THE USE OF A MOUSE MODEL TO DETERMINE THE INFLUENCE OF DIET AND GENETICS ON VITAMIN D STATUS, VITAMIN D DEFICIENCY, AND GLYCEMIC CONTROL

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ABSTRACT

Elizabeth K. Hutchins: The Use of a Mouse Model to Determine the Influence of Diet and Genetics on Vitamin D Status, Vitamin D Deficiency, and Glycemic Control
(Under the direction of Folami Ideraabdullah)

Well-known for its importance in calcium homeostasis, there is evidence that vitamin D also plays a role in the maintenance of glycemic control. In this study, we utilized a panel of Collaborative Cross inbred mice to investigate the extent to which vitamin D status, vitamin D deficiency, and glycemic regulation are influenced by diet and genetic background. We detected a dietary treatment effect on 25(OH)D but not on $1,25(OH)_2D$. We detected genetic effects on vitamin D status, deficiency, and on glycemic control. Interestingly, strains with lower vitamin D had lower markers of glycemic control. Lastly, dietary depletion of vitamin D led to lower liver *GAPDH* expression, which was also associated with lower markers of glycemic control. These data not only validate a relationship between vitamin D status and glycemic regulation, but provide evidence that genetic contribution to that status is an important factor in understanding risk of associated physiological outcomes.

To Mom, I could not have done this without you and Dad.
Thank you for your unwavering support.

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LIST OF ABBREVIATIONS

AGS	American Geriatric Society
CC	Collaborative Cross
CYP	Cytochrome P450
DBP	Vitamin D Binding Protein
DRIP	Vitamin D Receptor Interacting Protein
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GTT	Glucose Tolerance Test
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
IOF	International Osteoporosis Foundation
IOM	Institute of Medicine
IR	Insulin Receptor
MAP	Mitogen-Activated Protein
MZF-1	Myeloid Zinc Finger-1
NAFLD	Non-Alcoholic Fatty Liver Disease
PBMC	Peripheral Blood Mononuclear Cell
PLC	Phospholipase C
PTH	Parathyroid Hormone
qPCR	Quantitative Polymerase Chain Reaction
RXR	Retinoid X Receptor
SNP	Single Nucleotide Polymorphism
UCP	Uncoupling Protein
UVB	Ultraviolet B
VDD	Vitamin D Deficient
VDR	Vitamin D Receptor

VDRE	Vitamin D
VDS	Vitamin D Sufficient
WT	Wild-Type

CHAPTER 1: INTRODUCTION

Vitamin D: Sources, Metabolism, and Activity

Vitamin D is one of the thirteen essential vitamins needed for normal human physiological function and is found in the body in two forms: Vitamin D_2 (ergocalciferol) and Vitamin D_3 (cholecalciferol). While ergocalciferol is obtained in the diet via plants, cholecalciferol is obtained in the diet via meat products, fortified goods, and supplements. Arguably the best source of vitamin D is vitamin D_3 synthesized by the body when exposed to sunlight. Vitamin D biosynthesis begins with a provitamin, 7-dehydrocholesterol, in the epidermis of the skin. When exposed to ultraviolet B (UVB) radiation, 7-dehydrocholesterol photolyzes to previtamin D_3, which then isomerizes to form vitamin D_3[1,2]. Once in the circulation, vitamin D binds to a specific transporter known as vitamin D binding protein (DBP), facilitating transport of the vitamin in blood stream throughout the body[3].

In order to become biologically active, vitamin D must go through multiple hydroxylation events by a series of cytochrome P450 (CYP) enzymes. The first step of vitamin D activation occurs in the liver where carbon 25 is hydroxylated by CYP2R1 forming 25(OH)D (calcidiol)[4–6]. This form of vitamin D is the predominant circulating form of the vitamin due to its longer half-life[7] . The final activation event occurs in the kidney, where 25(OH)D is hydroxylated at the 1α carbon position by CYP27B1 yielding the active metabolite, $1,25(OH)_2D$ (calcitriol)[8–10]. Finally, other members of the CYP enzyme class are responsible for the excretion of vitamin D by hydroxylation of 25(OH)D and $1,25(OH)_2D$ in the carbon 24 position[11,12] (**Figure 1**).

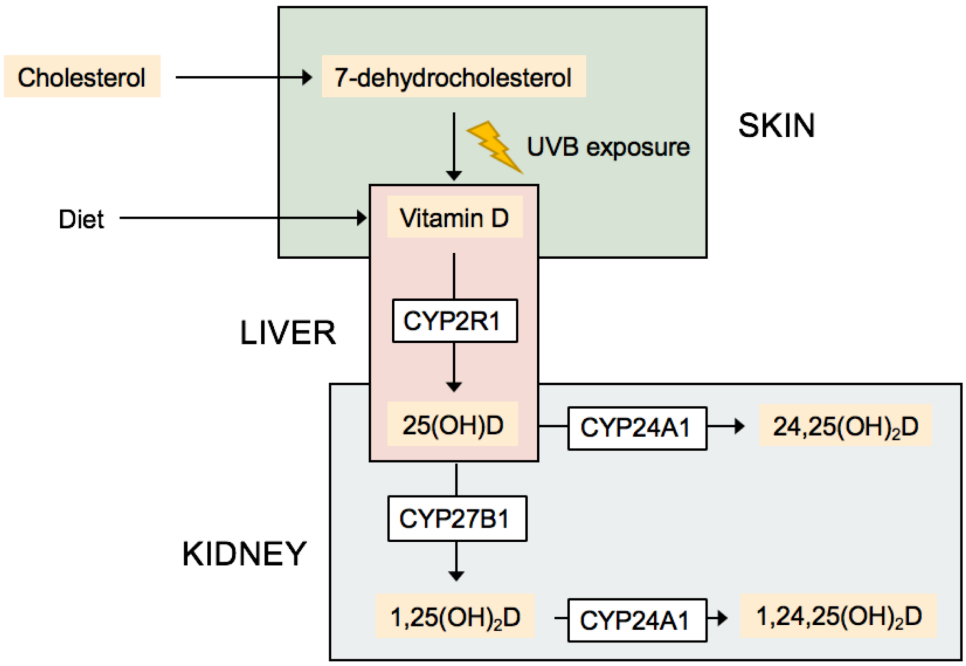

Figure 1. Vitamin D Metabolism. Vitamin D synthesis and production of vitamin D metabolites.

Vitamin D is not just a vitamin; it is a prohormone. Once fully activated, vitamin D acts as a member of the steroid hormone superfamily and is exported from the kidneys back into circulation to affect target tissues. Cells in target tissues contain the vitamin D receptor (VDR), a nuclear hormone receptor specific for $1,25(OH)_2D$[13]. Once the activated ligand binds to this receptor in the cytoplasm, it is translocated to the nucleus and heterodimerizes with the retinoid X receptor (RXR)[14], leading to the recruitment of co-activators and the DRIP (D receptor interacting protein) complex[15]. The ligand/receptor complex then binds a specific DNA sequence on the target gene known as the vitamin D response element (VDRE), allowing the regulation of downstream transcription[16–18] **(Figure 2)**.

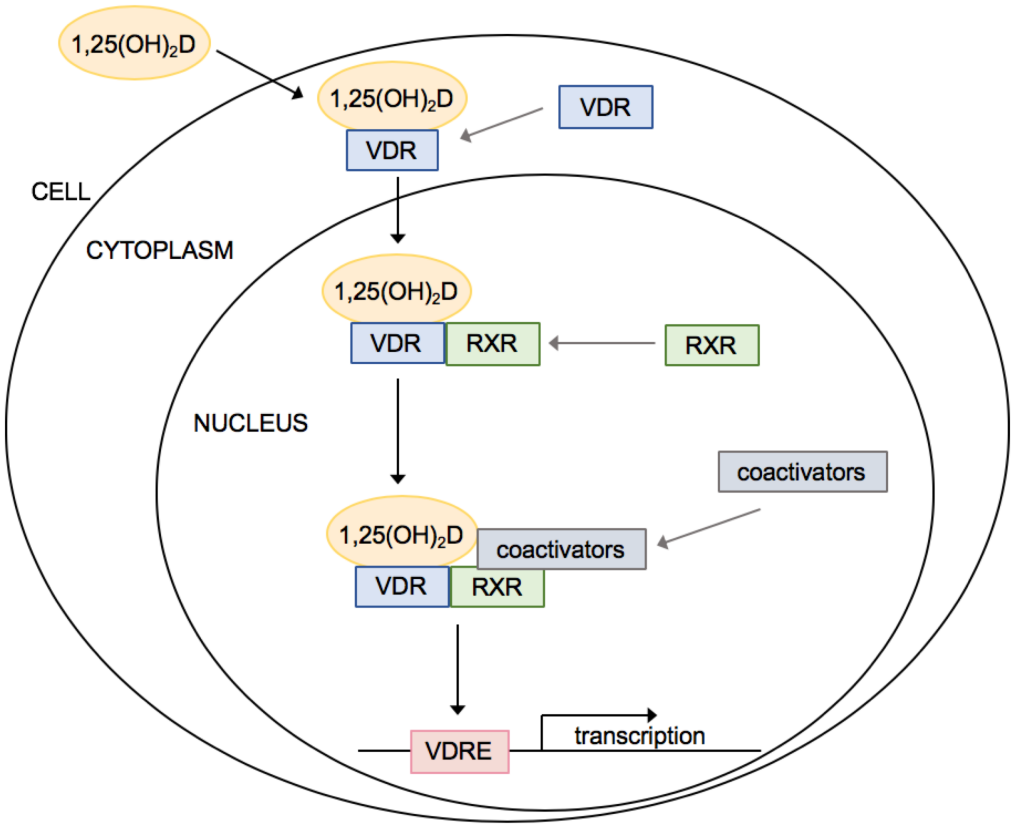

Figure 2. Vitamin D as a Steroid Hormone. Mechanism of vitamin D's hormonal action allowing transcriptional regulation.

While vitamin D is known classically to act through the VDR receptor, research has shown that this model is not comprehensive of vitamin D's full range of action. Evidence shows that $1,25(OH)_2D$, while still having important genomic functions, also can stimulate rapid membrane reactions independent of VDR[19]. Studies have proposed that this non-genomic rapid activity is mediated by $1,25(OH)_2D$-stimuted activation of tyrosine kinase, causing downstream tyrosine phosphorylation of effector enzymes in signal transduction pathways such as phospholipase C (PLC) and mitogen-activated protein (MAP) kinase[20,21]. This research provides evidence that vitamin D plays an intricate role in human physiology that is still not fully understood.

Vitamin D Deficiency: Relevance to Public Health

Vitamin deficiency refers to the absence of a vitamin to the extent that it has the potential to negatively impact normal physiological function. Despite knowing that vitamin D plays an important biological role, there is no consistent recommendation for assessing vitamin D deficiency. Additionally, recommendations are based on vitamin D's role in calcium metabolism without regard to vitamin D's other physiological role. Although $1,25(OH)_2D$ is the active metabolite in vitamin D metabolism, serum 25(OH)D is generally used for estimation of deficiency due to its longer half-life. While 25(OH)D is consistently recommended as the unit of quantification of deficiency, recommendations for required levels of the metabolite are not as consistent. The Institute of Medicine (IOM) supports the recommendation of using 25(OH)D at a minimum concentration of 20 ng/mL to determine risk of vitamin D deficiency[22], while other institutions and researchers believe 30 ng/mL is what defines risk[23]. There is an urgent need to better understand vitamin D metabolism and deficiency across populations in order to better establish both measures of deficiency and implications of deficiency in all individuals.

The primary cause of vitamin deficiencies is lack of dietary consumption. In the case of vitamin D this is likely due to the absence of vitamin D as a substantial source in many foods, particularly in non-meat products. Therefore, UVB radiation is arguably the best source of vitamin D, and regular sunlight exposure has decreased globally due to both changes in lifestyle and the connection between UV exposure and skin cancer. Additionally, factors such as season and darker skin pigmentation can lead to decreased biosynthesis of vitamin D from UVB rays[24,25]. It is also important to consider age and sex when thinking about vitamin D's role in bone metabolism. The International Osteoporosis Foundation (IOF) and American Geriatric Society (AGS) recommend 25(OH)D levels of at least 30 ng/mL in elderly populations in order to minimize risk of falls and fractures[26,27].

Because vitamin D metabolism involves a complex series of enzymatic steps as well as transcriptional regulation leading to diverse outcomes, genetic regulation likely is an important

consideration in understanding vitamin D status and the physiological effects of that status. While standards have been established to define deficiency is in the human population, deficiency rates vary widely depending on race and genetic background[28]. Overall deficiency rates were found to be around 40% in the United States in 2011, but deficiency rates in African Americans and Hispanics were exorbitantly higher than that of the overall population[29]. Additionally, association studies have identified potentially causal single nucleotide polymorphisms (SNPs) in enzymes involved in vitamin D metabolism[30–32]. These differences in deficiency rates tell us that there are blatant differences in vitamin D metabolism among genetic backgrounds, generating a question of whether these variations in metabolism implicate variations in the risk of health outcomes.

Vitamin D deficiency is a global problem, with deficiency rates exceeding eighty percent in some human populations[33]. While it has been consistently established that Vitamin D acts with parathyroid hormone to maintain calcium homeostasis and downstream bone health[34], the vitamin has also been implicated for its role in immunity [35–39], cell proliferation and differentiation[40–43], and nutrient metabolism[44,45]. Research has provided evidence that these additional roles facilitate an interaction between vitamin D status and the etiology of many diseases. Multiple sclerosis has been repeatedly found to be associated with vitamin D status among human subjects, with low levels of vitamin D increasing the risk for the development of multiple sclerosis[46,47]. Cell culture studies have mechanistically displayed that vitamin D plays an immunomodulatory role in multiple sclerosis by regulating T cell homeostasis[48]. Cell culture studies have also shown that many types of tumors have expressed vitamin D receptors and cancer cell progression is responsive to vitamin D supplementation[49–51]. Vitamin D has a known role in the development of cardiovascular disease. Mechanisms by which vitamin D regulates nitric oxide production and endothelial muscle function have been proposed based on rodent studies and cell culture research [52,53]. Longitudinal and cross-sectional human studies have consistently shown an association between all three types of diabetes and vitamin D status[54–58].

While the results of these studies are compelling in the argument that vitamin D has a role in glycemic control, evidence of mechanistic action in cell culture and rodent studies needs to be expanded upon.

Glucose Homeostasis and Glycemic Control

Glucose is the body's main form of energy. Not only is glucose consumed in the diet, but can be produced by the liver via gluconeogenesis and glycogenolysis, and in the muscle via glycogenolysis. While gluconeogenesis in the liver uses substrates such as lipids and proteins to produce glucose, glycogenolysis uses glycogen, the stored form of glucose, to provide the body with glucose. In order to produce energy from glucose, the cells must perform glycolysis, breaking down the glucose molecule to generate ATP. While glucose is easily one of the most important metabolites in nutrient metabolism, it can be detrimental to the body's organs if left uncontrolled. Glycemic control refers to the body's ability to maintain blood glucose homeostasis. In order to prevent fluctuations that can lead to cell and organ damage, insulin and glucagon act as master hormonal regulators of blood glucose levels. Both regulators are secreted from the pancreatic islet of Langerhans. Insulin is secreted from the β-cells and has a hypoglycemic effect, while glucagon is secreted from the α-cells and has a hyperglycemic effect.

Insulin, arguably the most vital hormone for glucose regulation, is an anabolic peptide hormone secreted in response to glucose stimulation in the pancreatic β-cells. After entering the β-cell through a glucose transporter on the plasma membrane, glucose goes through glycolysis producing pyruvate. The pyruvate-producing step of glycolysis generates ATP from ADP, increasing the ATP/ADP ratio in the cell and leading to the closure of the potassium-gated channels[59,60]. The cellular depolarization of the membrane resulting from the closure of these channels causes calcium-gated channels to open, and increased intracellular calcium triggers the exocytosis of insulin from the β-cell[61–63]. It is important to note, however, that fatty acids and amino acids can also prompt the secretion of insulin in a similar manner. Once secreted into the bloodstream from the pancreas, insulin must be recognized by the cells of the body in order to

have a physiological effect. Insulin serves as a ligand to the insulin receptor (IR) which is located on the plasma membrane of cells[64–66]. The insulin receptor is a member of the tyrosine kinase receptor superfamily, meaning that binding of insulin causes autophosphorylation of the tyrosine residues of the receptor[67–69]. Through a canonical pathway of phosphorylation of substrates inside the cell, GLUT transporters are translocated to the membrane surface allowing the uptake of glucose into the cell, and therefore the lowering of glucose in the blood. This stepwise signaling also promotes the use and storage of glucose, fatty acids, and amino acids.

Public health efforts have contributed to the tackle of communicable diseases that were once such a burden to humanity. However, the ability to fight these diseases has permitted the drastic increase in the global prevalence of non-communicable diseases. In the 2011 Political Declaration on the Prevention and Control of Non-communicable Diseases, diabetes was one of the four non-communicable diseases targeted by world health leaders [70]. Diabetes Mellitus, a disease characterized by hyperglycemia (i.e. uncontrolled blood glucose levels), is characterized into three types each with different etiologies: Type I, Type II, and Gestational Diabetes Mellitus[71]. Type I Diabetes is classified as an autoimmune disease, where immune-mediated destruction of the pancreatic β-cells osccurs[72]. Therefore, insulin cannot be produced and perform its function in lowering blood sugar. Type II Diabetes is also characterized by insulin's inability to perform its hypoglycemic action. Conversely, hyperglycemia in Type II Diabetes is driven by resistance of peripheral cells to insulin's action rather than the body's inability to produce insulin. Hyperinsulinemia and hyperglycemia will lead to a cycle of β-cell destruction in these patients as well[73]. Gestational Diabetes is a unique form of diabetes that occurs during pregnancy when β-cells cannot compensate for increased glucose metabolism needs during gestation, leading to potential complications for the developing fetus as well as the mother. While diabetes is the disease most characterized by loss of glycemic control, it is important to consider that glycemic dysregulation is a hallmark of many other including

cardiovascular disease, cancers, non-alcoholic fatty liver disease (NAFLD), Alzheimer's disease, and Parkinson's disease, among others[74–79].

Role of Vitamin D in Glycemic Control

It is well known that the development of diabetes and other metabolic diseases is influenced by the interaction of environmental, genetic, and epigenetic factors[80–85]. However, until all aspects of disease etiology are better understood, prevention and treatment cannot be fully optimized. Epidemiological studies have consistently shown an association between low serum levels of vitamin D and all 3 types of diabetes: Type I Diabetes[86], Type II Diabetes[87], and Gesational Diabetes Mellitus[88]. Additionally, vitamin D supplementation in all forms of diabetes has been demonstrated to improve glycemic control[89–91].

There have been various proposed mechanisms that link vitamin D's role in regulation of glycemic control. Chronic-low grade inflammation is a key part of the development of metabolic diseases such as diabetes. Vitamin D may serve as a protective factor, as studies demonstrate that the vitamin decreases pro-inflammatory cytokines while increasing anti-inflammatory cytokines that are involved in the progression of chronic inflammation[92,93]. Vitamin D may influence cytokine and chemokine expression in the pancreatic islets, allowing decreased β-cell apoptosis and maintenance of insulin secretion[93]. VDR is abundant in many cells throughout the body, including the pancreatic β-cell[94]. By focusing on VDR in the pancreatic β-cell, it has been suggested that expression of the receptor transcriptionally upregulates the voltage-gated calcium channels leading to increased insulin secretion[95]. Vitamin D has also been linked to a role in insulin sensitivity. Research has shown that vitamin D plays a role in the regulation of the renin-angiotensin system by reducing renin and therefore angiotensin II, contributing to the prevention of cardiovascular disease[96–98]. However, this system relates to the development of diabetes as well, as angiotensin II decreases the sensitivity of peripheral cells to insulin by inhibiting the phosphorylation cascade that's vital for insulin and glucose signaling[99].

GAPDH: Relation to Vitamin D and Glycemic Control

GAPDH is a gene that codes for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein. GAPDH is an enzyme well known for its involvement in the glycolytic pathway, converting glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate while producing an NADH molecule in the sixth step of glycolysis[100] **(Figure 3)**. This is an important role in energy production and glycemic control. GAPDH is also involved in other processes vital to cell survival and homeostasis, including contributing to the regulation of microtubule bundling[101] and membrane fusion[102] in the cell. Additionally, GAPDH has been implicated as a regulator of transcription[103] and translation[104]. Relating to the cell cycle, studies have also found that GAPDH is involved in programming cell death, particularly in the event of oxidative stress[105–107]. There is significant evidence that the mechanism behind GAPDH-mediated cell death is due to the nitric oxide-induced *S*-nitrosylation of GAPDH, which causes inactivation of the enzyme and eventual cell death[108–110]. Interestingly, it has been shown that vitamin D stimulates nitric oxide production[52].

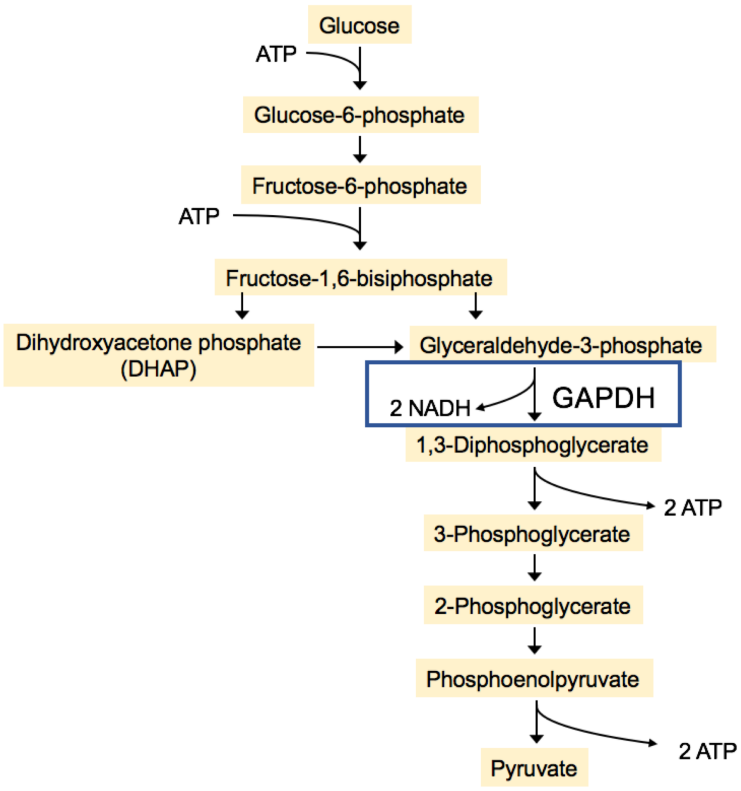

Figure 3. GAPDH in Glycolysis. Enzymatic regulatory role that GAPDH plays in glycolytic breakdown of glucose. All arrows represent enzymatic steps. Blue box displays where GAPDH plays active enzymatic role.

There is evidence that vitamin D may play a role in the regulation of energy metabolism and glycolysis. For example, VDR-null mice have higher expression of uncoupling proteins (UCPs) and increased rate of β-oxidation in white adipose tissue, as well as increased energy expenditure, oxygen consumption, and carbon dioxide production when compared to wild-type (WT) mice[111]. Consistent with these findings, an association study in humans showed that those with lower vitamin D status had increased respiration, ATP production, proton leak, background glycolysis, and glycolytic reserve in peripheral blood mononuclear cells (PBMCs)[112]. Research investigating vitamin D's role in skeletal muscle energy metabolism has provided evidence that vitamin D may play a role in increasing mitochondrial oxidative phosphorylation in skeletal muscle[113,114]. While minimal research has been done directly linking vitamin D to *GAPDH* expression, it has been demonstrated that vitamin D may upregulate myeloid zinc finger-1

(MZF-1), which binds to the *GAPDH* promoter and upregulates expression of the protein [115]. Although *GAPDH* is typically used as a normalization control in gene expression studies due to its consistent expression in all tissues[116], *GAPDH* expression may actually be dysregulated depending on vitamin D status.

Overall Objective

To use genetically divergent Collaborative Cross (CC) inbred mice to determine the influence of diet and genetic background on vitamin D status, potential for vitamin D deficiency, and glycemic status.

Study Hypothesis

I hypothesize that both genetic background and dietary deficiency of Vitamin D will impact vitamin D availability, with lower availability increasing risk of physiological impacts of deficiency and glycemic dysregulation.

CHAPTER 2: METHODS

Animals and Diet

Animal handling was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* under the corresponding animal use protocol at the University of North Carolina at Chapel Hill. Collaborative Cross (CC) inbred strains CC001/Unc (CC001), CC006/TauUNC (CC006), CC011/Unc (CC011), CC017/Unc (CC017), CC026/GeniUNC (CC026), and CC032/GeniUnc (CC032) mice were obtained from the UNC Systems Genetics Core Facility (Chapel Hill, NC)[117]. Mice were maintained in a low stress environment at 22°C, 50% humidity, and on a 12:12 hour light-dark cycle. Mice were provided water and fed rodent chow *ad libitum*. As displayed in **Figure 4**, animals were fed a standard chow diet (2400 IU vitamin D_3 /kg diet) until 21-22 weeks of age when they were randomly assigned a purified vitamin D sufficient diet (VDS) (2200 IU vitamin D_3/kg diet) or a purified vitamin D deficient diet (VDD) (0 IU vitamin D_3/kg diet). Mice remained on diet approximately 6 weeks, until euthanasia.

Body Composition and Glucose Tolerance Testing

Food intake and body weight were monitored weekly throughout the course of the study. Body composition was assessed before (week 0 of dietary treatment) and after (week 5 of dietary treatment) the study via EchoMRI at the Animal Metabolism Phenotyping Core (Chapel Hill, NC). Glucose tolerance tests (GTTs) for assessing metabolic parameters including glucose tolerance and fasting blood glucose were also performed before (week 0 of dietary treatment) and after (week 5 of dietary treatment) the study. For GTTs, mice were fasted for 6 hours and glucose was injected intraperitoneally at 2g/kg per lean body mass. A glucometer (AccuCheck) was used to

measure blood glucose concentrations via tail sampling over a 2 hour period (0, 15, 30, 45, 60, 120 minutes).

Tissue and Serum Collection

Mice were euthanized via CO_2 inhalation for tissue and serum collection at week 6 of dietary treatment. Whole blood was collected via submandibular bleed and cardiac puncture immediately after euthanasia. Serum was prepared from blood and snap-frozen in liquid nitrogen. Submandibular serum was used for quantification of insulin, calcium, and parathyroid hormone, while cardiac puncture serum was used for quantification of vitamin D metabolites. The liver was removed, weighed, and sectioned into three (3) pieces. The left and right medial lobes were used for liver histology, while the remainder was snap-frozen in liquid nitrogen and pulverized into a homogenous mixture for gene expression analysis.

Quantification of Metabolites, Insulin, Calcium, Parathyroid Hormone, and HOMA-IR

Cardiac puncture serum was used for the measurement of vitamin D metabolites. In samples in which there was not enough cardiac puncture serum, cardiac puncture serum and submandibular serum were pooled. Seven vitamin D metabolites were quantified via mass spectrometry at the UC Davis Lipid Analytical Core Facility (Davis, California) including 25(OH)D2, 25(OH)D3, $1,25(OH)_2D2$, and $1,25(OH)_2D3$. Different forms of vitamin D (D2 &D3) were combined in all statistical analysis of the metabolites.

Submandibular serum collected at euthanasia was used for the quantification of insulin, calcium, and parathyroid hormone. Insulin concentrations were quantified using UltraSensitive Mouse Insulin ELISA kit (CrystalChem) according to manufacturer's protocol. Serum parathyroid hormone (PTH) was measured using Mouse PTH 1-84 ELISA kit (Immutopics) according to manufacturer's instructions and serum calcium was measured using Calcium Colorimetric Assay kit (BioVision). The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated using fasting glucose measured during GTT at diet week 5 and fasting insulin

measured from serum collected during harvest at diet week 6. Calculation used was HOMA-IR = {[fasting insulin (μU/ml)] × [fasting glucose (mmol/l)]}/22.5[118].

Quantitative PCR (q-PCR)

Total RNA was extracted from pulverized liver using Trizol reagent. RNA was treated with DNAse and reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems); 2.5-5 ng cDNA was used for each assay. q-PCR reactions were performed using the TaqMan Fast Advanced Master Mix (Applied Biosystems) and the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Efficacy of q-PCR was calculated using standard curves within each PCR run.

Statistical analysis

JMP Pro software version 12.2.0 (SAS, NC) was used to compute all statistical analyses. In order to bin continuous data into categorical variables, R 'Statar' package was used (https://cran.r-project.org/package=statar). Before each statistical test, data normality was assessed using a Shapiro-Wilk goodness-of-fit test while equal variance was assessed using Bartlett's test. Data that fulfilled both assumptions of normality and equal variance were analyzed using parametric tests (t-test, ANOVA, adjusted least squares regression). Data that failed the goodness-of-fit test were analyzed using non-parametric tests (Wilcoxon/Kruskal-Wallis test). Data that failed both the goodness-of-fit and variance tests were analyzed using the Median test. Tukey post-hoc was run on ANOVA, Kruskal-Wallis, and Median models that revealed significant results. In order to determine confounders for regression models, statistical analyses were run to determine what independent factors impacted the study outcomes on their own. Those that were significant were included in regression models. Statistical analyses used are indicated in figure legends. For all comparisons, p-value <0.05 are considered statistically significant.

CHAPTER 3: IMPACT OF SIX WEEKS ADULT DIETARY VITAMIN D DEPLETION ON VITAMIN D STATUS, POTENTIAL FOR DEFICIENCY, AND SUBSEQUENT GLYCEMIC OUTCOMES

Introduction

To assess the impact of chronic vitamin D deficiency on vitamin D status among adult CC mice, we measured vitamin D status and glycemic control in a population of six (6) strains of Collaborative Cross (CC) inbred mice (**Figure 4**). Adult mice were treated with vitamin D sufficient (VDS) or vitamin D deficient diet (VDD) over 6 weeks. To determine vitamin D status, serum was collected and total 25(OH)D2/3 and 1,25$(OH)_2$D2/3 were measured via mass spectrometry. Dietary depletion of vitamin D did not affect body weight, fat mass, lean mass, or total food intake (**Figure 5**).

	Diet				
	VDS N=		VDD N=		
Strain	Female	Male	Female	Male	Total
CC001	3	3	3	3	12
CC006	3	3	3	3	12
CC011	3	3	3	3	12
CC017	3	0	3	0	6
CC026	3	3	3	3	9
CC032	3	0	3	0	9
Totals	18	12	18	12	60

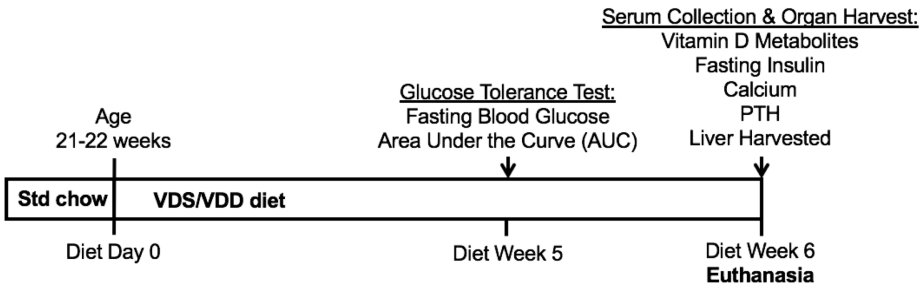

Figure 4. Study Design and Treatment Scheme. Male and female adult mice (age 21-22 weeks) from 6 CC strains were treated with either with VDS (Vitamin D sufficient diet, 1000 IU/kg Vitamin D) or VDD (vitamin D deficient diet, 0 IU/kg Vitamin D) for ~6 weeks. Mice were fed standard (Std) chow prior to treatment. Glucose tolerance tests were performed at week 5 of diet treatment, and organs were harvested and serum was collected at week 6 of diet treatment.

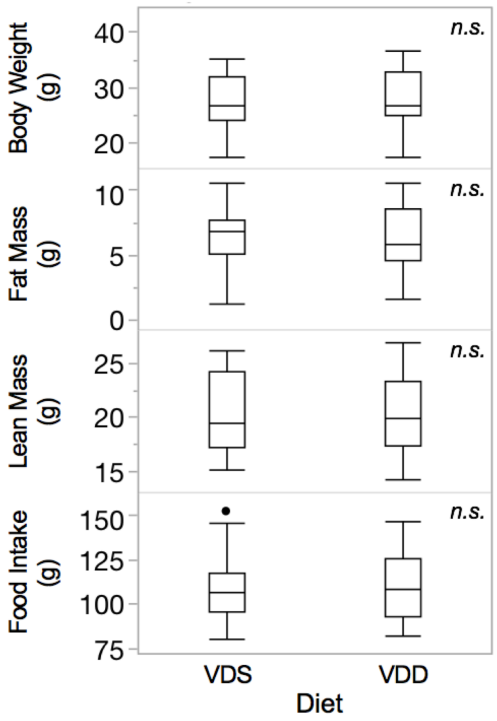

Figure 5. Effects of Dietary Depletion of Vitamin D on Potential Confounders. Box and whiskers plots with morphological outcomes between treatment groups across an entire population. Diet effect on each glycemic outcome determined by Wilcoxon test.

Six Weeks Dietary Depletion of Vitamin D Reduces Serum 25(OH)D Levels but Not Serum 1,25$(OH)_2$D Levels or Clinical Markers of Vitamin D Deficiency in CC Mice

VDD mice had significantly lower serum 25(OH)D concentrations in comparison to VDS mice (average percent decrease of 76.27%, **Figure 6A).** However, 1,25$(OH)_2$D concentrations were not significantly different between VDD and VDS treated mice (**Figure 6A**). We next determined whether VDD decreased 25(OH)D was sufficient to induce a physiological response characteristic of vitamin D deficiency. Because vitamin D's most well understood role in the body is its co-action with parathyroid hormone (PTH) in calcium homeostasis, calcium and PTH are reliable markers of vitamin D deficiency. We measured calcium and PTH and found no significant difference between dietary treatment groups in either clinical marker. (**Figure 6B**). Therefore, despite a significant reduction in the marker for vitamin D status, these mice do not

exhibit the classical signs of vitamin D deficiency. This is most likely due to maintenance of $1,25(OH)_2D$ levels in VDD treated mice.

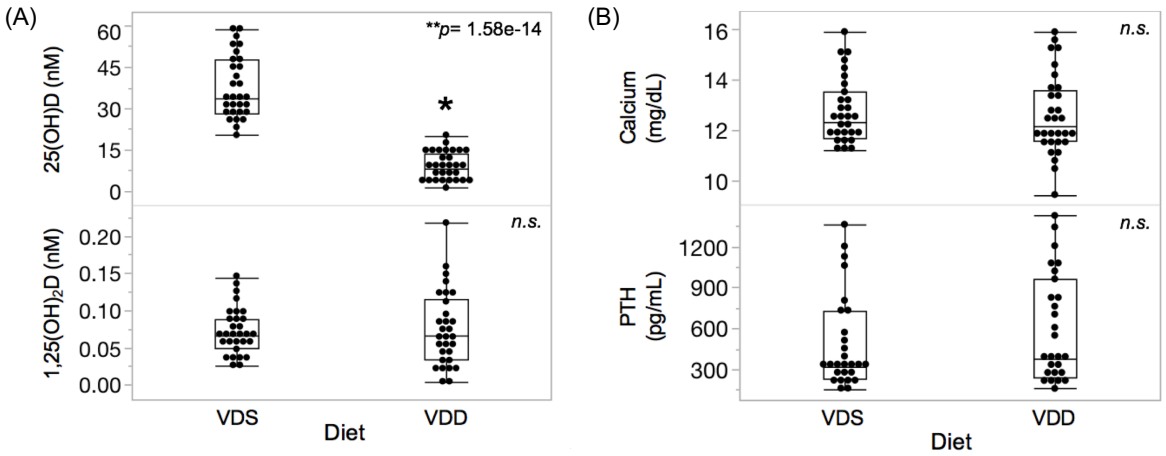

Figure 6. Effects of Dietary Depletion of Vitamin D on Vitamin D Status. Box and whiskers plots across an entire population. Each dot represents a single mouse. (A) Serum 25(OH)D and 1,25(OH)D measured by mass spectrometry for each diet. Main effect of diet on metabolites determined by Median test. (B) Circulating levels of classic markers of vitamin D status for each diet. Main effect of diet on calcium and parathyroid hormone determined by Wilcoxon test.

Six Weeks Adult Dietary Depletion of Vitamin D Does Not Affect Glycemic Control in CC Mice

In order to assess the role of dietary vitamin D availability in glycemic control, we compared glycemic status between the treatment groups. Insulin is one of the key regulators of glucose homeostasis, making circulating insulin and glucose two of the best indicators of glycemic status. In addition, glucose tolerance represents how well the body responds to a glucose stimulus which is a vital part of glycemic control. We saw no significant differences in fasting insulin, fasting glucose, glucose tolerance, or HOMA-IR between the VDS and VDD treated mice (**Figure 7**).

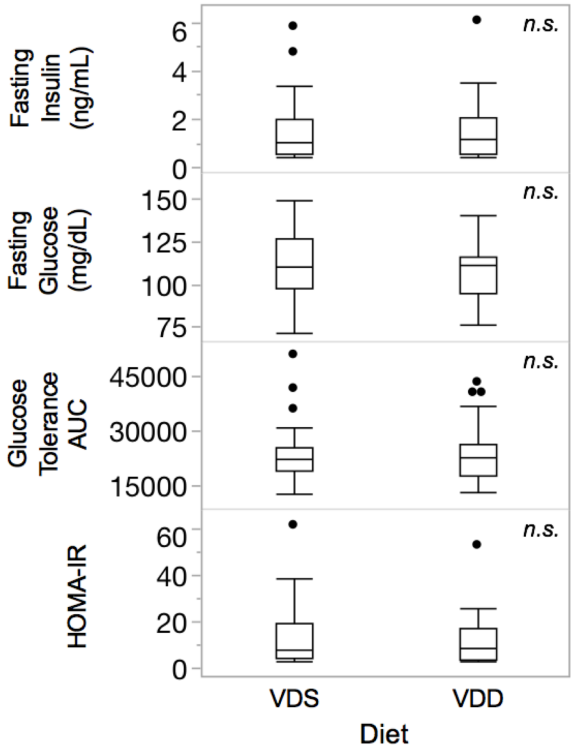

Figure 7. Effects of Dietary Depletion of Vitamin D on Glycemic Status. Box and whiskers plots with markers of glycemic status between treatment groups across an entire population. Diet effect on each glycemic outcome determined by Wilcoxon test.

Vitamin D Availability Determined by Metabolites Does Not Affect Physiological Outcomes Related to Vitamin D Deficiency or Glycemic Control

To better understand how vitamin D availability affects physiological outcomes across the population regardless of dietary treatment, we used vitamin D metabolite levels (25(OH)D2/3 and 1,25(OH)D2/3) to represent vitamin D availability. Because vitamin D metabolite data was non-normal, we binned data into tertiles and used only the top and bottom tertiles as [High & Low] measurements for statistical analysis (**Figure 8A**). To assess the effect of vitamin D availability on vitamin D deficiency, we again used calcium and PTH as indicators of a physiological response to deficiency. No significant effect was detected between vitamin D availability and either clinical marker of vitamin D deficiency (**Figure 8B**). We then used the binned metabolite data to assess for a potential relationship between vitamin D availability and glycemic control. A significant effect was found between 25(OH)D availability and fasting

glucose using a Wilcoxon test, with the lower 25(OH)D group having lower fasting glucose levels (p=1.10e-2). However, once adjusted for sex and fat mass, the relationship between 25(OH)D and glucose was no longer significant (p=5.94e-2). There was no significant relationship between either of the metabolite levels and any of the remaining markers of glycemic control that we measured (**Figure 8C**).

(A)

Metabolite	25(OH)D		$1,25(OH)_2D$	
Group	High	Low	High	Low
N=	19	20	19	21
Mean ±SD (nM)	43.55 ±9.35	5.82 ±2.91	0.120 ±0.033	0.033 ±0.014

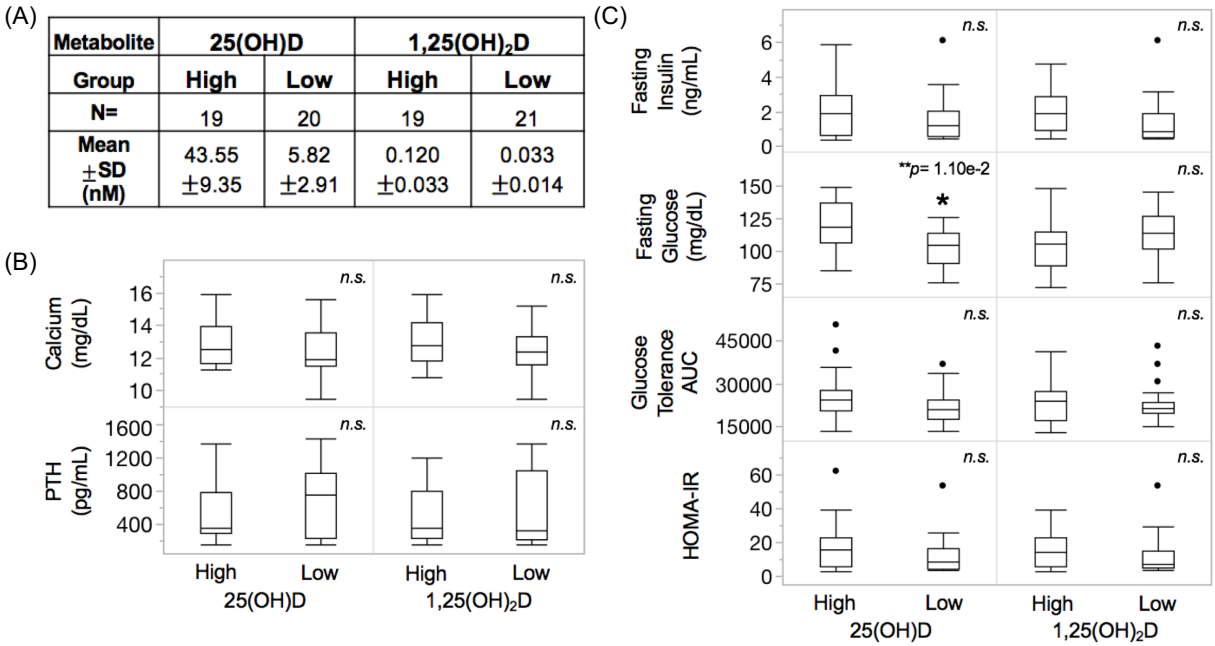

Figure 8. Effects of Vitamin D Status on Clinical Markers of Vitamin D Status and Glycemic Control. Box and whiskers plots across an entire population. (A) Vitamin D metabolites were binned into [High & Low] status by individual sample across an entire population in order to investigate the effects of metabolite status on physiological outcomes. (B) Clinical markers of vitamin D status between levels of vitamin D availability for each metabolite. Effect of the availability of vitamin D metabolites on markers of vitamin D status determined by Wilcoxon test. (B) Markers of glycemic control between levels of vitamin D availability for each metabolite. Effect of availability of vitamin D metabolites on glycemic control determined by Wilcoxon test. While a significant effect was found for fasting glucose using the Wilcoxon test, when adjusted for sex and fat mass the regression model yielded a non-

CHAPTER 4: ROLE OF GENETIC STRAIN IN DETERMINING VITAMIN D STATUS, POTENTIAL FOR DEFICIENCY, AND SUBSEQUENT GLYCEMIC OUTCOMES

Introduction

In this chapter, I describe our studies to characterize the role of genetic strain differences in vitamin D status and measure the physiological consequences of strain specific effects.

Genetic Strain Background Influences Baseline Vitamin D Status as Well as Response to Dietary Deficiency in Adult CC Mice

Among the six strains of CC mice (sample information found in **Figure 4**), vitamin D status was determined using plasma concentrations of 25(OH)D2/3 and $1,25(OH)_2D2/3$ metabolites. To determine the effect of strain on vitamin D status under dietary sufficient vs insufficient conditions, we compared the strain effect for the VDS to that of the VDD treatment group. VDS treated strains (N=30) exhibited variable baseline levels of 25(OH)D and $1,25(OH)_2D$. However, it was only statistically significant for $1,25(OH)_2D$ (**Figure 9A**). In the VDD group (N=30), there were significant strain differences in both 25(OH)D and $1,25(OH)_2D$ (**Figure 9B**). There was additionally a strain effect for the response to VDD (measured as percent change in metabolite status) for both 25(OH)D and $1,25(OH)_2D$ (**Figure 9C**). Consistent with the diet-induced changes in metabolites when all strains were combined (**Figure 6A**), 25(OH)D decreased across all strains while $1,25(OH)_2D$ decreased in some strains while increasing in others.

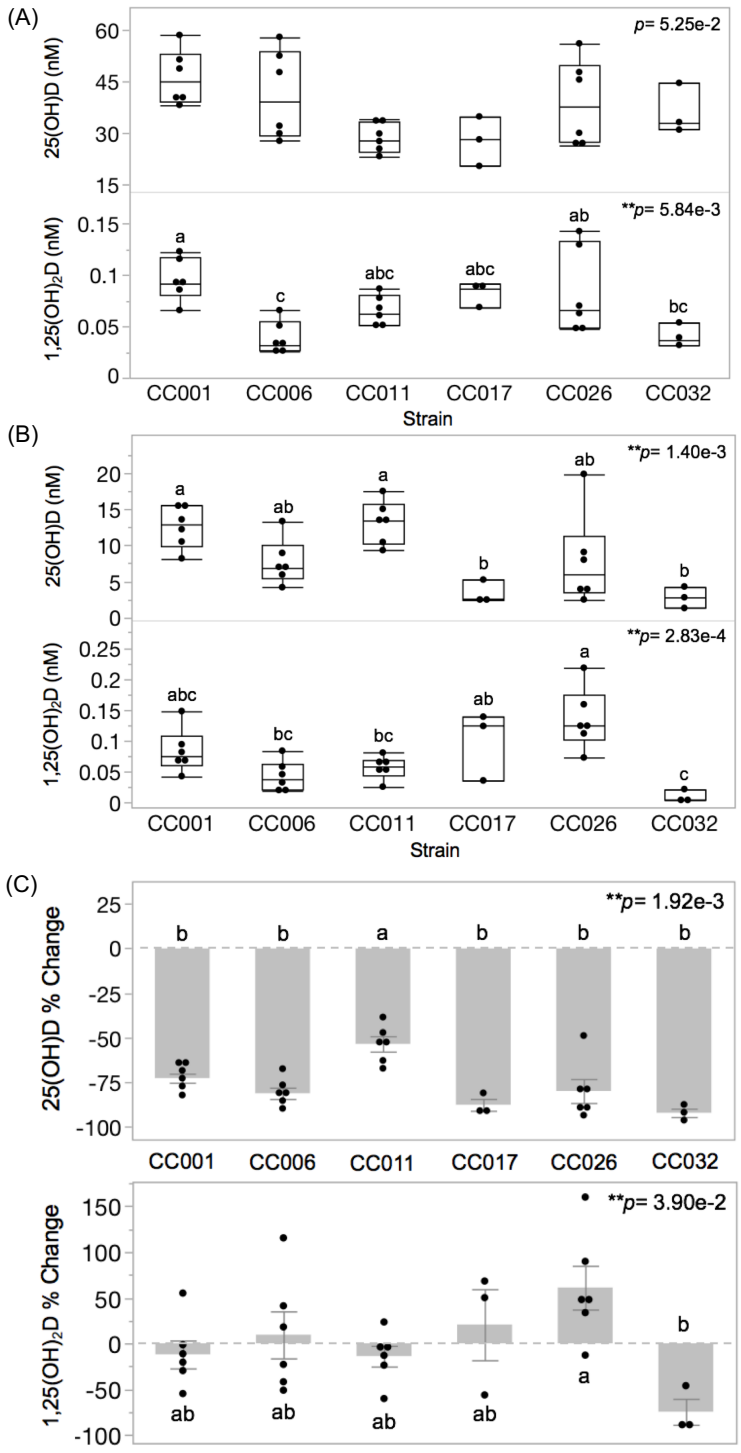

Figure 9. Effects of Strain on Vitamin D Metabolite Levels. Each dot represents a single mouse. Letters denote strains that differ significantly [a ≠ b = ab] as determined by Tukey posthoc. Serum 25(OH)D and 1,25$(OH)_2$D measured by mass spectrometry. (A) Box and whisker plot of metabolite levels in a VDS population. Main effect of strain on metabolites in the VDS population measured by Kruskal-Wallis test. (B) Box and whisker plot of metabolite levels in a VDD population. Main effect of strain on metabolites in the VDD population measured by ANOVA. (C) Bar graph of percent change in serum vitamin D metabolite concentrations caused by VDD [$(VDD^{Sample}-VDS^{Mean})/VDS^{Mean}$], separated by strain. Main effect of strain on percent change of metabolites caused by VDD determined by Kruskal-Wallis test.

Genetic Strain Background Influences Clinical Markers of Vitamin D Deficiency in Adult CC Mice

To establish the role of genetics in determination of markers of physiological vitamin D deficiency without accounting for strain-determined vitamin D status, we investigated strain

effects on calcium and PTH. Strain had a significant effect on calcium in both the VDS population (**Figure 10A**) and the VDD population (**Figure 10B**), but did not have an effect on PTH in either population. Contrastingly, strain did not have a significant effect on calcium response to dietary deficiency but did have an effect on percent change in PTH (**Figure 10C**).

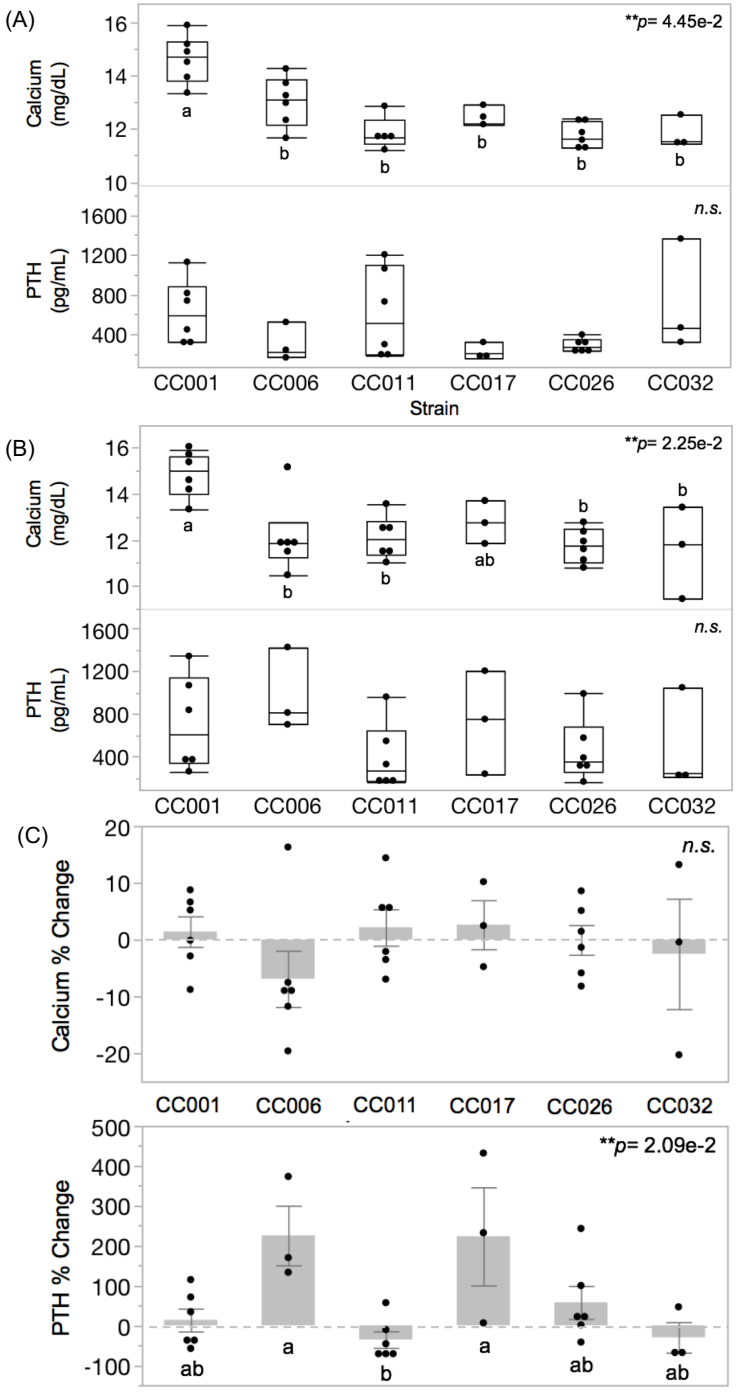

Figure 10. Effects of Strain on Diagnostic Markers of Vitamin D Deficiency. Each dot represents a single mouse. Calcium and PTH measured by ELISA. (A) Box and whisker plot of calcium and PTH in a VDS population. Main effect of strain on clinical markers of vitamin D deficiency in the VDS population measured by Median test. (B) Box and whisker plot of calcium and PTH in a VDD population. Main effect of strain on clinical markers of vitamin D deficiency in the VDD population measured by Kruskal-Wallis test. (C) Bar graph of percent change in clinical markers of vitamin D deficiency caused by VDD [$(VDD^{Sample}-VDS^{Mean})/VDS^{Mean}$], separated by strain. Main effect of strain on percent change of markers caused by VDD determined by Kruskal-Wallis test.

Genetic Strain Background Influences Glycemic Status in Adult CC Mice

In order to understand the direct effect of genetic background on glycemic control, we measured the strain effect on glycemic markers separately in a VDS and VDD population. In the VDS population, we detected a significant strain effect for fasting insulin, glucose tolerance, and HOMA-IR (**Figure 11A**). Interestingly, we observed significant strain effects in the same outcomes in the VDD population (**Figure 11B**), providing us with strong evidence that genetic background plays a consistent role in glycemic status across all populations. Although strain had a significant effect on these numerous important markers of glycemic status in both populations, there were no detected significant strain effects on percent change in any of the glycemic outcomes in response to dietary deficiency (**Figure 11C**).

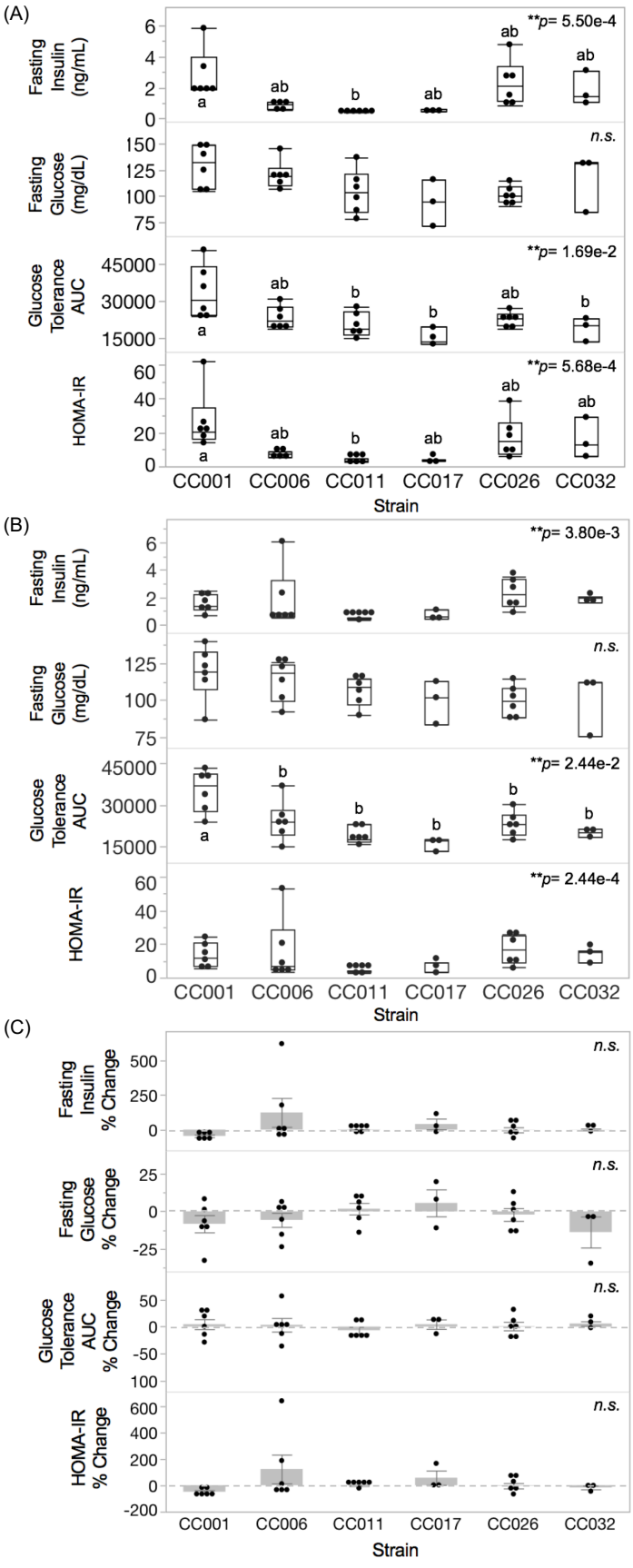

Figure 11. Effects of Strain on Glycemic Status. Each dot represents a single mouse. (A) Box and whisker plot of fasting insulin, fasting glucose, glucose tolerance, and HOMA-IR in a VDS population. Main effect of strain on clinical markers of vitamin D deficiency in the VDS population measured by Kruskal-Wallis test. (B) Box and whisker plot of fasting insulin, fasting glucose, glucose tolerance, and HOMA-IR in a VDD population. Main effect of strain on clinical markers of vitamin D deficiency in the VDD population measured by Median test. (C) Bar graph of percent change in clinical markers of vitamin D deficiency caused by VDD [(VDD^{Sample}-VDS^{Mean})/VDS^{Mean}], separated by strain. Main effect of strain on percent change of markers caused by VDD determined by Median test.

Strain Vitamin D Status is Not Associated with Clinical Markers of Vitamin D Deficiency in Adult CC Mice

To determine if genetic influence on vitamin D status plays a role in physiological health outcomes, the six strains were binned into two (2) status categories [High & Low] for both 25(OH)D2/3 and 1,25$(OH)_2$D2/3 based on the median metabolite level for each strain. To ensure that the relationships being investigated were due to strain-determined vitamin D status and not dietary availability of vitamin D, this was done separately for each dietary treatment group (**Figure 12**).

(A)

Treatment Group	VDS				VDD			
Vitamin D Metabolite	25(OH)D		1,25$(OH)_2$D		25(OH)D		1,25$(OH)_2$D	
Group	High	Low	High	Low	High	Low	High	Low
Strains (Median ±SD)	CC001 (44.93±8.04)	CC011 (27.69±4.43)	CC001 (0.092±0.021)	CC006 (0.032±0.016)	CC001 (12.91±2.89)	CC017 (2.57±1.60)	CC001 (0.076±0.036)	CC006 (0.038±0.025)
	CC006 (39.27±13.29)	CC017 (28.14±7.24)	CC017 (0.087±0.012)	CC011 (0.063±0.014)	CC006 (6.83±3.18)	CC026 (5.97±6.40)	CC017 (0.124±0.056)	CC011 (0.059±0.018)
	CC026 (37.84±12.50)	CC032 (32.82±7.42)	CC026 (0.067±0.042)	CC032 (0.037±0.012)	CC011 (13.52±3.00)	CC032 (2.78±1.49)	CC026 (0.124±0.049)	CC032 (0.005±0.010)
N=	18	12	15	15	18	12	15	15
Totals: Median ±SD	**43.32** ±11.27	**30.04** ±6.42	**0.087** ±0.029	**0.051** ±0.019	**11.37** ±3.82	**3.93** ±5.05	**0.112** ±0.048	**0.046** ±0.026

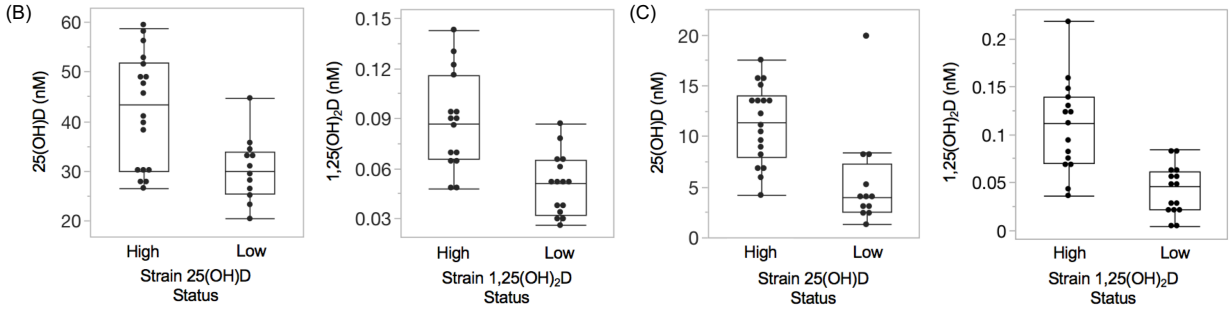

Figure 12. Strain Vitamin D Metabolite Status. (A) The 6 strains were binned into 2 status categories [High & Low] for both 25(OH)D and 1,25$(OH)_2$D based on the strain median metabolite status, separate for each dietary treatment group. Metabolite measurements in nM. (B) Distribution of samples in strain status categories for both metabolites in VDS treated mice. Each dot represents an individual sample. (C) Distribution of individual samples in strain status categories for both metabolites in VDD treated mice. Each dot represents an individual sample.

In order to further understand natural variations in vitamin D metabolism when in a sufficient state, we looked at the effect of vitamin D strain status on clinical markers of vitamin D deficiency in the VDS group. No significant difference was found in calcium or PTH levels

between the high and low status strains for either metabolite (**Figure 13A**). We also investigated the role of strain status in the VDD group to establish if genetic background offers a protective role in the development of physiological deficiency in a deficient state. Once again, no significant difference was detected in calcium or PTH levels between the strains of high and low status for either vitamin D metabolite (**Figure 13B**).

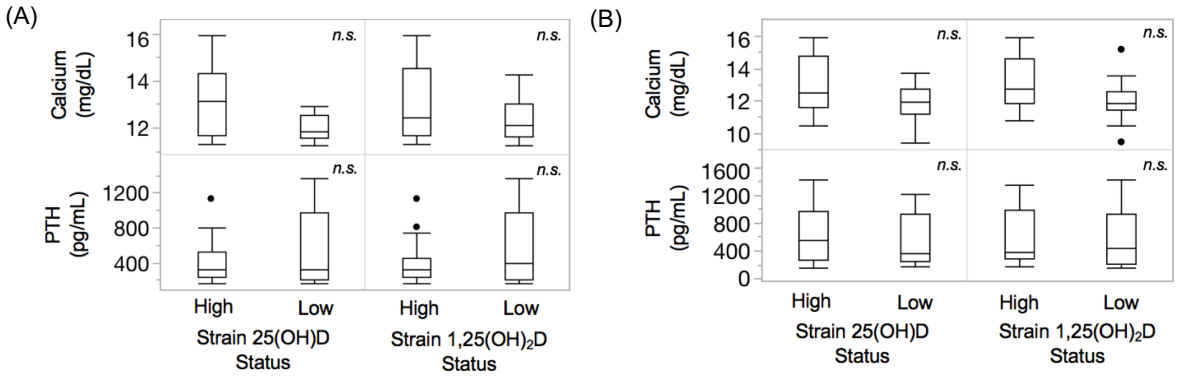

Figure 13. Effects of Vitamin D Strain Status on Clinical Markers of Vitamin D Deficiency. Box and whiskers plots across the VDS and VDD populations. Vitamin D metabolite strain availability categorized in each population into groups [High & Low] for 25(OH)D and 1,25($OH_{)2}$D. (A) Clinical markers of vitamin D status between vitamin D metabolite strain availability groups in the VDS treatment group. Effect of the strain vitamin D metabolite status on markers of vitamin D deficiency in VDS treated mice determined by Median test. (B) Clinical markers of vitamin D status between vitamin D metabolite strain availability groups in the VDD treatment group. Effect of the strain vitamin D metabolite status on markers of vitamin D deficiency in VDD treated mice determined by Wilcoxon test.

Strain-Specific Low Vitamin D Status is Associated with Glycemic Control in Both Vitamin D Sufficient and Vitamin D Deficient Populations of Adult CC Mice

To investigate how genetically determined vitamin D status may have an impact on glycemic outcomes, we looked at the effect of strain status of each vitamin D metabolite on our measured markers of glycemic status (strain statuses can be found in **Figure 12**). In the VDS treatment group, both low 25(OH)D strain status and low 1,25(OH)D strain status were associated with changes in glycemic outcomes. Interestingly, strains with low 25(OH)D had significantly lower fasting insulin, glucose tolerance AUC, and HOMA-IR, while the strains with low 1,25$(OH)_2$D had significantly lower fasting insulin and HOMA-IR (**Figure 14A**). We next measured the impact of genetically determined vitamin D status on glycemic status in a deficient

state using the VDD treatment group. While 25(OH)D status was significantly associated with improved fasting blood glucose with the low status group associated with lower fasting glucose, we did not detect a significant effect of 1,25$(OH)_2$D status on glycemic status (**Figure 14B**).

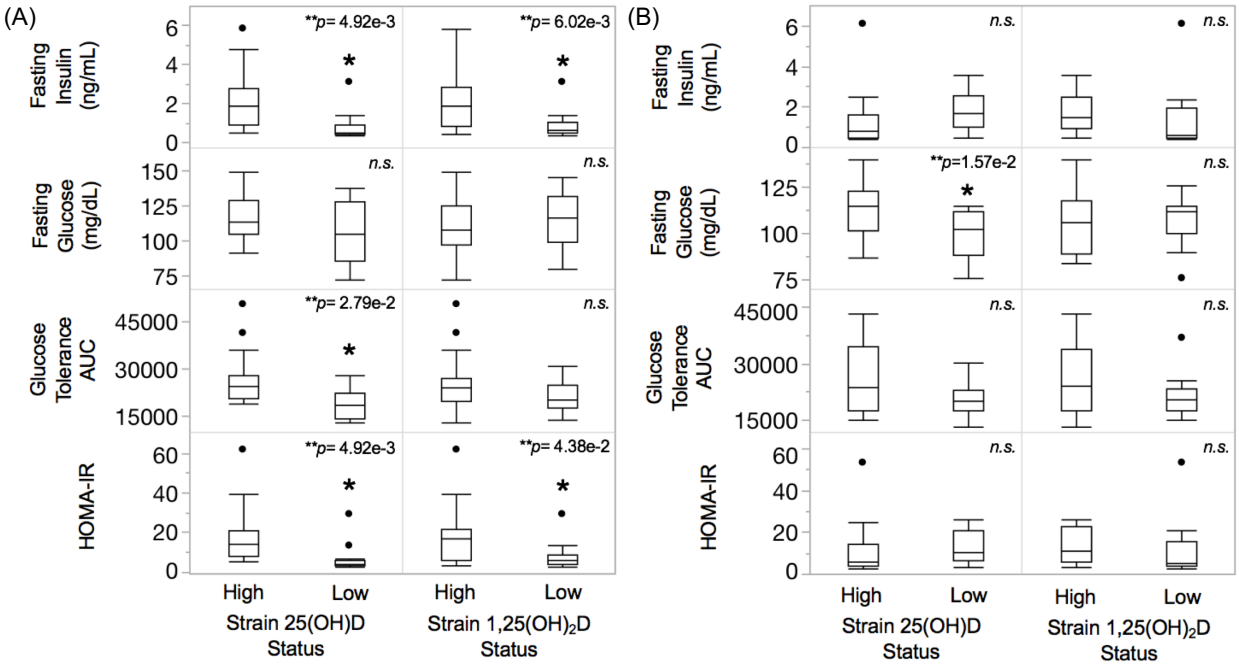

Figure 14. Effects of Vitamin D Strain Status on Glycemic Status. Box and whiskers plots across the VDS and VDD populations. Vitamin D metabolite strain availability categorized in each population into groups [High & Low] for 25(OH)D and 1,25$(OH_)_2$D. (A) Markers of glycemic status between vitamin D metabolite strain availability groups in the VDS treatment group. Effect of the strain vitamin D metabolite status on glycemic control in VDS treated mice determined by Median test. (B) Markers of glycemic status between vitamin D metabolite strain availability groups in the VDD treatment group. Effect of the strain vitamin D metabolite status on glycemic control in VDD treated mice determined by Wilcoxon test.

CHAPTER 5: ROLE OF GAPDH IN THE RELATIONSHIP BETWEEN VITAMIN D STATUS AND SUBSEQUENT GLYCEMIC OUTCOMES

Introduction

In this chapter, I explain the experiments that were performed in an attempt to develop a potential mechanistic explanation to understand the differences in glycemic outcomes across a population.

Six Weeks Adult Dietary Depletion of Vitamin D Reduces Liver GAPDH Expression in CC Mice

To establish a relationship between dietary treatment group and genetic expression, liver was collected in adult CC mice after 6 weeks of dietary treatment. Liver *GAPDH* expression was quantified via q-PCR, normalized to *ARRP0*. As hypothesized, dietary treatment group was associated with liver *GAPDH* expression, as the VDD group had significantly lower expression than the VDS group (average percent decrease of 22.10%, **Figure 15**).

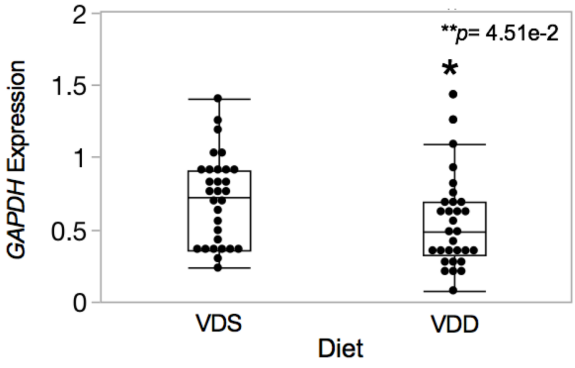

Figure 15. Effects of Diet on Liver *GAPDH* Expression. Box and whiskers plots across an entire population. Each dot represents a single mouse. Liver *GAPDH* expression normalized to *ARRP0*. Main effect of diet on liver *GAPDH* expression determined by Wilcoxon test.

Strain Has an Effect on Liver GAPDH Expression but Only in a Vitamin D Sufficient Population of Adult CC Mice

To better understand the changes that we observed in liver *GAPDH* expression, we assessed the strain effect on *GAPDH* expression. A significant strain effect was found on liver *GAPDH* expression in the VDS treatment group (**Figure 16A**). However, no such effect was

found in the VDD treatment group (**Figure 16B**) and no strain effect was detected for the change in liver *GAPDH* expression between dietary treatment groups (**Figure 16C**). This provided sufficient evidence that all strains normalize to the same low levels of *GAPDH* expression in a vitamin D deficient state (as seen in **Figure 15**), and the relationship we observe between *GAPDH* expression and glycemic status is likely due to dietary availability of vitamin D rather than genetic regulation.

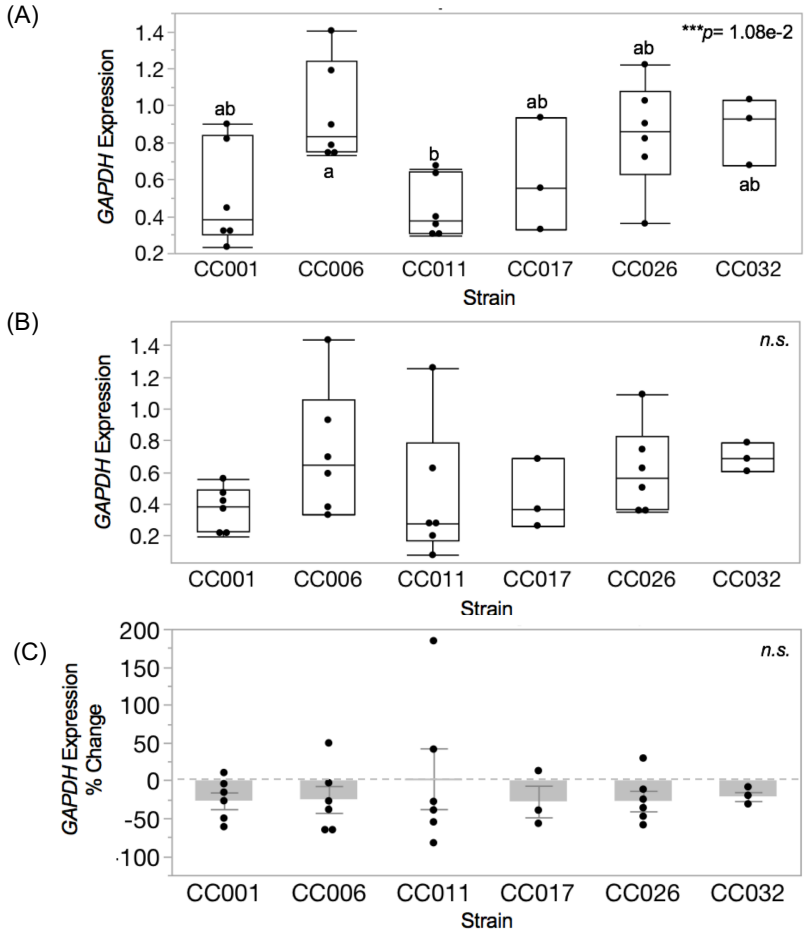

Figure 16. Effects of Strain on Liver *GAPDH* Expression. Each dot represents a single mouse. Liver *GAPDH* expression normalized to ARRP0. (A) Box and whisker plot of liver *GAPDH* expression in a VDS population. Main effect of strain on *GAPDH* expression in the VDS population measured by ANOVA. Letters denote strains that differ significantly [a ≠ b = ab] as determined by Tukey posthoc. (B) Box and whisker plot of liver *GAPDH* expression in a VDD population. Main effect of strain on clinical markers of vitamin D deficiency in the VDD population measured by Kruskal-Wallis test. (C) Bar graph of percent change in liver *GAPDH* expression caused by VDD [$(VDD^{Sample}-VDS^{Mean})/VDS^{Mean}$], separated by strain. Main effect of strain on percent change of markers caused by VDD determined by Median test.

Liver GAPDH Expression is Associated with Glycemic Status in Adult CC Mice

In order to investigate the mechanism of the relationship between vitamin D status and changes in glycemic status, we measured the association between liver *GAPDH* and markers of glycemic control, including fasting insulin, fasting glucose, glucose tolerance, and HOMA-IR. To control for the non-normal *GAPDH* data in the population, we binned data into tertiles and used

only the top and bottom tertiles as [High & Low] measurements for statistical analysis (**Figure 17A**). A significant effect was detected between liver *GAPDH* expression and fasting insulin as well as HOMA-IR. Contrary to what was expected, mice in the lower *GAPDH* expression group had lower fasting insulin and HOMA-IR (**Figure 17B**).

(A)

Group	High	Low
N=	19	20
Mean ±SD	1.01±0.21	0.29±0.07

(B)

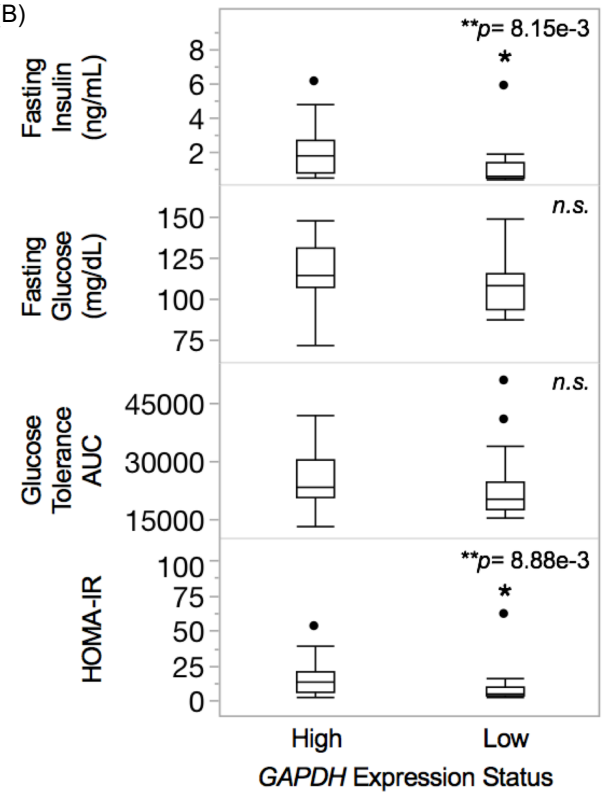

Figure 17. Effects of Liver *GAPDH* Expression on Glycemic Status. (A) Liver *GAPDH* expression was binned into [High & Low] status by individual sample across an entire population in order to investigate the effects of liver *GAPDH* expression on glycemic status. (B) Box and whiskers plots with markers of glycemic status separated by liver *GAPDH* expression status. Main effect of *GAPDH* expression status on each glycemic outcome determined by Wilcoxon test.

CHAPTER 6: DISCUSSION AND CONCLUSIONS

This study utilized a design combining dietary treatment scheme and strain genetic background to show that vitamin D availability has effects on physiological outcomes related to glycemic control. Through recombinant inbreeding from genetically distinct founder strains, the Collaborative Cross models the levels and genomic distributions of genetic diversity in a human population[119]. We were able to demonstrate that in this divergent population of adult CC mice, vitamin D metabolite status is determined both by dietary treatment and strain genetic background. Furthermore, we were able to establish a relationship between vitamin D availability and glycemic status.

We identified a significant effect of six weeks of adult dietary depletion of vitamin D on serum 25(OH)D availability. However, despite a 76.27% decrease on average in 25(OH)D, we interestingly did not observe any significant changes in the active metabolite, $1,25(OH)_2D$, between dietary treatment groups. Additionally, we detected no significant changes in clinical markers of vitamin D deficiency (PTH and calcium) between the dietary treatment groups nor markers of glycemic status (fasting glucose, fasting insulin, glucose tolerance, and HOMA-IR). While measurement of 25(OH)D is used to determine risk of deficiency in human populations[22], this study provides evidence that quantification of serum 25(OH)D may not fully implicate physiological deficiency. It is possible that the lack of physiological response to dietary vitamin D depletion was due to the short time period that the mice were on dietary treatment (6 weeks), after being exposed to dietary vitamin D sufficiency for the first 21-22 weeks of life. While it has been shown that it only takes 3 weeks of dietary vitamin D depletion for mice to become deficient defined by the IOM guidelines, the dietary treatment in these mice began at 4 weeks of

age rather than 21-22 weeks of age[120]. For adult mice, as were used here, that harbor substantial fat depots, it is possible that the fat-soluble vitamin D metabolites would require a longer time to deplete. Most studies do not measure 1,25$(OH)_2$D as its levels are much lower than 25(OH)D and its short half-life make it less stable. Thus, 1,25$(OH)_2$D is difficult to accurately measure with standard mass spectrometry methods and is not the standard metabolite used to assess deficiency[121].

We observed significant strain effects on vitamin D metabolites and clinical markers of vitamin D deficiency in a sufficient and deficient state, as well as strain-specific responses to dietary deficiency on these metabolic indicators of vitamin D metabolism. While genetic-based differences in vitamin D status have been identified in human populations across different ethnicities [28,29], there are no differences in assessing deficiency in a clinical setting across genetic backgrounds[22]. In addition to having a direct effect on various markers vitamin D metabolism, strain genetic background also had an effect on glycemic status, consistent with evidence that glycemic control is strongly in part genetically determined[122–124]. Interestingly, strains with lower vitamin D status determined by both metabolites, 25(OH)D and 1,25$(OH)_2$D, were also the strains with decreased insulin resistance in both a dietary sufficient and dietary depleted population. This suggests that the genetically-determined differences in vitamin D status may have an effect on glycemic status and health related outcomes. Further research is required to confirm.

In an attempt to develop a mechanistic link between vitamin D availability and glycemic control, we measured expression of liver *GAPDH*, a key enzyme and rate limiting step in glycolysis/gluconeogenesis. Literature provides evidence that vitamin D is involved in energy metabolism[111–114], potentially by way of inducing *GAPDH* expression[115]. As hypothesized, there was a significant VDD-induced decrease in *GAPDH* expression. Because GAPDH is one of the essential enzymes in breaking down glucose via glycolysis, we expected that decreased expression in the liver would lead to increased serum glucose. We hypothesized that other

markers of glycemic status would also be elevated due to increased levels of glucose. However, we discovered that a decrease in *GAPDH* expression was associated with a decrease in insulin and HOMA-IR. Interestingly, there was no significant *GAPDH* expression effect on fasting glucose. This may have been due to the fact that the animals were fasted prior to testing glucose. Although this is a necessary step in order to eliminate confounding of consumption of food, we must consider the physiological result of fasting. During fasting and low glucose states, the body is producing glucose via gluconeogenesis and glycogenolysis, rather than breaking it down via glycolysis. Therefore, it is not extremely surprising that we may not have seen the glycemic effects of potential changes in glycolysis due to changes in *GAPDH* expression in these mice.

This study provides novel evidence that vitamin D availability, determined by both diet and genetic background, is important for glycemic control. We propose that strain genetic background plays a direct role in the regulation of vitamin D metabolism and its association with glycemic outcomes, potentially via modulation of glycolytic enzymes such as liver *GAPDH* expression. Surprisingly, we found that strains with lower vitamin D status as well as mice with lower *GAPDH* expression actually had outcomes signifying increased glycemic control. We believe that this may be due to fasting of the mice as well as a potential differential effect of dietary response in vitamin D2 versus D3. It is important to consider that we do not have clinical definitions for “optimal” glycemic status in mice. While significantly lower insulin, glucose, and HOMA-IR may seem beneficial, these may either not substantially alter health across the population, or may be detrimental. Regardless of the necessary meaning of the physiological outcomes, we believe that *GAPDH* is playing some regulatory physiological role based on vitamin D status.

While our study provides original insight to the role of strain genetic background in vitamin D availability and its association with glycemic control, there were some potential limitations that could affect the final application & interpretation of the data. First, the relatively

small sample size in the original study design limits the power of our statistical analyses to detect small changes, therefore increasing the potential of false negatives. Additionally, as the high rates of vitamin D deficiency in the human population are not exclusive to adults, our study design utilization of only adult CC mice detracted the ability to understand how developmental vitamin D status could influence physiological outcomes both during development and as an adult. The most important limitation in this study was likely the fact that the mice did not show physiological signs of vitamin D deficiency. While we were still able to detect some physiological outcomes that were associated with vitamin D status, we cannot fully understand the implications of deficiency based on this study.

This study allowed us to identify not only that the metabolite used for measuring clinical vitamin D deficiency (25(OH)D) may not be fully accurate in predicting physiological deficiency, but also that the way we think about vitamin D deficiency needs to be transformed. Possibly most importantly, is that it is vital that we try to better understand the genetic regulation of vitamin D metabolism. Not only were we able to detect genetic differences in vitamin D status both in a sufficient and depleted state, but we were able to determine that risk for functional health consequences may be different based on that genetically-determined vitamin D metabolism. Through future studies, hopefully this question can be further addressed in order to improve our understanding of how genetics plays a role in the regulation of vitamin D and its associated physiological outcomes.

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