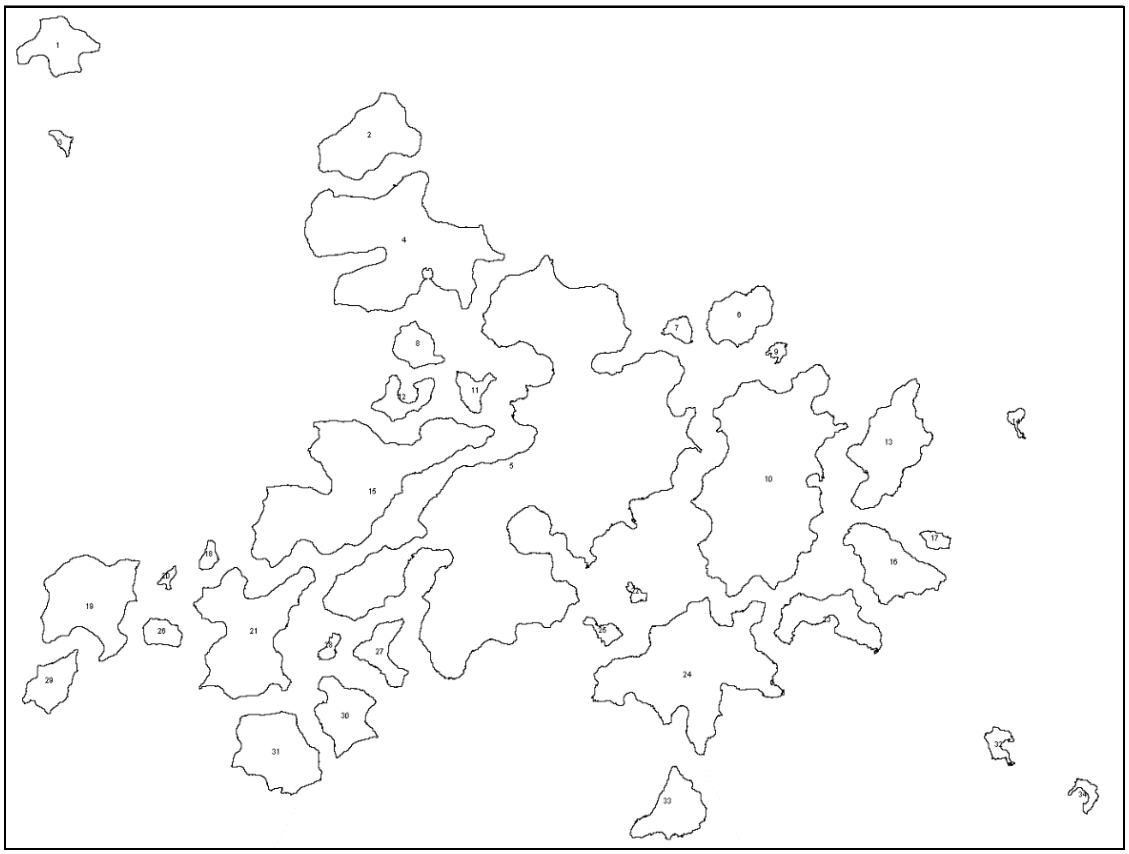


Figure 21: Alveoli count of lung from rat 14. Rat 14 fed a VAM diet. Alveoli count is 34 alveoli. The image was taken from tissue sections stained with H&E, and analyzed using iLastik and ImageJ. Each alveoli is labeled with a number.

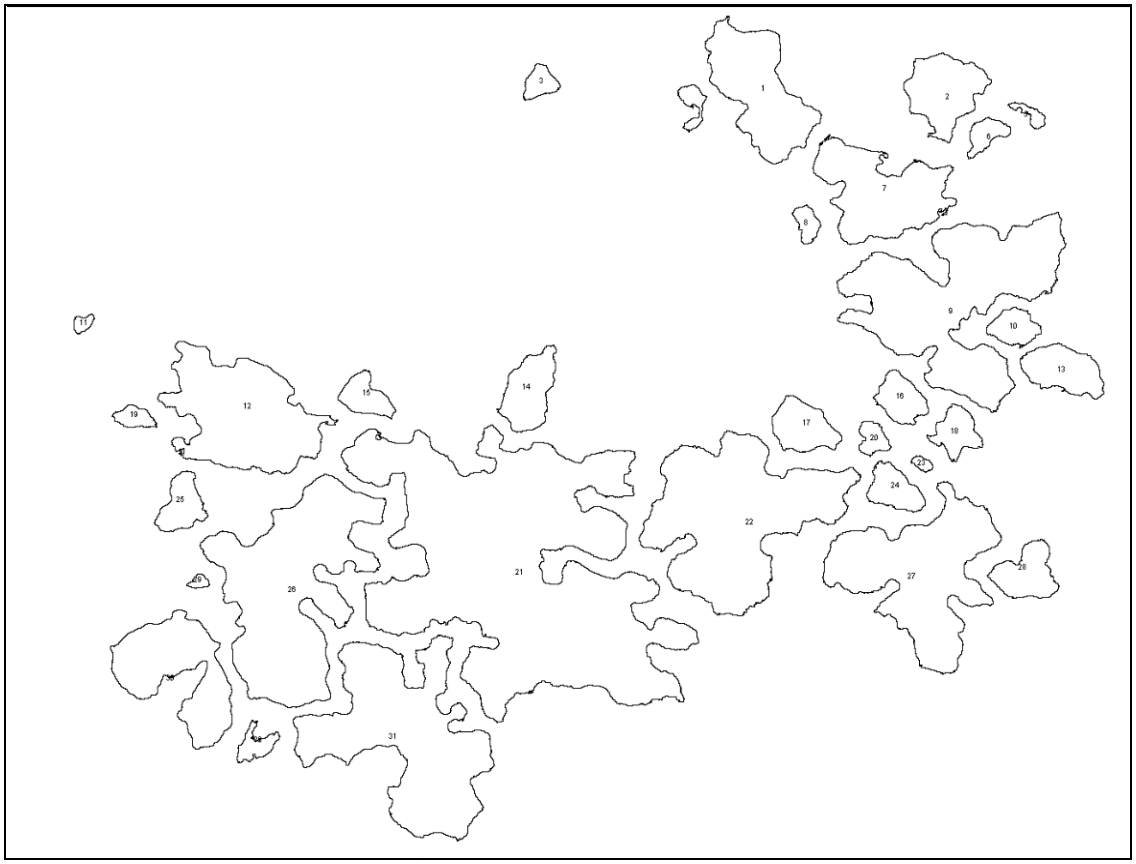


Figure 22: Alveoli count of lung from rat 15. Rat 15 was fed a VAM diet. Alveoli count was 32 alveoli. The image was taken from tissue sections stained with H&E, and analyzed using iLastik and ImageJ. Each alveoli is labeled with a number.

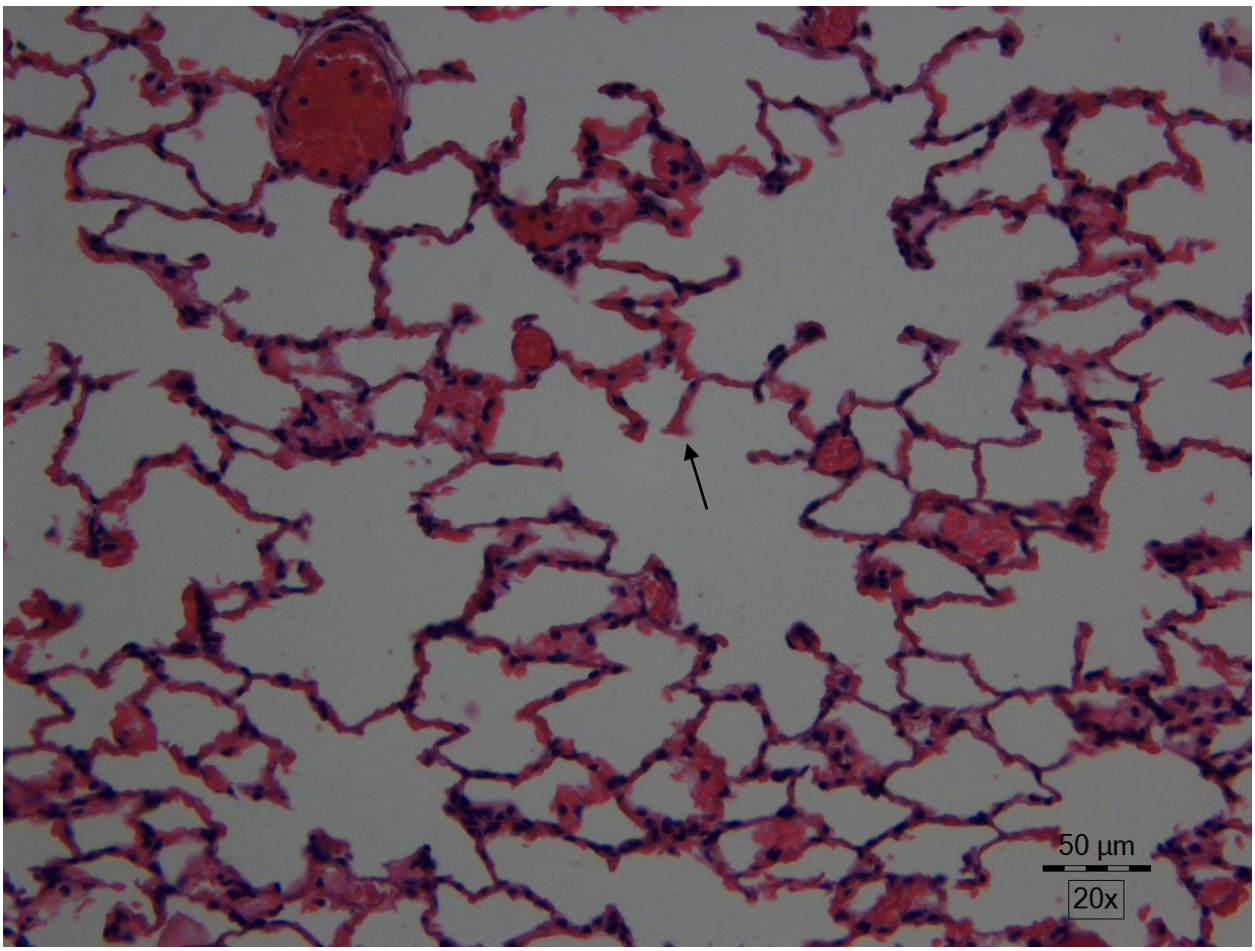


Figure 23: H and E staining of lung tissue (rat 12) of rat fed a VAA diet. Tissue was magnified using 20x lens. White space is alveoli. Red represents red blood cells and capillary beds. Purple represents the alveoli wall. The arrow is pointing to a septal crest.

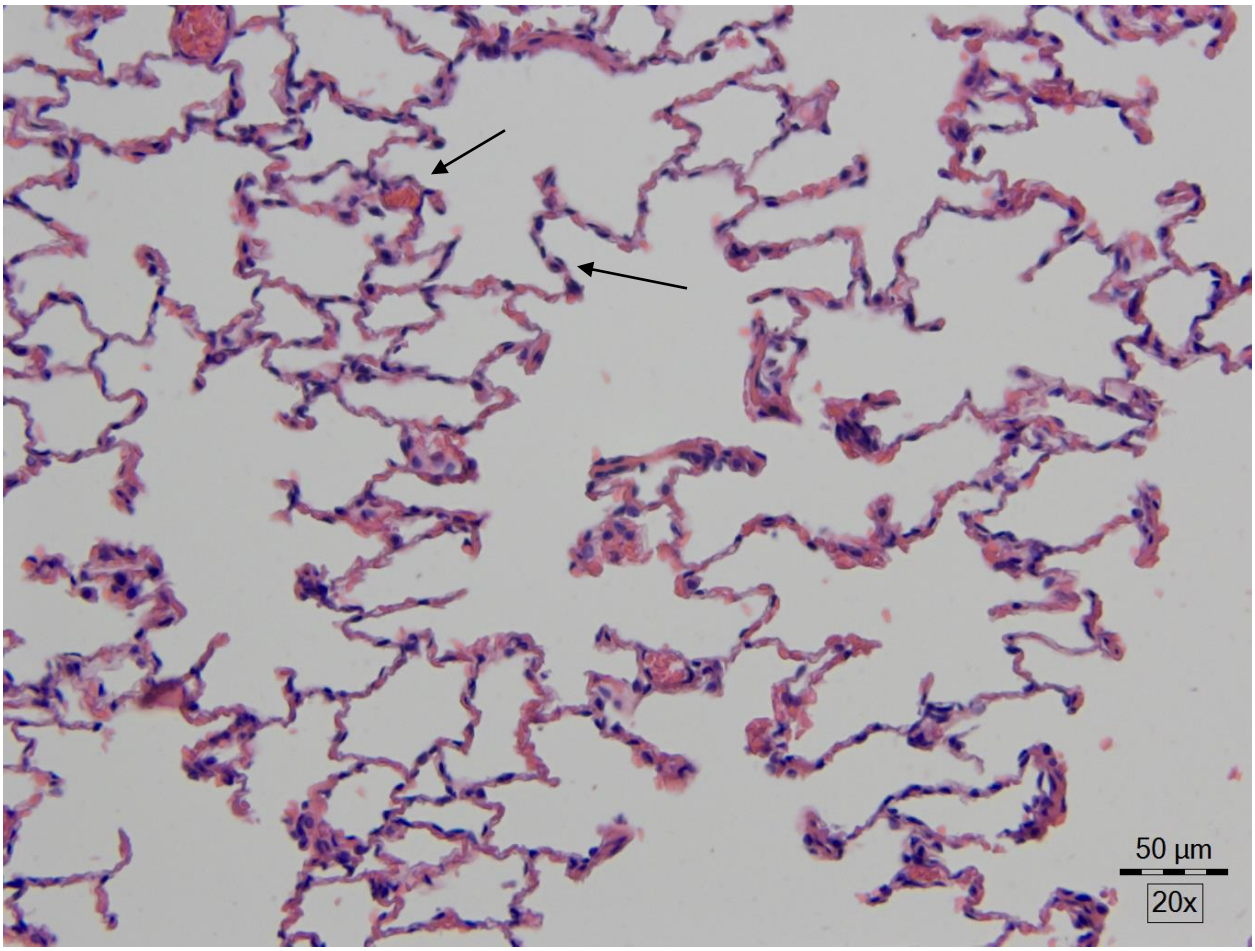


Figure 24: H and E staining of lung tissue (rat 13) of rat fed a VAA diet. Tissue was magnified using 20x lens. White space is alveoli. Red represents red blood cells and capillary beds. Purple represents the alveoli wall. One arrow is pointing to a red blood cell (red) and the other arrow is pointing to alveoli wall (purple).

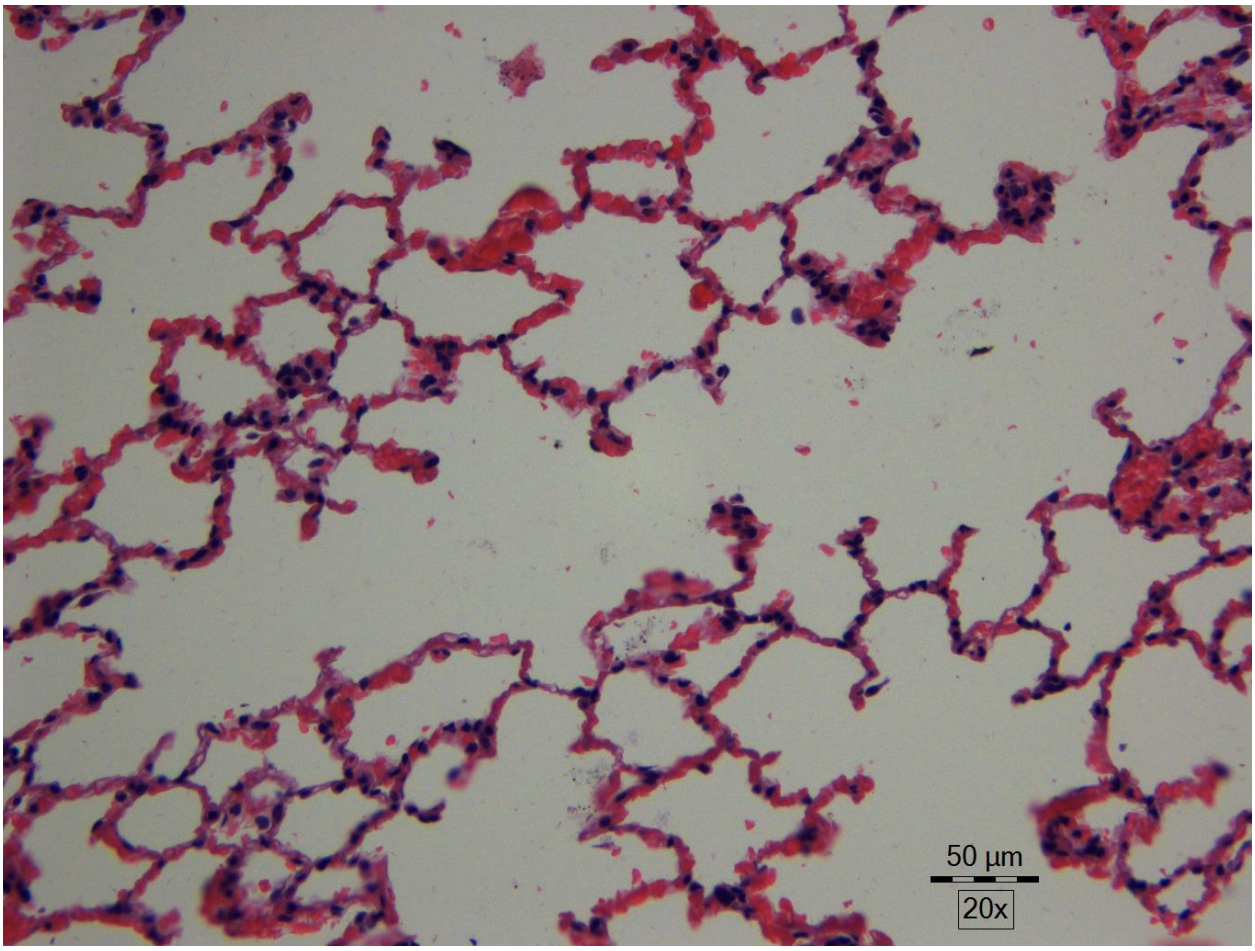


Figure 25: H and E staining of lung tissue (rat 14) of rat fed a VAM diet. Tissue was magnified using 20x lens. White space is alveoli. Red represents red blood cells and capillary beds. Purple represents the alveoli wall.

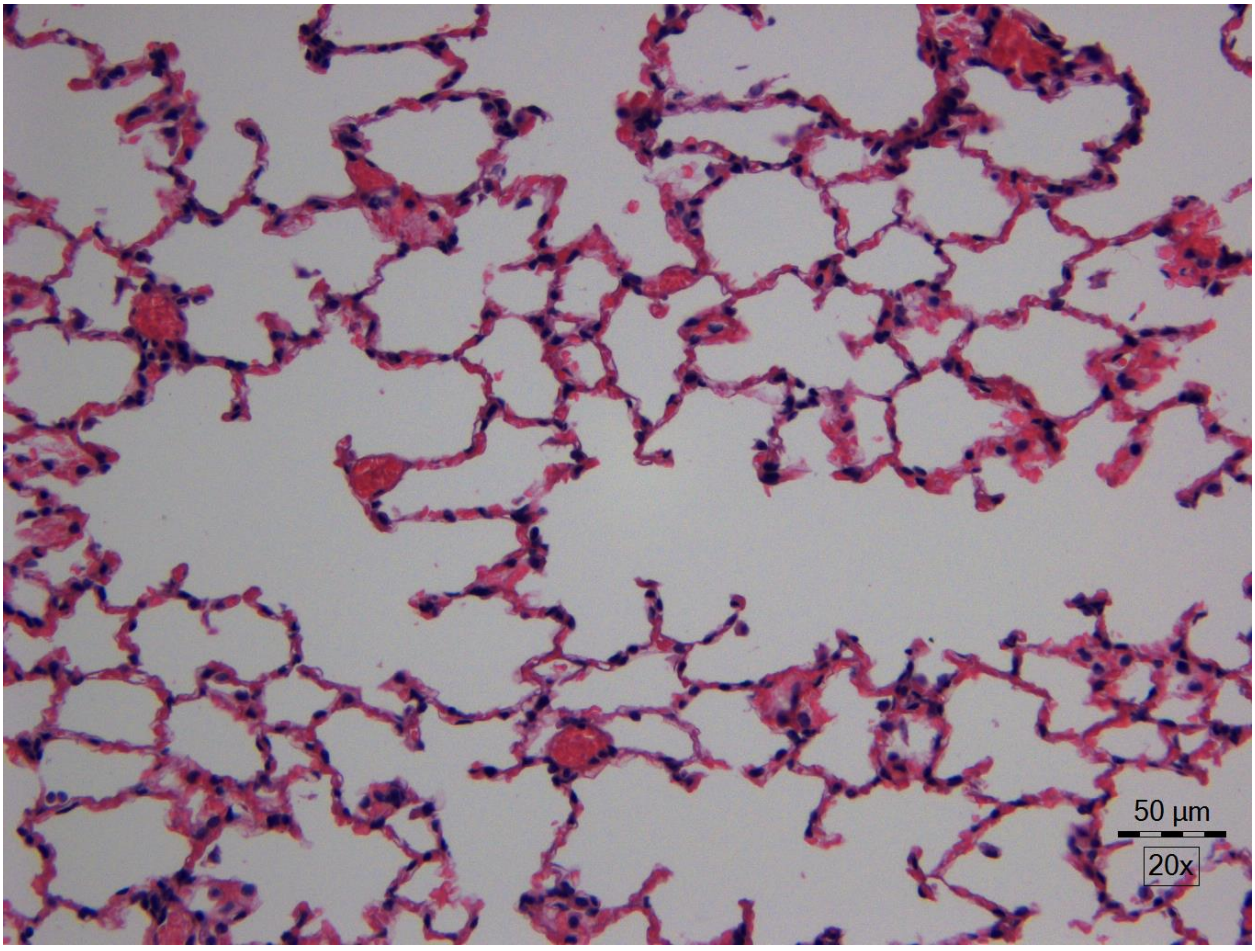


Figure 26: H and E staining of lung tissue (rat 15) of rat fed a VAM diet. Tissue was magnified using 20x lens. White space is alveoli. Red represents red blood cells and capillary beds. Purple represents the alveoli wall.

Bibliography

1. World Health Organization. 2009. Global Prevalence of Vitamin A Deficiency Populations at Risk. Geneva: World Health Organization.

2. Guyer, MacDroman, Martin, Peters, Strobino. Annual summary of vital statistics-1997. *Pediatrics*. 1998. 102:1333–1349.

3. Ross AC, Harrison EH. Vitamin A: Nutritional Aspects of Retinoids and Carotenods. Handbook of Vitamins, 4th Ed. CRC Press; Boca Raton: 2006.

4. Gropper, S.S., Smith, J.L., Groff, J.L. Advanced Nutrition and Human Metabolism. 5th Ed. Wadswoth: 2008.

5. D'Ambrosio DN, Clugston RD, Blaner WS. Vitamin A metabolism: An update. *Journal of Nutrients*. 2011. 3(1):63-103.

6. Wu L, Ross AC. Acidic retinoids synergize with vitamin A to enhance retinol uptake and STRA6, LRAT, and CYP26B1 expression in neonatal lung. *The Journal of Lipid Research*. 2010. 51(2): 378-387.

7. O'Byrne, S.M, Wongsiriroj, N., Libien, J., Vogel, S., Goldberg, I.J., Baehr, W., Palczewski, K., Blaner, W.S. Retinoid absorption and storage is impaired in mice lacking lecithin: retinol acyltransferase (LRAT). *The Journal of Biological Chemistry*. 2005. 280:35647-35657.

8. Ross AC, Ambalavanan N, Zolfaghari R, Li NQ. Vitamin A combined with retinoic acid increases retinol uptake and lung retinyl ester formation in a synergistic manner in neonatal rats. *Journal of Lipid Research.* 2006. 47:1844-18

9. Ross AC, Li NQ, Wu L. The Components of VARA, a Nutrient-Metabolite Combination of Vitamin A and Retinoic Acid, Act Efficiently Together and Separately to Increase Retinyl Esters in the Lungs of Neonatal Rats. *Journal of Nutrition.* 2006. 136:2803-2807.

10. Food and Agriculture Organization of the United Nation Food and Agriculture Organization of the United Nation. Human Vitamins and Mineral Requirements: Chapter 7. http://www.fao.org/docrep/004/Y2809E/y2809e0d.htm

11. Van Golde, LMG. Potential Role of Surfactant Proteins A and D in Innate Lung Defense. *Journal of Neonatal Biology*. 1995. 67:2-17.

12. Lei T, Wei Y. Effects of Vitamin A on Lung Development in the Rat from Early Age to Adulthood. *Journal of Pediatrics*. 2008. 121: S152.

13. Sommer, Alfred. *Vitamin A Deficiency and its Consequences: A field guide to detection and control.* 3rd ed. WHO. 1995.

14. Baybutt R., Hu L, Molteni A. Vitamin A deficiency injuries lung and liver parenchyma and impairs function of rat II pneumocytes. *The Journal of Nutrition*. 2000. 130(5): 1159-1165.

15. Checkly W, West KP, Wise RA, Wu L, LeClerg SC, Khatry S, Katz J, Christian P, Tielsch JM, Sommer A. Supplementation with vitamin A early in life and subsequent risk of asthma. *European Respiratory Journal*. 2011. 38(6):1310-9.

16. Jobe AH. The New Bronchopulmonary Dysplasia. *Current Opinion in Pediatrics*. 2011. 23(2):167-172.

17. Starcher BC. Elastin and the lung. *Thorax* 1986. 41:577-585.

18. Hoeben A, Landuyt B, Highley S, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular Endothelial Growth Factor and Angiogenesis. *Pharmacological Reviews*. 2004. 56(4): 549-580.

19. Voelkel NF, Vandivier RW, Tuder RM. Vascular Endothelial Growth Factor in Lung. *American Journal of Physiology: Lung Cellular and Molecular Physiology.* 2006. 290(2):209-221.

20. Leiling Chen, Thomas Acciani, Tim Le Cras, Carolyn Lutzko, and Anne-Karina T. Perl "Dynamic Regulation of Platelet-Derived Growth Factor Receptor α Expression in Alveolar

Fibroblasts during Realveolarization." *American Journal of Respiratory Cell and Molecular Biology.* 2012 47(4): 517-527.

21. Kheradmand F, Rishi K, Werb Z. Signaling through the EGF receptor controls lung morphogenesis in part by regulating MT1-MMP-mediated activation of gelatinase A/MMP2. *Journal of Cell Science*. 2002. 115:839-848.

22. Leslie, K.O., Mitchell, J.J., Woodcock-Mitchell, J.L., Low, R.B. Alpha smooth muscle actin expression in developing and adult human lung. *Differentiation*. 1990. 44:143-149.

23. Whitsett JA, Weaver TE. Hydrophobic Surfactant Proteins in Lung Function and Disease. *The New England Journal of Medicine.* 2002. 347. 2141-2148.

24. Hamvas A, Cole FS, deMello DE, Moxley M, Whitsett JA, Colten HR, Nogee LM. Surfactant protein B deficiency Antenatal diagnosis and prospective treatment with surfactant replacement. *The Journal of Pediatrics*. 1994. 125(3):356-361.

25. Veness-Meehan KA, Pierce RA, Moats-Staats B, Stiles AD. Retinoic acid attenuates O_2-induced inhibition of lung septation. *American Journal of Lung Cell Molecular Physiology*. 2002. 283:971-980.

26. Guimarãea H, Guedes MB, Rocha G, Tomé T, Albino-Teixeira A. Vitamin A in prevention of bronchopulmonary disease. *Current Pharmaceutical Design*. 2012. 18(21):3101-13.

27. Esteban-Pretel G, Marin MP, Renau-Piqueras J, Sado Y, Barber T, Timoneda J. Vitamin A deficiency disturbs collagen IV and laminin composition and decreases matrix metalloproteinase concentrations in rat lung. Partial reversibility by retinoic acid. *The Journal of Nutitional Biochemistry.* 2013. 24(1):137-145.

28. Londhe VA, Maisonet TM, Lopez B, Shin B, Huynh J, Devaskar SU. 2013. Retinoic Acid Rescues Alveolar Hypoplasia in the Calorie-Restricted Developing Rat Lung. *Am. J. Resp. Cell Mol. Biol*. 2013. 48(2): 179-187.

29. James ML, Ross AC, Bulger A, Phillips III JB, Ambalavanan N. 2010. Vitamin A and Retinoic Acid Act Synergistically to Increase Lung Retinyl Esters During

Normoxia and Rescue Hyperoxic Lung Injury in Newborn Mice. *Pediatric Research*. 2010. 67(6): 591-597.

30. Bourbon J, Bocherat O, Chailley-Heu B, Delacourt C. Control Mechanism of Lung Alveolar Development and their Disorders in Bronchopulmonary Dysplasia. *Journal of Pediatric Research*. 2005. 57(5): 38R-46R.

31. Chailley-Heu B, Chelly N, Leliévre-Pégorier M, Barlier-Mur A, Merlet-Bénichou C, Bourbon, JR. Mild Vitamin A Deficiency Delays Fetal Lung Maturation in the Rate. *American Journal of Respiratory Cell and Molecular Bio*logy. 1999. 21:89-96.

32. Antipastis C, Ashworth CJ, Grant G, Lea RG, Hay SM, Rees WD. Effctes of maternal vitamin A status on fetal heart and lung/l change sin expression of key developmental genes. *The American Journal of Physiology*. 1998. 275: 1184-1191.

33. Bielsalski HK, Nohr D. Importance of vitamin A for lung function and development. *Molecular Aspects of Medicine*. 2003. 24(6):431-440.

34. Bielsalsk HK. The significance of vitamin A for the development and function of the lung. *Forum of Nutrition*. 2003. 56: 37-40.

35. Montesano R, Soulié. Retinoids induce lumen morphogenesis in mammary epithelial cells. *Journal of Cell Science*. 2002. 115: 4419-31.

36. Ross AC. Chapter 17: Diets in Vitamin A Research. *Methods of Molecular Biology*. 2010; . 652:295-313.

37. Labno, Christine. University of Chicago. Integrated Light Microscopy Core.

38. Ross AC, Li NQ. Retinol Combined with RA Increases Retinol Uptake and Esterification in the Lungs of Young Adult Rats when Delivered by the Intramuscular as well as Oral Routes. 2007. *The Journal of Nutrition*. 137(11): 2371-2376.

39. Chan V, Greenough A, Chessman P, Gamsu HR. (1993) Vitamin A levels at birth of high risk preterm infants. *Journal of Perinatal Medicine*. 1993. 21(2): 147-151.

40. Hustead VA, Gutcher GR, Anderson SA, Zachman RD. Relationship of vitamin (retinol) status to lung disease in the preterm infant. *Journal of Pediatrics*. 105(4): 610-615.

41. Zolfaghari A, Ross AC. Lecithin: Retinol Acyltransferase Expression is Regulated by Dietary Vitamin A and Exogenous Retinoic Acid in the Lung of Adult Rats. *The Journal of Nutrition*. 2002. 132: 1160-1164.

42. Molenaar R, Knippenberg M, Goverse G, Olivier BJ, de Vos AF, O'Toole T, Mebius RE. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. *Journal of Immunology*. 2011. 186(4):1934-1942.

43. Maden M, Hind M. Retinoids in lung development and regeneration. *Current Topics in Developmental Biology*. 2004. 61:153-189.

44. Grummer MA, Zachman RD. Retinoic acid and dexamethasone affect RAR-β and surfactant protein C mRNA in the MLE lung cell line. *The American Journal of Physiology*. 1998. 274: L1-7.

45. Mclaren DS. Vitamin A deficiency disorders. *Journal of Indiana Medical Association*. 1999. 97(8): 320-323

ACADEMIC VITA

ALEXIS V. HYCZKO

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EDUCATION

The Pennsylvania State University — **University Park, PA**
The Eberly College of Science, B.S. in Biology, Vertebrate Physiology — Fall *2014*
The College of Health and Human Development, Minor in Nutrition
Schreyer Honors College Scholar
London Study Tour 2012, Theater course/International Program

The Victoria University of Wellington — **Wellington, New Zealand**
Coursework in Genetics, Evolution, and Maori studies — *July 2013 – November 2013*

RESEARCH EXPERIENCE

Dr. Virginia Stallings, Children's Hospital of Philadelphia — **Philadelphia, PA**
Division of Nutrition and Gastroenterology — *Summer 2012*
Research Intern

- Organized, entered, and checked data from two clinical studies, located in Philadelphia and Botswana, testing the effects of Vitamin D on HIV positive patients

Dr. A. Catharine Ross, Pennsylvania State University — **University Park, PA**
Undergraduate Researcher — *Spring 2013 – Fall 2014*

- Studying the effects of Vitamin A on lung development using a rat model

EMPLOYMENT EXPERIENCE

Office of Governmental Affairs, Pennsylvania State University — **University Park, PA**
Suite Hostess — *Fall 2010-2012*

- Represented Penn State's student leadership body, directed visitors to pre-game banquet, guided visitors to football game seats, and resolved any issues to make football game day events run smoothly

Diana Gruendler, English Professor, Pennsylvania State University — **University Park, PA**
Teaching Assistant — *Fall 2011*

- Created a blog, taught students how to use the blog through a PowerPoint presentation with fellow TA's, and graded the students' weekly blog post

Dr. Connie Rogers, Nutrition Professor, Pennsylvania State University — **University Park, PA**
Teaching Assistant for Nutrition Metabolism Course — *Fall 2014*

- Help students during office hours, guide review sessions, and help grade exams

SUMMER ACADEMIC PROGRAMS

Children's Hospital of Pittsburgh — **Pittsburgh, PA**
Externship through the Eberly College of Science — *May 2011*

- Completed two-day shadowing experience with Penn State alumnus, Dr. Kathryn Torok, in Pediatric Rheumatology

Thomas Jefferson University Hospital — **Philadelphia, PA**
Externship through the Eberly College of Science — *May 2012*

- Completed two-day shadowing experience with Amy Bustin, Physician Assistant in Neurology

Primary Care Scholars Program — **Hershey, PA**
Penn State College of Medicine — *May 2014*

- Participated in a two-week educational program. The first week consisted of learning about important issues in health-care delivery and primary care, medical school admissions, financial aid and the medical school's curriculum process. The second week involved gaining clinical experience in primary care by shadowing pediatricians from Gordon-Klinow Pediatric Associates.

Summer Clinical Preceptorship Program **Hershey, PA**
Penn State College of Medicine *June 2014*
- Participated in a two week shadowing program in which I shadowed doctors within Family and Community Medicine, Pediatrics, Orthopedics, Surgery, and Internal Medicine

COMMUNITY SERVICE

Thomas Jefferson University Hospital
Volunteer **Philadelphia, PA**
- Surveyed patients in the Emergency Room for the Research Department and completed paperwork for nurses *July - August 2010*
- Assisted nurses and Child Life Specialist in the Pediatric Unit *July - August 2011*

Mount Nittany Medical Center **State College, PA**
Volunteer in Emergency Room *Summer 2013*
- Wheeled patients to X-ray and CT scans, changed bedding, replenished stocks of food and bedding

ACTIVITIES

Clubs and Societies **University Park, PA**
- Alpha Epsilon Delta, The Health Pre-Professional Honors Society *September 2010 – Fall 2014*
  - Distinguished Membership; THON committee, Public Health Fair Committee
- Phi Eta Sigma National Honor Society *February 2011 – Fall 2014*
- Victoria International Leadership Program (New Zealand) *July 2013 - November 2013*
- Mentoring with Honors Program *November 2013 – Fall 2014*
  - Matched with and accomplished Schreyer scholar alum who shares similar career goals and has given me advice on how to pursue my own

Leadership **University Park, PA**
- FTCAP Mentor *May 2012*
  - Helped to run FTCAP for incoming Schreyer Honors scholars by giving presentations to students about academics in the honors college, guiding scholars and parents to various activities, and answering any questions from the scholars and parents

- Co-founder of "Pause for Paws"
  - Organized an event to bring dogs from a local animal shelter to students the week before finals as a source of stress relief *April 2013*

HONORS AND AWARDS

Dean's List *Fall 2010, Spring 2011, Fall 2011, Fall 2012, Spring 2013, Fall 2013, Spring 2014*

A. Whitney Frankenberry scholarship *2010-2011, 2011-2012, 2012-2013, 2013-2014*

Summer Research Grant from Schreyer Honors College *Summer 2013*

Summer Schreyer Ambassador Travel Grant *Fall 2013*

Louis A. Martarano Endowment for Education Abroad *Fall 2013*