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The Vitamin D Pathway in the Nonhuman Primate Beta Cell

By

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CERTIFICATE OF APPROVAL

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Abstract

Background: Studies have proposed that serum vitamin D concentrations may affect the pancreatic beta-cell. There is an inverse relationship between serum vitamin D levels and glucose homeostasis and evidence that serum vitamin D levels are independently predictive of developing prediabetes or type 2 diabetes [14,21,23]. Obesity is associated with low serum vitamin D levels, which may contribute to metabolic conditions such as diabetes and cardiovascular disease [3]. Functions of vitamin D are often associated with the maintenance of bone quality and calcium absorption. Yet, alternative functions of vitamin D and associated enzymes in the beta cell have been proposed, including possible influences on insulin secretion, resistance, and sensitivity [2, 22]. These circular associations between vitamin D deficiency, insulin secretion, hyperglycemia, and obesity suggest a relationship may exist but do not prove cause and effect. Clinical studies of vitamin D have been underpowered and often complicated by confounders such as adiposity, gender, and diet [4,13,24]. Most animal studies of vitamin D in the pancreas have been completed in murine models. Nonhuman primate studies may help elucidate the role of vitamin D on glucose homeostasis because they provide a more physiologically related model and may eliminate some confounding factors common in human clinical studies [2,12]. The purpose of this study was to investigate the expression of the vitamin D signaling cascade in the nonhuman primate pancreas.

Methods: Expression of the vitamin D receptor (VDR), and two key enzymes regulating the activation/degradation of vitamin D, CYP27B1, CYP24A1, and calbindin was measured in pancreatic tissue samples of 5 adult rhesus macaques on control diets and 5 obese adult rhesus macaques on a western style diet. RNA was isolated from whole pancreas homogenates and the expression of target genes was measured by quantitative PCR. Statistical Analysis was conducted on real-time qPCR data to quantify gene expression in the pancreas, and compare

targets of interest between the control group and obese group. Unpaired, two-sided, student's t-tests were used to compare differences in mRNA levels between NHP pancreas of the control group and obese group. Spearman correlations were used to test the relationship between expression of VDR, CYP27B1 and CPY24A1 with markers of glucose homeostasis and insulin sensitivity.

Results: qPCR analysis determined moderate expression of the VDR, calbindin, and CYP27B1 in the NHP pancreas but expression of CYP24A1 was undetected. There was a significant difference in VDR expression between control and obese groups (p= 0.009). The mean difference in VDR expression between the control and obese group was 1.771 ± 0.5121 (95%CI: 0.5900 to 2.952). There was a significant strong correlation between VDR and NHP AUC Glucose measurements (r_s= 0.84; p= 0.0037), and a trend towards a correlation between VDR and AUC Insulin measurements (r_s=.59; p =.08).

Conclusion: The study demonstrates that VDR, CYP27B1, and calbindin are moderately expressed in the NHP pancreas and that VDR expression was increased in the obese group. CPY24A1 was undetectable. The study also found that VDR expression had a significant positive correlation to AUC blood glucose, indicating that higher VDR expression was associated with a higher glucose excursion after an IV glucose load. However, our study did not detect significant differences in the expression of downstream proteins involved in vitamin D homeostasis (CYP27B1, CYP24A1, and calbindin) between the control and obese groups, indicating cytosolic metabolism of active vitamin D inside the pancreas may be minimal and serum vitamin D levels rather than VDR itself may be the primary driver of vitamin D signaling. Overall, these results suggest that VDR is upregulated in the NHP pancreas to compensate for higher insulin needs and low serum vitamin D to produce more insulin and maintain euglycemia.

Specific Aims

Vitamin D receptors have been identified in the beta cell of the pancreas, suggesting there may be a role for vitamin D in the regulation of insulin secretion and glucose homeostasis [1] [2]. However, there is controversy regarding lesser known functions of vitamin D, including the role of vitamin D in the pancreas. The exact role of vitamin D and its signaling cascade in the pancreas is unknown. Disorders of pancreatic beta cells include the inability to match insulin release with circulating glucose concentration due to impaired insulin secretion. Decreased insulin secretion is often observed in late stages of type 2 diabetes, and type 2 diabetes is associated with obesity. Obesity has also been associated with vitamin D deficiency. The increasing prevalence of obesity has led to a rise in morbidity and mortality from diabetes and cardiovascular disease [3-7]. These circular associations between vitamin D deficiency, insulin secretion, hyperglycemia and obesity suggest a relationship may exist, but do not prove cause and effect; further studies of the role of vitamin D in pancreatic beta cell function are needed.

Evaluating the association between vitamin D deficiency and glucose homeostasis is difficult in observational studies due to confounders such as adiposity (which decreases 25(OH)D bioavailability), gender, diet, and overall nutritional status [4 8]. Some longitudinal studies show that people with sufficient serum vitamin D levels have a reduced chance of developing diabetes [9-11]. Overall, clinical studies investigating the effects of vitamin D on glucose homeostasis have been inconsistent and underpowered, and a fundamental lack of knowledge regarding the vitamin D signaling cascade in the pancreas has hindered progress in this field. Current knowledge has been based almost exclusively on murine models [2 12-14].

Some limited data suggests that 1α,25-dihydroxyvitamin D3 (1,25(OH)D_3), the biologically active form of vitamin D, may work through both genomic and non-genomic mechanisms in the pancreas, affecting beta cell function and insulin secretion [2]. Diabetic rats treated with vitamin D analogs show significant changes in the regulation of the Vitamin D Receptor (VDR) and improved glucose parameters [15]. In vitro studies on isolated human and mouse pancreatic islets have shown that 1,25(OH)D_3 may increase insulin secretion. In addition, VDR mutant mice have impaired glucose tolerance and insulin secretion [16 17]. VDR and calbindin have been located in rat and human beta cells and appear to influence insulin secretion by regulating intracellular calcium [18 19]. Pancreatic expression of the enzyme 1 alpha-hydroxylase (CYP27B1), which converts 25(OH)D to 1,25(OH)D_3 is regulated by circulating levels of vitamin D. Patients with vitamin D deficiency may show decreased expression of CYP27B1 and glucose intolerance. Both CYP27B1 and 24-hydroxylase (CYP24A1) can affect the regulation and conversions of 25(OH)D and 1,25(OH)D_3 in some tissues. 1,25(OH)D_3 may activate VDR, which then may modulate the insulin gene and stimulate insulin secretion [13]. Several studies in murine models have found associations between serum vitamin D levels, insulin sensitivity and beta cell function, yet conclusions are inconsistent. Despite data in a variety of animal models and humans, no nonhuman primate data on the function of vitamin D in the pancreas currently exists [8 14 20].

The overall goal of this study is to measure the expression and localization of VDR, CYP27B1, CYP24A1, and calbindin in the pancreas of the nonhuman primate, and determine if obesity induced by a high fat diet alters expression of the pancreatic Vitamin D Receptor and related enzymes. Well-controlled primate studies offer physiological human similarities and may help clarify the role of vitamin D and its regulatory proteins on the vitamin D pathway in the beta cell [12 13]. We hypothesize that the VDR and critical regulatory proteins in the vitamin D pathway are expressed in the NHP pancreas, and regulated by obesity that is induced by a high fat diet. To

test this hypothesis, we will determine the expression and localization of VDR, CYP27B1, CYP24A1, and calbindin in pancreatic tissue samples of 5 adult rhesus macaques on control diets and 5 adult rhesus macaques that became obese on high fat diets. Expression of the genes will be measured in isolated RNA from whole pancreas homogenates by quantitative PCR. Localization of the protein will be measured using standard immunohistochemistry in paraffin embedded and sectioned pancreas. Our specific aims are:

Aim #1: Investigate the expression and localization of the VDR, CYP27B1, CYP24A1, and calbindin in the NHP pancreas and specifically the beta cell.

Hypothesis #1: The VDR, CYP27B1, CYP24A1, and calbindin are expressed in the NHP pancreas and localized to the beta cell on IHC.

Aim #2: Determine the effects of obesity induced by a high fat diet on the regulation of VDR, CYP27B1, CYP24A1, and calbindin in the NHP pancreas and beta cell.

Hypothesis #2: Expression studies from pancreas of NHP's fed a high fat diet that induced obesity will show an upregulation of VDR, and down regulation CYP27B1, CYP24A1, and calbindin. Changes in mRNA by qPCR will be associated with similar changes in staining intensity by IHC suggesting changes in protein concentrations in the tissue.

Establishing expression and localization of the vitamin D pathway in NHP pancreas and understanding the role a high fat diet may play in that expression pattern is an essential first step to understanding the role of vitamin D signaling in beta cell physiology. Future studies will be needed to address the effects of vitamin D deficiencies on beta cell function in the NHP. Ultimately, research using a more physiologically related model such as the NHP may help elucidate the role of vitamin D on insulin secretion in humans.

Background

Global levels of obesity and diabetes are rising along with increasing rates of vitamin D deficiency [7 21 22]. Obesity has been associated with hypovitaminosis D, and low circulating vitamin D may affect metabolic conditions such as diabetes and cardiovascular disease [3]. Several studies have proposed that serum vitamin D blood concentrations may have a direct or indirect effect on the pancreatic beta cell [14 23] . Although classically associated with maintenance of bone quality and calcium absorption, alternative functions of vitamin D have been proposed, including possible influences on insulin secretion, resistance and sensitivity [2 22]. However, the literature is equivocal. Vitamin D status can be a marker for poor health, and thus comparing vitamin D status in supplement trials is difficult. Further, clinical studies of vitamin D have been underpowered and complicated by confounders such adiposity, gender, and diet [4 13 24]. However, some studies show low serum vitamin D concentrations are independently predictive of developing prediabetes or type 2 diabetes, while other studies show no clear effect of vitamin D supplementation on glucose homeostasis [13 24 25]. More research is needed to help determine the functions of vitamin D in the beta cell and elucidate the effect of vitamin D on glucose homeostasis. Most animal studies have been completed in murine models. Studies in the NHP could provide beneficial information and eliminate some of the issues with confounding factors, however, no prior studies have investigated vitamin D proteins in the NHP beta cell [2 12] .

Vitamin D Definition and Source

Vitamin D is a fat-soluble vitamin, and the term vitamin D includes two different forms: ergocalciferol (D2) obtained from plant sources and cholecalciferol (D3) obtained from animal

sources. Vitamin D3 can be ingested or synthesized endogenously in the skin by ultraviolet light from sun exposure. Endogenous synthesis of vitamin D comprises about 80% of an individual's vitamin D source [26]. People receive about 20% of their vitamin D from a very limited number of dietary sources, including vitamin D fortified foods and supplements [23 26]. Foods naturally high in vitamin D include fatty fish like salmon, mackerel, and tuna, beef liver and eggs [22 23 26]. Foods that are fortified with synthetic forms of vitamin D include orange juice, dairy products, ready-to-eat breakfast cereals and nutritional yeast [3]. People often consume vitamin D through dietary supplements made from both plant and animal sources [27] .

Vitamin D Metabolism and Classic Function

There are several mechanistic steps to transform dietary and endogenous vitamin D to the active form of the vitamin, 1,25(OH)D_3. Vitamin D3 is synthesized in the skin when ultraviolet light causes 7-dehydrocholesterol to be converted to pre-vitamin D3, and then it is isomerized to become vitamin D3 and then enters into blood by vitamin D binding protein (VDBP). After ingestion, dietary vitamin D is incorporated into chylomicrons, released into the lymphatic system, enters into the venous blood at the thoracic duct and ultimately transported to the liver [2 5 28]. In the liver, vitamin D is hydroxylated by the enzyme 25-hydroxylase (CYP2R1) to 25-hydroxyvitamin D (25(OH)D), which is the major circulating form of vitamin D. 25(OH)D is further hydroxylated in the kidney by enzyme 1-alpha-hydroxylase (CYP27B1) to become 1,-25-dihydroxyvitaminD3 (1,25(OH)D_3), the active vitamin D metabolite [5]. It is important to point out that in the kidney, 1-alpha-hyroxylase is regulated by several different enzymes including parathyroid hormone, fibroblast growth factor and calcitriol [2]. The conversion of 25(OH)D to 1,25(OH)D_3 by the enzyme 1-alpha-hydroxylase can take place in other target tissues throughout the body. The subsequent release of active 1,25(OH)D_3 can act by paracrine or autocrine mechanisms [2]. 1-alpha-hydroxylase (CYP27B1) enzyme is expressed in other cells, such as the

pancreatic islets. In the islet, it is not regulated by parathyroid hormone or fibroblast growth factor, but instead regulated by circulating levels of 25(OH)D and 1,25(OH)D_3 is catabolized by 24-hydrxyolase (CYP24A1) [2]. The active vitamin D metabolite, 1,25(OH)D_3, binds to the Vitamin D Receptor (VDR) which has many different biological functions [1 29]. The downstream effects of binding to the VDR include increased absorption of calcium in the gut and regulation of calcium and phosphate serum concentrations to maintain bone homeostasis [1]. Other functions of vitamin D signaling include cell growth, neuromuscular, immune and inflammation functions [29]. The Vitamin D Receptor and the local activation of 25(OH)D to the active 1,25(OH)D_3 is found in many tissues throughout the body. The expression of the VDR and conversion of 25(OH)D to its active form 1,25(OH)D_3 may be influenced by circulating blood levels of 25(OH)D, and may also be regulated differently in different target organs [2 29].

Vitamin D Homeostasis

Associations between vitamin D homeostasis and chronic diseases have spurred research to examine the relationship between circulating vitamin D and the pathogenesis of some chronic diseases. Serum 25(OH)D is the most stable form of vitamin D in circulation and is the best way to evaluate overall vitamin D status. Although 1,25(OH)D_3 is the active form of vitamin D, it only has a half-life of fifteen hours and therefore does not give the best indications of vitamin D status [29]. Recommended ranges of adequate serum vitamin D can vary. The Institute of Medicine (IOM) proposed that serum 25(OH)D levels above 20 ng/mL (>50 nmol/L) are adequate and The Endocrine Society recommends serum 25(OH)D levels above 30 ng/mL (≥75 nmol/L) [29]. Normal target ranges for serum vitamin D can usually be met by a daily intake of 400-800 IUs of dietary vitamin D and some sun exposure. The RDA for vitamin D is 600 IU for most adults and increases to 800 IU for ages 70 and older [29 30].

Both hypovitaminosis D and hypervitaminosis D have potential adverse effects [29]. Effects of hypovitaminosis D are mainly associated with problems in calcium and phosphorus and bone metabolism causing rickets, osteomalacia and osteoporosis [27 34]. Recently, more studies have been conducted that show associations with hypovitaminosis D and increase risk of cardiovascular disease, cancer, type 1 diabetes and type 2 diabetes [27 31]. An expert committee of the Food and Nutrition Board (FNB) concluded that serum 25(OH)D levels below 50 nmol/L (>20 ng/mL) are generally considered inadequate for bone and overall health and blood serum levels below 30 nmol/L (<12 ng/mL) are associated with vitamin D deficiency that can lead to bone conditions such as rickets and osteomalacia [29 32 33]. Major factors affecting hypovitaminosis D are sunlight exposure, skin color, BMI, season, latitude, and deficient dietary intake of Vitamin D [27 31 34]. Many populations around the world do not consume the recommended dietary vitamin D intake of 400-800 IU/day [26] . The prevalence of vitamin D deficiency may be increasing because of factors that limit the endogenous production of vitamin D, such as low sun exposure, seasonal difference, and latitude [35-38]. People with darker skin are at a higher risk of vitamin D deficiency due to the reduced ability of ultraviolet light to penetrate the skin to convert vitamin D into its necessary bioactive form [35 37 39]. Hypervitaminosis D is mainly caused by excess intake of vitamin D dietary supplementation. High serum vitamin D levels may cause problems such as hypercalcemia which can cause nausea and vomiting, weakness, frequent urination and may also progress to kidney and bone disease [29]. A 2013-2014 NHANES study showed an increase trend of high-dose vitamin D supplementation [40]. Rooney et al. reported that 3% of the US population exceeded the upper daily limit of Vitamin D of 4,000 IU/day, and may be at risk for adverse consequences, such as prostate cancer, pancreatic cancer, and all-cause mortality [40] .

Vitamin D Associations with Glucose Homeostasis

Inverse associations between serum vitamin D levels and glucose homeostasis has led to interest in the function of vitamin D in the beta cell, including potential roles in insulin secretion, insulin sensitivity and resistance [23 41]. Cross-sectional studies, cohort studies and meta-analyses have demonstrated an inverse relationship between some measures of glycemic control, the risk of type 2 diabetes and serum vitamin D concentrations. Correlation does not prove cause and effect, but does suggest vitamin D may be involved with insulin secretion and insulin sensitivity. However, the mechanism of action has not been determined [9-11 24 42-44].

Insulin Secretion

In vivo and *in vitro* studies suggest that vitamin D may directly and indirectly affect insulin secretion [2 13 45-47]. Glucose Stimulated Insulin Secretion (GSIS) tests in isolated rat pancreas show that Vitamin D deficiency causes impaired insulin secretion in the beta cell [16 45 46]. Norman et al. compared isolated rat pancreases from vitamin D deficient and vitamin D sufficient groups. *In vitro* tests showed a typical insulin release from vitamin D sufficient pancreas, and impaired insulin secretion from vitamin D deficient perfused rat pancreases in response to glucose [46]. Glucose Stimulated Insulin Secretion (GSIS) tests performed on beta cells show that treatment with 1,25(OH)D_3 combined with high levels of glucose increased insulin secretion in INS1E cells (insulin secreting rat beta cell line), but that cells pre-treated with 25(OH)D versus cells not pre-treated with 25(OH)D under regular conditions did not show any significant differences. This suggests that glucose stimulated insulin secretion may be related to the type of vitamin D metabolite [45].

Cross sectional and clinical studies also report associations between serum vitamin D and insulin secretion and glucose homeostasis [48-50]. A landmark clinical cross-sectional study of 488

participants examined the associations of 25(OH)D concentrations with insulin action and insulin secretion in persons with pre-diabetes. The study measured insulin resistance by Steady State Plasma Glucose (SSPG) and HOMA-IR and insulin secretion were measured by HOMA-B. The results suggest that people with pre-diabetes who were also vitamin D deficient were more insulin resistant than those with pre-diabetes who had sufficient vitamin D serum levels. Vitamin D status was further associated with low HOMA-IR in participants that were pre-diabetic, while no relationship was observed among participants with Normal Fasting Glucose (NFG). Finally, there were no significant mean differences in SSPG concentration and HOMA-IR and 25(OH)D status in the NFG group. Therefore, the study concluded that participants with pre-diabetes and vitamin D deficiency may have impaired beta cell functions and may be at higher risk for type 2 diabetes [48]. This finding was supported by a cross sectional study in 500 healthy patients with a mean age of 39.4 that looked at associations between low serum vitamin D and glucose parameters. Using HOMA-IR and HOMA-B to assess insulin sensitivity and insulin secretion, the study observed a significant inverse relationship between serum 25(OH)D concentrations and fasting glucose, insulin levels, and insulin sensitivity. However, calculations of HOMA index for β cells secretion remained unrelated to serum 25(OH)D in this population [49]. Overall, both clinical and *in vivo* studies show an inverse relationship between serum vitamin D and blood glucose levels. However, exactly how factors such as vitamin D deficiency, blood glucose levels, weight, gender, and the effects of vitamin D supplementation are related is not fully understood.

Insulin Sensitivity/Resistance

Several studies have specifically investigated the relationship between vitamin D and insulin sensitivity [8 14 42 51]. Chui et al studied 126 patients with normal glucose tolerance using a hyperglycemic clamp and an infusion of glucose to assess insulin sensitivity and beta cell function. The study found a positive correlation with insulin sensitivity but no independent

correlation between 25(OH)D serum levels and 1st and 2nd phase insulin release. However, there was an association between vitamin D and plasma glucose concentrations suggesting that plasma vitamin D concentrations may have a positive correlation with insulin sensitivity and negative effect of low serum vitamin D on beta cell function [14]. Two different studies looked at the relationships between serum 25(OH)D and insulin sensitivity among subjects with type 2 diabetes. Among middle-aged participants with type 2 diabetes, there was a significant negative correlation between serum 25(OH)D with fasting plasma glucose, HbA1c, and HOMA-IR. There was also a significant positive association with serum 25(OH)D with HOMA-B and QUICKI, measures of insulin sensitivity, among both subjects with normal glucose regulation and participants with type 2 diabetes. The authors suggest that sufficient serum vitamin D levels, defined as > 30 ng/ml, might lower the risk of developing insulin resistance and type 2 diabetes in middle-aged participants [42]. In another study of 395 newly diagnosed participants with type 2 diabetes, Matsuda ISI and HOMA-IR were used to measure insulin sensitivity and glucose to insulin release ratio was used to assess beta cell function. Although there was a significant inverse correlation of 25(OH)D and HOMA-IR and beta cell insulin secretion, the correlations were only observed in female participants and there was no association among the male participants [24]. Finally, in a study on non-diabetic youth, hypovitaminosis D was prevalent in 90% of the obese youth participants. Among these subjects, vitamin D was positively correlated with the Masuda index and negatively correlated with HOMA- B suggesting that participants with lower serum 25(OH)D were less insulin sensitive and had worse beta cell function and insulin secretion [51]. These studies suggest that lower vitamin D concentrations are correlated with measures of insulin resistance in subjects with normal glucose regulation, type 2 diabetes and obesity.

Vitamin D and Type 2 Diabetes Mellitus Risk

Several meta-analysis and prospective cohort studies report an inverse relationship between serum vitamin D and the risk of type 2 diabetes [10 11 42 44 50 52]. Song et al. conducted a meta-analysis of 21 studies examining the association between incidence of type 2 diabetes and serum 25(OH)D concentrations. There was a significant inverse relationship between serum 25(OH)D and incidence of type 2 diabetes. For each 10 nmol/L increase of 25(OH)D, there was a 4% lower chance of type 2 diabetes. Another important finding of this meta-analysis was that the current recommended baseline 25(OH)D levels >20 ng/mL (>50 nmol/L) was associated with a lower risk of type 2 diabetes [11]. A prospective cohort of 903 participants was followed for 12 years to assess the association between plasma 25(OH)D and 1,25(OH)D_3 concentration, and the development of type 2 diabetes or pre-diabetes. Participants with serum 25(OH)D levels >30 ng/ml had 1/3 lower incidence of diabetes then those with serum levels < 30ng/ml. Concentrations of serum 1,25(OH)D_3 were not associated with incidences of diabetes or pre-diabetes [43]. In this study, serum 25(OH)D levels had a strong inverse association with risk of type 2 diabetes and weak inverse association with risk of pre-diabetes [43]. Serum 25(OH)D can also be a marker for poor health status, and studies on 25(OH)D and glucose homeostasis can also be difficult to compare due to variations in study populations, and differences in assessment techniques for insulin sensitivity. Some overall similarities across multiple studies suggest serum 25(OH)D levels are positively correlated with beta cell function, insulin secretion and improved insulin sensitivity. These studies show conflicting results on the associations of 25(OH)D and plasma glucose levels and show some differences among correlations of 25(OH)D in male and female participants. Overall, higher serum 25(OH)D levels were associated with lower risk of type 2 diabetes [11 14 24 42 43 51].

Vitamin D and Association with Obesity

Cross sectional, observational and meta-analysis studies consistently report a high prevalence of vitamin D deficiency and an inverse correlation between vitamin D status and obesity [4 8 27 33 44]. NHANES data from 2011-2012 reported a significant difference in the rate of obesity between vitamin D deficient and sufficient groups. Adults with obesity have a 3.09 times greater prevalence of vitamin D deficiency compared to adults who are not obese [27]. Another study using the same NHANES data from 2011-2012 found significant differences in the rate of obesity and overweight groups between participants who were vitamin D insufficient and sufficient. This study showed that 45% of vitamin D deficient participants were obese versus 31% of vitamin D sufficient participants were obese. However, this study also showed that only 29.4% of the vitamin D deficiency group was overweight, and 33% of the overweight group was vitamin D sufficient. There were differences in the rate of people who were obese versus overweight and the prevalence of vitamin D deficiency [33].

There are several suggested mechanistic causes for the high prevalence of vitamin D deficiency in individuals who are obese. Mechanisms such as volumetric dilution, sequestered vitamin D in adipose tissue, reduced sun exposure, and altered carbohydrate metabolism have been studied as potential causal factors of reduced serum vitamin D levels in obesity. In addition, the effect of vitamin D deficiency as a contributor to obesity cannot be ruled out due to functions of the VDR in adipose tissue [38].

Volumetric and Sequestered Vitamin D

Some studies suggest that sequestered vitamin D in adipose tissue leads to volumetric dilution of vitamin D and may be the cause of the inverse relationship in obesity and vitamin D deficiency [38 53]. The volumetric theory refers to the volume of adipose tissue, liver, muscle, and

serum increases in obesity and the dilution of vitamin D in the extra mass of people with obesity resulting in less available serum 25(OH)D. Sequestration of vitamin D refers to the fat soluble and hydrophobic nature of vitamin D which allows it to be stored in adipose tissue as well vitamin D's inability to get back into circulation once it has been stored in adipose tissue. This sequestered vitamin D may be reflected in lower 25(OH)D status in obese individuals [53].

Obesity versus Glycemic Control

Examination of the relationship between vitamin D deficiency, obesity, and glycemic control determined that serum vitamin D levels may be more tightly associated with glycemic control versus obesity. Serum 25(OH)D levels were lower in pre-diabetes and diabetic groups compared to normal glucose control and independent of obesity level. There were no differences in 25(OH)D blood levels between BMI categories among subjects who had normal glycemic control versus pre-diabetes or diabetic groups, but there were significant differences in serum 25(OH)D levels between BMI groups with prediabetes or diabetes. The study also did an in-vitro analysis and compared VDR gene expression in adipose tissue from donors with different BMI levels. The differentiated preadipocytes were stimulated with active form of vitamin D and showed different VDR regulation responses between different adiposity levels. Upregulation of VDR was only induced in adipose tissue from obese donors, and no similar upregulation was observed among lean subjects [54].

Reduced Sun Exposure

Sun exposure, skin reflectance, and body composition directly affect serum 25(OH)D concentrations in adults who are overweight and obese. Piccolo et al. found that The Hall Model, which is often used to assess sun exposure, over estimates vitamin D intake in over weight and obese populations. To more objectively measure sun exposure, the group used an

adjusted regression model that more accurately explained the differences in post intervention 25(OH)D concentrations. Predictors of low serum 25(OH)D levels included sun exposure, skin reflectance, total fat mass, total lean mass, and intra-abdominal adipose tissue. Using this corrected regression model, they reported a direct correlation to less sun exposure due to additional clothing, less time outside and correlations of serum 25(OH)D status and higher levels of BMI [39]. This finding lead to the theory that the endogenous conversion of vitamin D is the same in both lean and obese people, but as vitamin D is converted, more is sequestered and diluted in high blood volume with increased adiposity [53]. The study also suggested that overweight and obese groups need more sun exposure time to meet the same levels of serum 25(OH)D as the lean group. Additionally, the authors suggest that within obese populations, sun exposure and potential sun exposure behavior are reduced with increasing levels of adiposity and further contribute to the risk of vitamin D insufficiency in obese populations [39].

Vitamin D in the Beta Cell

The vitamin D pathway is expressed in pancreatic beta cells and may help to maintain glucose homeostasis [2]. The Vitamin D Receptor is a ligand induced transcription factor and part of the steroid hormone nuclear receptor family [5 55]. 25(OH)D is the main circulating form of vitamin D and must be hydrolyzed to 1,25(OH)D_3 by 1,alpha-hydrolase (CYP27B1) to become 1,25(OH)D_3, the active form of vitamin D. 24-hydroxalase (CYP24A1) can convert 1,25(OH)D_3 back to the main circulating form of vitamin D, 25(OH)D [2 56 57] . The primary function of CYP24A1 is the degradation of vitamin D, which prevents toxic levels of 1,25(OH)D_3. It has been shown that the make-up and location of CYP24A1 varies between species, and its regulation is cell specific [5]. Human and rat pancreatic cells have shown the presence of VDR and CYP27B1, possibly indicating local production and regulation that might involve CYP24A1 and a negative feedback system [2 56 57]. Expression of CYP24A1 has proven to be low to undetectable in the human

pancreas, but can be upregulated in pancreatic endocrine cells in some disease conditions and inflammation [57]. Bland et al. reports the presence of calbindin, a calcium dependent binding protein, which may influence the secretion of insulin. There is evidence that the VDR, 25(OH)D, 1,25(OH)D_3, CYP27B1, calbindin are part of the vitamin D pathway in the rat and human pancreatic cells. Each component of the vitamin D cascade may have a unique role and regulation in the beta cell [2 5 18 56 57].

Several studies indicate that the VDR is expressed in mouse and human pancreas endocrine cells and some studies researched VDR expression in the beta cell to establish a relationship with the VDR and insulin secretion [17 18 47] . Kjalarsdottir et al. performed GSIS, microarray, and qPCR on human and mouse islets that were cultured with either vehicle or 1,25-dihydroxyvitamin-D3 to examine the vitamin D pathway in the beta cell. Islets cultured with 1,25-dihydroxyvitamin-D3 showed enhanced GSIS and increases in glucose-stimulated calcium influx. VDR was highly expressed in islets and glucose responsive. Microarray analysis identified a calcium channel gene upregulated by 1,25-dihydroxyvitami-D3 that modulates the beta cell to increase insulin secretion [16] . Zeitz et al. established a relationship of the VDR and insulin synthesis. Mice with a nonfunctioning VDR have impaired oral glucose tolerance and reduced insulin secretion and pancreas from mice with nonfunctioning VDR have reduced insulin mRNA content compared to mice with normal VDR function. However, there was no significant impairment in insulin response in the VDR mutant group under normal glucose conditions. These studies propose that functions of the VDR play a role in insulin secretion and confirm the presence of VDR in the beta cell [17].

Vitamin D may play an important role in the development and treatment of diabetes, and it is necessary to understand the effects of circulating active vitamin D and the possibility of local production of active vitamin D in the islet. RT–PCR, western blot, and immunohistochemistry in

isolated rat islets and MIN6 cells (insulin-producing mouse cells) confirmed the presence of 1-alpha-hydrolase mRNA and 1-alpha-hydrolase protein expression in insulin-secreting cell models. Bland et al. demonstrated the presence and activity of CYP27B1 in the islet using isolated rat islets and MIN6 cells incubated with 25(OH)D. The cells were then analyzed for $1,25(OH)D_3$ production and islet response times. Results confirmed CYP27B1 expression, CYP27B1 activity, and determined a rapid response to 25(OH)D in cells that produce insulin. The expression and activity of CYP27B1 in the islets and the rapid response of these cells to 25(OH)D may indicate the local production of active vitamin D, which may play a significant role in islet function [56].

Enzyme 24 hydroxylase (CYP24A1) catalyzes the conversion of 25-hydroxyvitamin D3 (25(OH)D3) and 1,25-dihydroxyvitamin D3 (1,25(OH)D3) into 24-hydroxylated products and begins the degradation of the active vitamin D molecule. It is thought to work in sync with CYP27B1 and is important in the negative feedback system to control levels of $1,25(OH)D_3$ [58]. Hummel et al performed both qRT-PCR and Immunohistochemistry on tissue from 11 human pancreases from patients with pancreatic ductal adenocarcinoma (PDAC). CYP24A1 mRNA expression was significantly increased in PDAC cells compared to adjacent non-tumor cells. In an IHC experiment of the same 11 human pancreases, CYP24A1 protein expression is also increased in ductal adenocarcinoma compared with other regions of pancreatic tissue. Hummel et al. concluded that the vitamin D system can become deregulated during the development of pancreatic ductal adenocarcinoma in both endocrine and exocrine cells. Also, both CYP24A1 mRNA and CYP24A1 proteins can be upregulated in pancreatic tumor cells and pancreatic cancer and chronic pancreatitis effects the negative feedback system of CYP24A1 in the islets [57].

In addition to the vitamin D metabolites and the VDR, calbindin (D28K), a calcium binding protein regulated by vitamin D, may play a role in insulin secretion in the beta cell. Prior studies

have located calbindin in beta cells and its effect on calcium may lead to exocytosis of insulin [2 18]. Reddy et al. used RIN 1046-38 cells, a rat beta cell line, and overexpressed calbindin to examine the effects on insulin expression. Rat calbindin was over expressed in a pancreatic beta cell line by transfection of calbindin and changes in cell line mRNA were determined by radioimmunoassay and Northern Blots. Cells were then grown in RPMI medium containing glucose and then insulin was measured by ELISA. The study determined that cells with highest level of calbindin expression had increased insulin mRNA and increased insulin content. The results of this study suggest that calbindin may stimulate insulin synthesis in beta cells [19].

Vitamin D Supplementation

Meta-analysis studies have looked at the effects of vitamin D supplementation on parameters such as BMI, fat mass, and blood glucose levels [53 59]. Clinical trials on vitamin D supplementation can be difficult, and results have been inconsistent causing some debate over the therapeutic benefits and appropriate dose of vitamin D supplementation. Some clinical studies show benefits of vitamin D supplementation in patients with obesity. People with obesity have a higher rate of vitamin D deficiency and need higher doses of vitamin D supplements to obtain sufficient serum vitamin D levels [30]. Although sufficient serum vitamin D levels are associated with improved glucose and insulin parameters and lower risk of type 2 diabetes, vitamin D supplements have not shown to lower the incidence rate of type 2 diabetes. A meta-analysis by Tang et al. included over 47 Randomized Control Trials of vitamin D supplementation in non-diabetic adults and indicated that vitamin D supplementation showed reduced fasting blood glucose, fasting insulin and HOMA-IR, increased serum 25(OH)D levels, and no effect on insulin secretion, beta cell function or overall risk of type 2 diabetes [59]. Another study by Jorde et al. looked at intervention with 20,000 IU/ of cholecalciferol per week vs placebo for 5 years and found no reduction in type 2 diabetes rate in patients with sufficient

serum vitamin D levels. However, no conclusion could be made about vitamin D supplementation in the vitamin D deficient group due to small sample size [60]. In a review of several different vitamin D supplementation studies, it was suggested that vitamin D supplementation may not provide protection against type 2 diabetes in vitamin D sufficient adults and more attention must be paid to vitamin D serum levels when assessing effects of vitamin D supplementation [61]. There may be benefits from the combination of vitamin D supplementation and a lower calorie diet on glucose parameters [53 62 63]. Cholecalciferol supplementations and a low-calorie diet improves insulin sensitivity in healthy patients with obesity and hypovitaminosis D. A study by Kalani et al. looked at three different vitamin D deficient treatment strategies for prebariatric surgery patients and found that high weekly oral doses of vitamin D supplement for longer duration of time improved serum vitamin D levels more than high single dose injections of vitamin D3 [63].

Relevance in Nonhuman Primates

The use of primate studies in obesity and diabetes research has proven advantages over rodent and other animal models. Nonhuman primates are closer genetically and have more physiologic similarities to humans than rodent models. The development of obesity, central adiposity and insulin resistance in nonhuman primates is more similar to humans than rodents, and the use of primates can provide a more controlled diet environment than human models. Models of nonhuman primates fed western style diets have been established and have been used to study disease pathology and prevention as well as pharmaceutical interventions. Some studies have used the obese NHP model that is fed a high fat diet aimed at causing obesity because they often develop different ranges of metabolic symptoms. A subset of these animals will develop increased adiposity, insulin resistance and dyslipidemia and is often referred to either high fat sensitive group or the obese group. In a study that examined the effect of high fat diet in

nonhuman primate fetal off spring of mothers fed a high fat diet, some but not all the adult female monkeys fed a high fat diet became obese and insulin resistant. All of the off spring from nonhuman primate mothers fed a high fat diet had signs of nonalcoholic fatty liver disease and increased body fat compared with control offspring [64]. Using pancreatic islets from NHP tissue has been incredibly useful in the pathology of many diseases and can provide insight about functions of insulin and glucose that are critical to research on treatment of obesity and type 2 diabetes [12].

Conclusion

Studies consistently report associations between low serum 25(OH)D levels and both type 2 diabetes and obesity, yet there are still many unanswered questions about the non-skeletal functions of vitamin D. Many studies have looked at the function of VDR and enzymes related to insulin secretion and its relations to serum 25(OH)D levels. The vitamin D cascade includes the VDR and several vitamin D metabolites that are regulated by different factors and physiological states. Understanding how vitamin D tissue uptake, regulation, and functions affect insulin secretion in the beta cell is important. Determining the effects of supplementation in clinical studies is difficult due to inconsistencies comparing baseline serum levels, body weight, adiposity, age, gender, and skin color. Effective dose, delivery and vitamin D supplementation that impact glucose metabolism is unclear. In vivo and in vitro studies on insulin secretion and vitamin D are mainly conducted in murine models, which makes conclusions about the mechanistic functions of vitamin D in the beta cell less relatable to human conditions. Determining expression and localization of the vitamin D pathway in NHP pancreas would allow a clearer insight of the effects of obesity induced by a high fat diet on expression pattern on the vitamin D pathway and may be an important link to vitamin D signaling and beta cell physiology.

Methods

Animals and Diet

All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center (ONPRC). The care and housing of the rhesus macaques was provided by the Obese Resource at the Oregon National Primate Research Center (ONPRC), Oregon Health & Science University. Adult rhesus macaques were fed a control diet (CTR) or high fat diet (HFD) ad libitum. The diet composition for the two diets are provided in Table 1. NHP Control and High Fat Diet Nutrients. Monkeys were placed in colony groups fed either the control or HFD around 9 years of age for 2-3 years. The purpose of the HFD is to induce obesity in that research group. Five adult male monkey pancreas from animals fed the control diet and 5 from the HFD were used in this study.

Table 1 - NHP Control and High Fat Diet Nutrients

Nutrients	Control	HFD
Total Kcal/g	3.72	4.72
CHO % Total Kcals	59	45
PRO % Total Kcals	26	19
FAT % Total Kcals	15	36
Vitamin D IU/gram	6.6	3

Table 1. NHP Control and High Fat Diet Nutrients provides detailed information of the total Kcal/g, percent of total calories from carbohydrate, percent of total calories from protein, and percent of total calories from fat in the NHP control diet and the NHP high fat diet.

Sample Collection

Pancreas tissue was previously harvested from 5 control and 5 obese adult male rhesus macaque groups and frozen until analysis. The samples were analyzed using quantitative RT-PCR (qPCR) for this project.

qPCR Preparation

RNA Isolation

The following materials and equipment were used for RNA Isolation.

Materials: Qiagen Rneasy Mini Kit (Qiagen cat# 74106), Rnase-Free Dnase Kit (Qiagen cat#79254), 2ml Eppendorf tubes with 3mm stainless steel beads (McMaster-Carr cat#9291K42), Trizol (Life Tech cat# 15596-018), 70% EtoH, RNase free water

Equipment: Qiagen Tissue Lyser II, Centrifuge

RNA was isolated from NHP pancreas with Trizol (Life Tech cat# 15596-018) and Qiagen RNeasy Mini Kit (Qiagen cat# 74106). The previously collected NHP pancreas samples were placed on dry ice and 10-30 mg samples were cut from each pancreas and placed in separate 2ml Eppendorf tubes with 5 mm stainless steel beads (McMastre-Carr Cat# 9291K45). 1ml of Trizol was added to each sample and homogenized using a Qiagen TissueLyserII. Samples were centrifuged for 10 minutes at 4 degrees at 12,000g, and the middle pink layer containing RNA was pipetted into new 1.7ml tubes with caps. 200ul chloroform was added to the pink layer and shaken by hand for 15 seconds, allowed to sit for 2-3 minutes and then centrifuged for 15 minutes at 4 degrees at 14,000g. 400-450ul of supernatant from each sample was pipetted into new 1.7ml tubes and then 400ul of 70% EtOH was pipetted into each tube and mixed. 600ul of RNA/EtOH mixture was transferred to the pink spin columns from the Qiagen Kit (Qiagen Rneasy Mini Kit cat#74106), centrifuged for 30 seconds at 10,000g and then flow-through was discarded. 350ul RW1 was added to the center of each column and centrifuged for 30 seconds, then flow-through was discarded. 10ul of DNase1 and 70ul of RDD were added and mixed with each RNA sample (Rnase-Free Dnase Kit, Qiagen cat#79254). 80ul of master mix was added to

each column and then let sit for 15 minutes at room temperature. 350ul RW1 was added to each column and centrifuged for 30 seconds at 10,000g, and flow-through was discarded. 500ul RPE was added to each column and centrifuged for 30 seconds at 10,000g and flow-through was discarded. Each column was put into a new 2ml collection tube and centrifuged for 1 minute at 14,000g, and then the sample column was collected and the other collection tubes were discarded. Columns with sample were placed in new 1.5 ml tube with cap, 40ul Rnase-free water was added directly to column membrane (center of column) and centrifuged for 1 minute at 10,000g. Eluted RNA was collected and put back into the column and centrifuged 1 minute at 10,000g. Each RNA sample was then quantified using 2ul of RNA on a Spectrophometer ND100.

Reverse Transcription (cDNA Synthesis)

The following materials and equipment were used for reverse transcription.

Materials: Random Primers (Promega Cat# C1181), 385 well plates, 5x M-MLV 1st strand reaction buffer (Promega Cat# M5313), 100uM PCR Nucleotide Mix (or dNTP) (Promega Cat# C1145), Rnasin (40u/ul) (Promega Cat#N2111), 200u/ul M-MLV Reverse Transcriptase Enzyme (Promega Cat#M170B) , 0.1 M DTT (Promega Cat#P1171)

Equipment: Spectrophometer ND1000 and incubator.

Isolated RNA samples were analyzed on a spectrophotometer to determine the amount of RNA ng/ul. The expected concentration of RNA was 500-2500 ng/ul and the quality should be between 1.98 and 2.10 after isolation. Total RNA was recorded from the Spectrophotometer ND1000 and used to calculate the needed volume of each RNA sample for cDNA preparation. Each sample was converted from RNA ng/ul to RNA ug/ul per sample. Exact RNA concentrations, calculations, and dilutions can be found in Table 2. RNA Concentrations and Water Dilutions for cDNA preparation. RNA samples were diluted to a total volume of 24.5ul using previous

calculated amount of RNA samples, 1ul Random Primer, and water. See Table 3. Reagents and Volume added to PCR Tube with RNA, Random Primer and Water for exact volume of RNA, random primers, and water for each tube that was prepared. RNA, Random Primers and water were mixed by pipetting and then incubated at 65 degrees for 5 minutes and then placed on ice. Samples were briefly centrifuged to collect solution at the bottom of tubes. A total volume of 15ul, including the following reagents was added to the PCR tube: 5X M-MLV 1st strand reaction buffer, 100uM PCR Nucleotide Mix, Rnasin (40u/ul), M-MLV RT (200u/ul), .1 M DTT. Exact volumes of each reagent is shown in Table 4. Reagents and Volume added to PCR Tube with RNA, Random Primer and Water. Total contents of 40ul was briefly centrifuged and incubated at 37 degrees for 60 minutes, and then incubated at 95 degrees for 5 minutes to stop the reaction. Total reaction volume was 40ul which resulted in 50ng/ul of cDNA of each sample. Note: Sample #7 and sample #10 were diluted by 100ul extra water to total 136ul.

Table 2 - RNA Concentrations and Water Dilutions for cDNA preparation

Sample #	Animal ID	Tissue	RNA ng/ul	RNA ug/ul	2ug RNA	Added H2O
1	19836	Pancreas	2196.04	2.19604	0.9	22.6
2	19843	Pancreas	2187.08	2.18708	0.9	22.6
3	21032	Pancreas	1103.57	1.10357	1.8	21.7
4	22459	Pancreas	2021.88	2.02188	1	22.5
5	23527	Pancreas	1174.64	1.17464	1.7	21.8
6	19267	Pancreas	2156.78	2.15678	0.9	22.6
7	20169	Pancreas	687.86	0.68786	2.9	20.6
8	21125	Pancreas	2048.23	2.04823	1	22.5
9	21937	Pancreas	2550.21	2.55021	0.8	22.7
10	21969	Pancreas	464.12	0.46412	4.3	19.2
water			0	0	0	0

Table 2. RNA Concentrations and Water Dilutions for cDNA Preparation provides detailed information on each experiment sample number, corresponding animal ID of each sample, amount of RNA ng/ul in each sample, conversion or RNA ug/ul to RNA ug/ul, amount of RNA in 2 ug of each sample and the amount of added water to for each cDNA sample preparation.

Table 3 - Volume of RNA, Random Primer, and Water		
Material	Volume	Unit
2ug of total RNA	calculated from RNA	ul
Random primers (0.5ug/ul)	1	ul
Nanopure H2O	up to 23.5	ul
Total for above	24.5	ul

Table 3. Volume of RNA, Random Primer, and Water lists volume of RNA, volume of random primers, and water used in each random primer cocktail for qPCR experiments.

Table 4 - Reagents and Volume added to PCR Tube with RNA, Random Primer and Water		
Material	Volume	Unit
5X M-MLV 1st strand reaction buffer	8	ul
100uM PCR Nucleotide Mix	2.5	ul
Rnasin (40u/ul)	1	ul
M-MLV RT (200u/ul)	2	ul
.1 M DTT	2	ul
Total:	15.5	ul

Table 4. Reagents and Volume added to PCR Tube with RNA, Random Primer and Water lists volume of each qPCR reagent that is mixed with qPCR tube, RNA, random primers for sample preparation.

qPCR

The following materials were used for the qPCR protocol:

Materials: previously made cDNA, 2 ml micro tubes (2.0 ml Eppendorf catalog # 022364376), TaqMan Master Mix (SsoAdvanced Universal Probes Super Mix; Bio-Rad catalog #1725284), .2 ml PCR tubes (Genemate .02 mL flat cap PCR tubes Bio express catalog # C33282), VWR 384 Well Plate (Bio-Rad Catalog # HSP381).

Equipment: Bio RAD CFX384 Real Time PCR Machine (C1000 Touch Thermal Cycler System)

The previously made cDNA samples were collected and used in following qPCR protocol. The number of samples included: 10 samples of cDNA from the selected NHP pancreases from the same location within the pancreas, 1 positive control tissue for each primer probe and 1 set of

standard curves. Positive controls tissues were selected based on known expression levels stated in the Human Protein Atlas [65]. NHP small intestine tissue was used for a positive control for VDR and NHP kidney tissue was used for positive controls for genes CYP27B1, CYP24A1, and CALB1. The probes were from a premade qPCR gene expression assay kit directed against either human or NHP sequences from Thermofisher Scientific. Homology between species was checked in order to confirm a high degree of confidence using NCBI BLAST between human amplicon sequence and rhesus macaque gene sequence. Samples were analyzed using a Bio RAD CFX384 Real Time PCR Machine (C1000 Touch Thermal Cycler System). Standard curves on serial dilutions of cDNA (1:2, 1:4, 1:5, 1:6, 1:10, 1:20, 1:50, 1:100) were performed using cDNA from pooled pancreas samples consisting of the 5 control and 5 obese male animals, and measured in triplicates for each primer probe being tested. The housekeeping gene 18s, a ribosomal RNA gene, was used for normalization in all samples. Cocktails were prepared for standard curves, experimental tests for each primer probe and 18s housekeeping gene for each study group.

To prepare cDNA samples for qPCR, samples were thawed for several hours at 4 degrees, vortexed, and then briefly centrifuged. 2ul of each sample was loaded in triplicate into the VWR 384 well plate, followed by 8ul of primer probe cocktail (primer probe, master mix, water). Water was used as a negative control. Two separate plates were prepared. One plate was loaded with standard curves for each primer/probe and positive controls and another plate was loaded with primer/probe cocktails for 18S, calbindin, CYP24A1, CYP27B1, and VDR. A clear cover was pressed and sealed onto the plate, then plates were vortexed, and centrifuged at 1000g for 1 minute. Plates were loaded into Bio RAD CFX384 Real Time PCR Machine (C1000 Touch Thermal Cycler System) for analysis.

Statistical Analysis

Statistical Analysis was conducted on real-time qPCR data to quantify gene expression in the NHP pancreas, and compare targets of interest in NHPs fed a control diet with NHPs fed a high fat diet. Multiple steps were completed to quantify mRNA concentrations in each NHP sample. Steps to determine accurate Ct values included averaging triplicate Ct values, exclusion of outliers, generation of the standard curve, and assay evaluation for efficiency and correlation. Relative Quantification was conducted to calculate gene expression using the 2Delta Delta Ct Method (calculation of fold change) and Pfaffle Calculations to adjust for efficiency [66]. Unpaired, two-sided, students t-tests were used to compare differences in mRNA concentrations between NHP pancreas of control diet group and HFS diet group.

Determine Accurate Ct Values

Average of Triplicate Ct Values

Ct values from serial dilutions of NHP samples were measured in triplicate then averaged for each target gene of interest and 18S. Ct values of experimental samples were run at 1:5 dilution, were measured in triplicate, and then averaged.

Exclusions and Outliers

Ct values were considered outliers if Ct values were 1.0 Ct value larger or smaller than the other samples and excluded from the respective averaged triplicate sample. mRNA quantity that did not meet the detection threshold or if the Ct value was shown as undetermined were excluded from the triplicate average.

Generate Standard Curve

A standard curve was generated using the averaged triplicate Ct values of a ten-fold serial dilution (1:2, 1:4, 1:5, 1:6, 1:10, 1:20, 1:50, and 1:100) for each gene of interest and 18S. The standard curve was plotted with mean Ct values on the Y-axis and log cDNA on the X-axis.

Assay Evaluation

PCR efficiency was evaluated using a ten-fold serial dilution with the target assays and calculated by generating the slope of the standard curve. The slope of the standard curve was used to generate a PCR efficiency value using the formula $10^{(-1/m)}$ -1. An efficiency value ranging from 1.8 to 2.0 or between 90-100% ($-3.6 \geq$ slope ≥ -3.3) represents good probe efficiency for detecting message transcript. Ct values of the ten-fold serial dilution should be 3.3 cycles apart, indicating there is a 2-fold change for each change in Ct. A PCR assay with a slope below -3.6 is considered to have poor efficiency.

Relative Quantification (2Delta Delta Ct Method)

The relative quantification method, 2 Delta Delta Ct Method was used to calculate relative changes in gene expression represented by the equation ΔΔCt = ΔCt (treated sample) - ΔCt (untreated sample). Relative quantification was used to relate the Ct value of the target transcript of the obese group to the Ct value of the target transcript of the control group. The 2 Delta Delta Method was calculated using the following steps. The mean Ct value was calculated for each target gene of interest and 18S (as discussed above). Data was normalized by calculating the Delta Ct for each sample using the formula ΔCt = Ct (gene of interest (GOI)) – Ct (housekeeping gene). The normalized Ct values are the Delta Ct values and were calculated by (mean Ct GOI obese group - mean Ct of obese group 18S) and (mean Ct GOI control - mean Ct control 18s). The creation of a control average (calibrator/reference sample) was made by

calculating the average Ct values for each gene of interest which allows the results to be presented relative to the control average Ct values. Calculation of the Delta Delta Ct (ΔΔCt) values for each sample was done using the formula: ΔΔCt = ΔCt (sample) - ΔCt (control). The Delta Delta Ct value was calculated by (mean Ct GOI obese group - mean Ct 18S obese group) - (mean Ct control GOI - mean Ct control 18S). The fold gene expression (fold over change) value was calculated by using the following formula, Fold gene Expression = 2 ^ – (ΔΔCt). The formula assumes near perfect doubling of mRNA during the replication phase of qPCR (explaining the 2 to the power of Delta Delta Ct value) and was the measure used to describe how much the quantity of mRNA in the obese group changes relative to the quantity of mRNA in the control group (Fold Change). The Pfaffle Calculation was used to account for differences in gene efficiencies (accounts for non-perfect doubling). Calculated efficiencies (E) from the standard curve were used with calculations that compare the Ct difference of 18s (reference gene) between the Ct value of gene of interest and the control Ct value with the Ct difference of the target gene between the control Ct value. The Pfaffle Calculation (E= efficiency) Gene Expression Ratio is:

$$\text{Pfaffle Calculation} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (control-sample)}}}{(E_{\text{ref}})^{\Delta Ct \text{ ref (control-sample)}}}$$

Fold Change with corrected Ct values from Pfaffle Calculation values were used to generate unpaired, two-tailed, student T-Tests in GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla) to compare effects of high fat diet and control diets and difference between groups. Significance is reported as p=0.05.

Results

Animal Descriptive Data

Characteristics of the NHP Control Group (n= 5) and the NHP Obese Group (n= 5) were measured at the time of necropsy and include body weight, fasting glucose, insulin, triglycerides and cholesterol. See Table 5. NHP Control and Obese Group Characteristics for mean and standard deviation for each measurement. Changes in glucose levels in response to an intravenous glucose load was calculated as Area Under the Curve Glucose (AUC Glucose). Area Under the Curve Insulin (AUC Insulin) was calculated from insulin values during the ivGTT and represents the total insulin secretory response to a glucose bolus. The standard deviations for each variable was not substantially different (>2 fold) between the control and the obese group. Therefore, the data was analyzed using a 2 sample, 2 sided t-test with equal variance and Spearman's Correlation Tests. There were no significant differences between the NHP control group versus the obese group in regards to age (11.2 ± 1.304 vs 11.4 ± 1.673; p = 0.791), fasting blood glucose (57.4± 4.037 vs 64 ± 3.38a; p = 0.322), or fasting blood insulin (20.66 ± 14.79 vs 358 ± 469.5; p = 0.183). In contrast, there were significant differences between the NHP control group and obese group in body weight (11.6 ± 1.654 vs 20.59 ± 2.349; p = <.001), Area Under the Curve Glucose (10363 ± 963.9 vs 13043 ± 1374; p =.007), or Area Under the Curve Insulin (6399 ± 3788 vs 51778 ± 25479; p= 0.0043), Percent Body Fat (23.35 ± 8.507 vs 35.89 ± 4.415; p= 0.019), Total serum Triglycerides (49.75 ± 8.421 vs 261.8 ± 78.83; p = 0.001) and Total Serum Cholesterol levels (125 ± 41.27 vs 218.2 ± 43.51; p= 0.0138).

Table 5 - NHP Control and Obese Group Characteristics		
	NHP Control	NHP Obese
	N = 5	N = 5
Characteristics	Mean ± SD	Mean ± SD
Age at Necropsy	11.2 ± 1.304	11.4 ± 1.673
Weight at Necropsy (Kg)	11.6 ± 1.654	20.59 ± 2.349
Fasting Glucose (mg/dl)	57.4± 4.037	64 ± 13.38
Fasting Insulin (µIU/ml)	20.66 ± 14.79	358 ± 469.5
AUC Glucose	10363 ± 963.9	13043 ± 1374
AUC Insulin	6399 ± 3788	51778 ± 25479
% Body Fat	23.35 ± 8.507	35.89 ± 4.415
Total Serum Triglycerides (mg/dl)	49.75 ± 8.421	261.8 ± 78.83
Total Serum Cholesterol (mg/dl)	125 ± 41.27	218.2 ± 43.51

Table 5. **Characteristics of NHP Control and Obese Group.** Data presented as mean +/- standard deviation of the mean. Measurements and samples were taken at time of necropsy.

Primer Probe Selection, Testing, Efficiency and Variance

Primer Probe Selection

Quantitative polymerase chain reaction (qPCR) gene expression assays were selected to measure expression of vitamin D genes and chosen because of homology with either human or NHP sequences. See Table 6. Assay Details, for gene name, primer probe name, primer probe number and homology, vendor, and catalog number of assays used in the experiments. Rh in the primer probe number indicates associated homology and indicates the oligo sequence is directed at rhesus genome and Hs indicates that sequence is directed at the human genome. For CYP24A1 a probe was chosen that was based on the human gene sequence. Sequence alignment for CYP24A1 demonstrates a 96% homology over the amplified region and only 1 sequence mismatch in the primer regions. Each primer probe was tested for efficiency and standardized to the housekeeping gene 18S. A primer probe with an efficiency rate between 1.8

and 2.0 Cycle Thresholds (Ct) or between 90-100% calculated by formula $E = 10^{(-1/m)} -1$, indicates an efficient assay.

Table 6 - Assay Details				
Gene	Primer Probe Name	Primer #	Vendor	Catalog#
VDR	Vitamin D Recptor	Rh02828247_m1	Thermo Fisher Scientific	4331182
CYP24A1	24-hydroxalase	Hs00167999_m1	Thermo Fisher Scientific	4331182
CYP27B1	Enzyme 1 alpha-hydroxalase	Rh02829696_m1	Thermo Fisher Scientific	4351372
CALB1	Calbindin	Rh02788814_m1	Thermo Fisher Scientific	4351372

Table 6. Assay Details. Gene name, primer probe name, primer probe number and homology, vendor, and catalog number of assays used in the experiments.

We generated a pool of cDNA from several samples of NHP pancreas and used the pooled cDNA to run a standard efficiency curve with each probe. Serial dilutions of pooled cDNA were created (1:100, 1:50, 1:20, 1:10, 1:5, 1:4, and 1:2) and the amount of mRNA concentration of each gene was determined by qPCR. In addition, we ran 18S as a control. Analysis of serial dilutions determined all experimental samples would be best run at a dilution of 1:5.

Testing of VDR Primer Probe

Triplicate Ct measures of each DNA dilution were averaged and plotted against the log cDNA (Figure 1). The efficiency of VDR PCR probe was calculated based on the slope of the linear relationship (m=-3.3) using the formula $10^{(-1/m)} -1$. The VDR PCR probe efficiency was 1.97 with a linear correlation of R^2=0.98. The efficiency value of the VDR PCR probe fell within the range of 1.8 to 2.0 and indicated 97% probe efficiency for detecting message transcripts. Ct values of the VDR assay were found to increase significantly with increase in log cDNA concentration dilutions ($p < .0001$). Every increase in log of cDNA led to a decrease of 3.3 in VDR Ct values (95% CI: decrease of 3.89 to 2.78). The log cDNA helps to explain approximately 98% of the variability of Ct values and indicates a strong linear relationship between cDNA dilutions from 1:2 to 1:100. Measurements of the VDR transcripts in pancreas from monkeys on normal

chow and high fat chow used a 1:5 dilution, within the linear range of the efficiency testing.

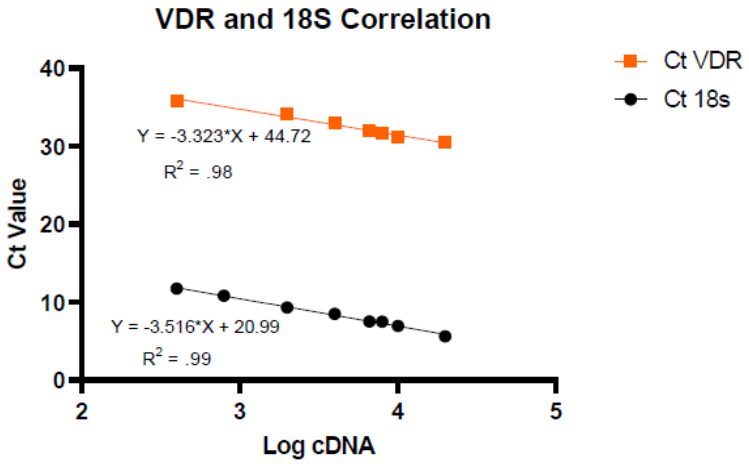

Figure 1. VDR and 18s liner regression correlations plotted by the average triplicate Ct measure of each DNA dilution plotted on the Y axis against the log cDNA plotted on the X axis. Efficiency for the VDR PCR probe was 1.97 with a linear correlation of $R^2 = 0.98$.

Testing of CYP24A1 Primer Probe

CYP24A1 mRNA was not detected in the NHP pancreas by the primer probe (Figure 2). However, the primer probe did detect CYP24A1 expression in the positive control, the NHP kidney. A CYP24A1 Ct value of 28.41 in the positive control and undetected Ct values in the NHP pancreas indicated an effective primer probe and undetectable CYP24A1 mRNA in the NHP pancreas.

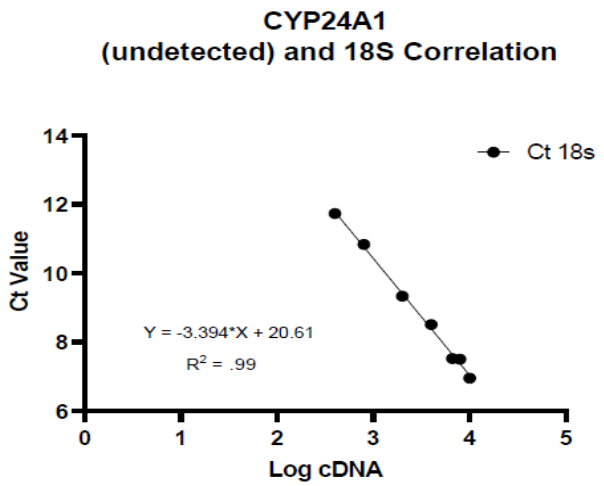

Figure 2. CYP24A1 and 18s linear regression correlations plotted by the average triplicate Ct measure of each DNA dilution plotted on the Y axis against the log cDNA plotted on the X axis and shows that CYP24A1 was undetectable in NHP pancreas.

Testing of CYP27B1 Primer Probe

Triplicate Ct measures of each DNA dilution were averaged and plotted against the log cDNA (Figure 3). The efficiency of CYP27B1 PCR probe was calculated based on the slope of the linear relationship (m=-4.2) using the formula $10^{(-1/m)}$ -1. The CYP27B1 PCR probe efficiency was is 1.73 with a linear correlation of R^2=0.95. The efficiency value of the CYP27B1 PCR probe did not fall within the range of 1.8 to 2.0 and had 73% efficiency which indicates the probe may not have good efficiency for detecting message transcripts. Ct values of the CYP27B1 assay were found to decrease significantly with increase in log cDNA concentration dilutions ($p < .0001$). Every increase in log cDNA led to a decrease of 4.2 in CYP27B1 Ct values (95% CI: decrease of 5.088 to 3.276). The log cDNA helped to explain approximately 95% of the variability of Ct values and indicates a strong linear relationship between Ct values cDNA dilutions from 1:2 to 1:100. Measurements of the CYP27B1 transcripts in pancreas from monkeys on normal chow and high fat chow used a 1:5 dilution, within the linear range of the efficiency testing.

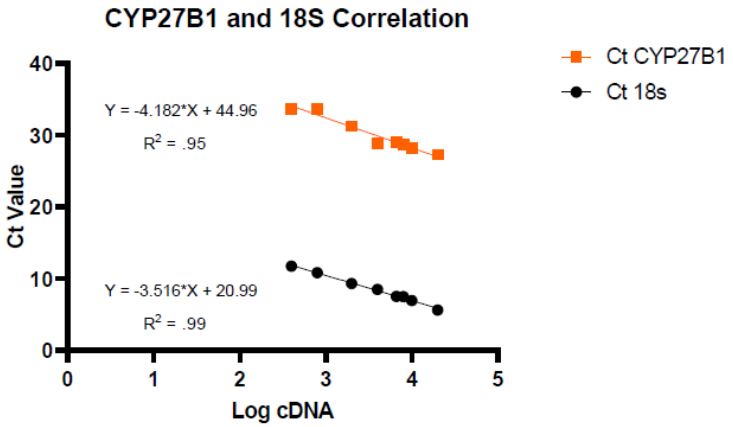

Figure 3. CYP27B1 and 18s liner regression correlations plotted by the average triplicate Ct measure of each DNA dilution plotted on the Y axis against the log cDNA plotted on the X axis. Efficiency for the CYP27B1 PCR probe was 1.73 with a linear correlation of $R^2 = 0.95$.

Testing of Calbindin Primer Probe

Triplicate Ct measures of each DNA dilution were averaged and plotted against the log cDNA (Figure 4). The efficiency of Calbindin PCR probe was calculated based on the slope of the linear relationship (m=-3.8) using the formula $10^{(-1/m)} -1$. The Calbindin PCR probe efficiency was 1.98 with a linear correlation of R^2=0.97. The efficiency value of the Calbindin PCR probe fell within the range of 1.8 to 2.0 and had 98% efficiency which indicated good probe efficiency for detecting message transcripts. Ct values of the Calbindin assay were found to decrease significantly with increase in log cDNA concentration dilutions ($p < .0001$). Every increase in log cDNA led to a decrease of 3.8 in Calbindin Ct values (95% CI: decrease of 4.529 to 3.180). The log cDNA helps to explain approximately 97% of the variability of Ct values and indicates a strong linear relationship between Ct values DNA dilutions from 1:2 to 1:100. Measurements of the Calbindin transcripts in pancreas from NHP's on normal chow and high fat chow used a 1:5 dilution, within the linear range of the efficiency testing.

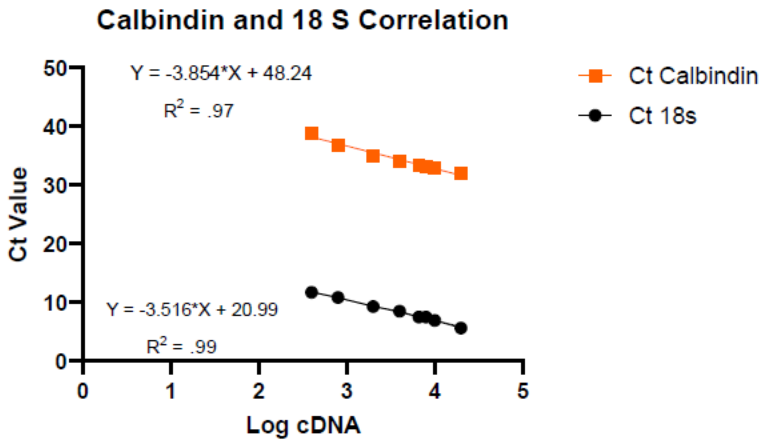

Figure 4. Calbindin and 18s liner regression correlations plotted by the average triplicate Ct measure of each DNA dilution plotted on the Y axis against the log cDNA plotted on the X axis. Efficiency for the calbindin PCR probe was 1.98 with a linear correlation of $R^2 = 0.97$.

Vitamin D Gene Expression and Diet Effects in NHP Pancreas

The expression of mRNA was examined by qPCR and initially quantified by Cycle Threshold (Ct) level defined as the number of cycles required for the fluorescent signal to cross the threshold. A positive reaction is detected by the accumulation of a fluorescent signal. The Ct value indicates the point in time during the cycle when the amplification of the probe is first detected. The more mRNA that is detected, the sooner a significant increase in fluorescence is seen. There is an inverse relationship between the Ct value and mRNA concentrations, and high Ct values indicate lower mRNA expression.

Ct values were then categorized into strong, moderate, or weak. Ct values less than 29 is a strong positive reaction indicating abundant mRNA expression. Ct values between 30-37 are positive reactions indicating moderate amount of mRNA expression. Ct values between 38-40 are indicative of weak reactions indicating little to no mRNA expression. Concentrations of mRNA levels shown by averaged Ct values for each gene are shown in Table 7. Average Ct Values of Target Gene in Control Group, Obese group, and Positive Control samples.

Table 7 - Average Ct Values of Target Gene in Control Group, Obese, and Postive Control Samples				
Sample Type	VDR	Calbindin	CYP24A1	CYP27B1
Control Group	33.75 (M)	35.28 (M)	undetermind	29.74 (M)
Obese Group	32.1 (M)	33.05 (M)	undetermind	29.38 (M)
Positive Control Sample	24.28 (S)	19.46 (S)	28.41 (S)	24.81 (S)
Positvie Control NHP Tissue	Jejunum	kidney	kidney	kidney

Table 7. Averaged triplicate measured Ct values for each primer probe tested in the control group, obese group and NHP positive control tissue for each primer-probe.

Differences in gene expression between control and obese groups were determined by two sample, two-sided, student's t-tests comparing fold change in gene expression were calculated using Ct values and the Relative Quantification Method. This method (2 ΔΔCT Method) is designed to normalize Ct values, control for assay efficiency, and calculate fold change of expression verses directly comparing Ct values. The fold change was calculated using the formula Fold Change = 2 ^ – (ΔΔCt) and was used to estimate the quantity of mRNA in each group.

Diet Effects of VDR Regulation

VDR is moderately expressed in both the control group and in the obese group. There was a significant difference in the mean fold change value of the VDR expression between control and obese groups (two-sample t-test; p= 0.009). The mean difference in VDR was 1.771 ± SEM 0.5121 (95%CI: 0.5900 to 2.952). VDR expression was higher in the obese group compared to the control group.

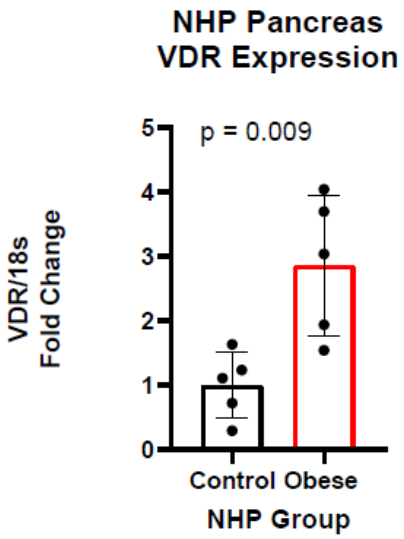

Figure 5. VDR Ct values normalized to 18s Fold Change Ct values of VDR obese group represented by the red bar and VDR control groups represented by black bar (p=.009, 95% CI= 0.5900 to 2.952).

Diet Effect of CYP24A1 Regulation in the NHP Pancreas

CYP24A1 mRNA levels were undetermined by qPCR analysis and did not show any level of expression in either the control or obese group and thus could not be tested for a diet effect. The effectiveness of the CYP24A1 PCR probe was confirmed with positive expression in the NHP kidney used as the positive control.

Diet Effect of CYP27B1 Regulation in the NHP Pancreas

CYP27B1 is highly expressed in both the control group and in the obese group. There was no significant difference in fold change between control and obese groups (two-sample t-test; p= 0.6). The mean difference in CYP27B1 expression was 0.2444 ± SEM 0.4828 (95%CI: -0.8689 to 1.358). CYP27B1 expression was similar between control and obese groups.

Figure 6. Cyp27B1 normalized to 18s Fold change Ct values of CYP27B1 obese group represented by the red bar and Cyp27B1 control groups represented by black bar (p= .626, 95% CI= 0.8689 to 1.358).

Diet Effect of Calbindin Regulation in the NHP Pancreas

Calbindin is moderately expressed in both the control group and in the obese group. There was no significant difference in the fold change of calbindin between control and obese groups (two-sample t-test; p= 0.19). The mean difference was 3.498 ± SEM 2.421 (95%CI: -2.085 to 9.080). Calbindin gene expressions are not significantly different between the obese group and the control groups.

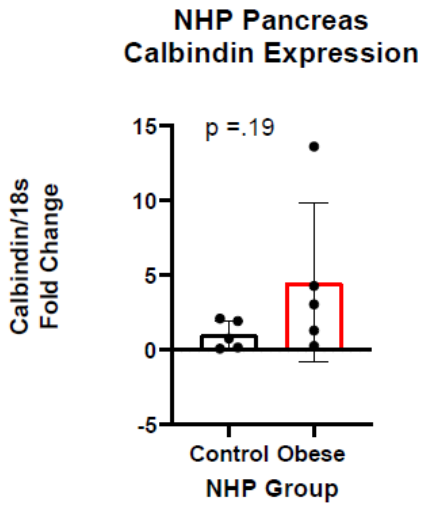

Figure 7. Calbindin normalized to 18s Fold Change Ct values of calbindin obese group represented by the red bar and calbindin control group represented by the black bar (p = .19, 95% CI= -2.085 to 9.080).

Correlation of Genes of Interest in NHP Pancreas and NHP Metabolic Markers

To determine if there is a relationship between gene expression of the vitamin D pathway and glucose homeostasis in the nonhuman primates, we correlated fold change in gene expression with the parameters measured prior to necropsy. The number of samples is small (n-10); a nonparametric Spearman correlation was performed for the following correlations because of the small sample size.

Correlation of NHP Pancreas VDR Ct values and NHP Metabolic Markers

There is a significant strong correlation between VDR fold change, and NHP AUC Glucose measurements (r_s= 0.84; p= .0037). In contrast, there was no significant correlation between VDR fold change and NHP measurements of AUC Insulin measurements (r_s=.59; p =.08), or Percent Body Fat (r_s= .50; p= .14).

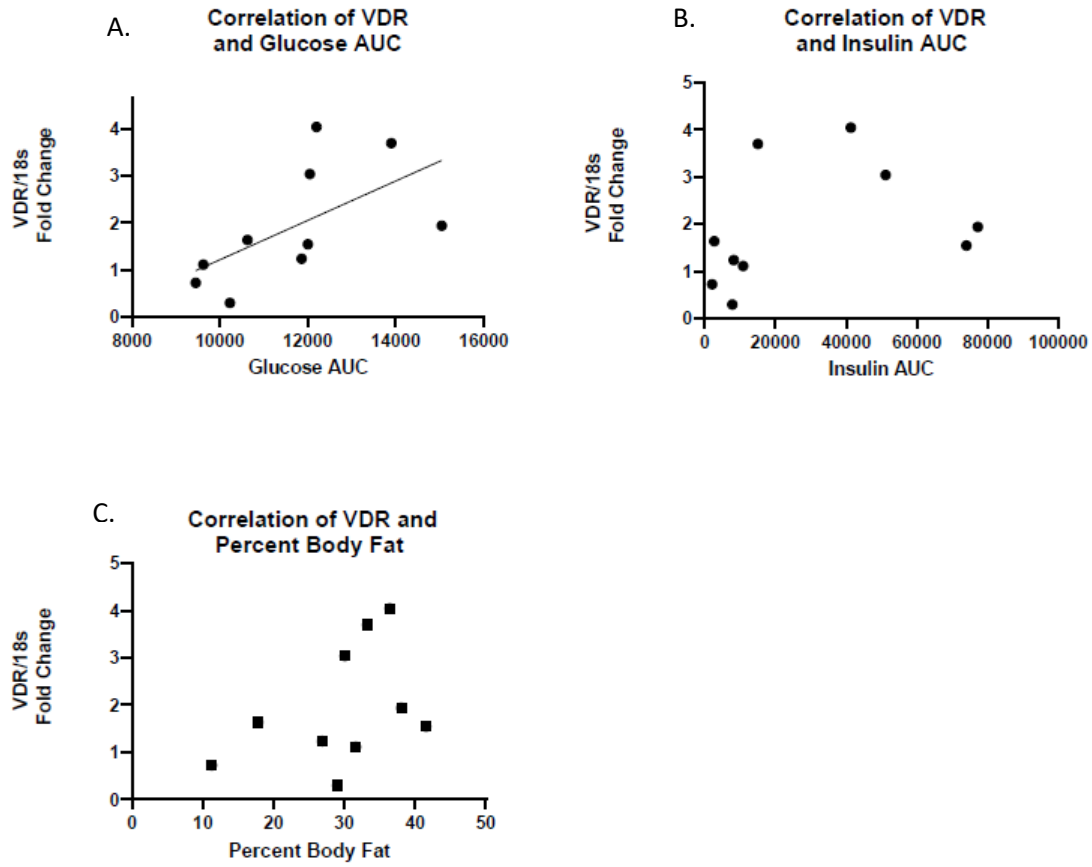

Figure 8. Correlation of NHP pancreas VDR and NHP metabolic markers**. A.** Indicates significant strong correlation between VDR Ct values and Glucose AUC (r_s=0.84; p= .0037). **B.** Indicates no significant correlation between VDR Ct values and AUC Insulin measurements (r_s=.59; p= .08). **C**. Indicates no significant correlation between VDR Ct values and Percent Body Fat (r_s=.50; p= .14).

Correlation of NHP Pancreas CYP27B1 Ct values and NHP Metabolic Markers

There are no significant strong correlation between CYP27B1 fold change and NHP AUC Glucose measurements (r_s= -0.042; p = 0.9184), NHP AUC Insulin (r_s = -.042; p= .91), or Percent Body Fat (r_s= .05; p= .89).

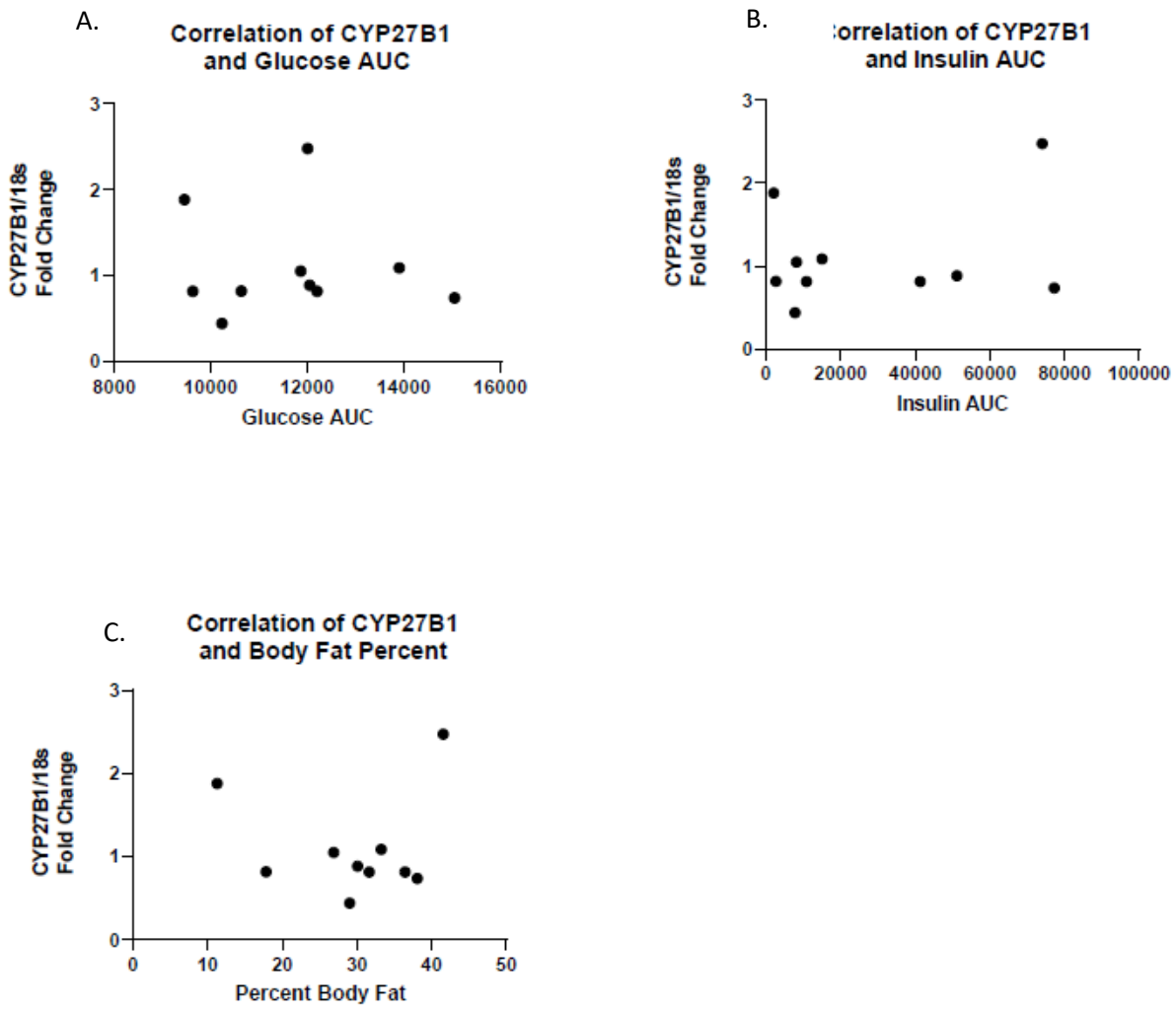

Figure 9. Correlation of NHP pancreas CYP27B1 and NHP metabolic markers. **A**. Indicates no significant correlation between CYP27B1 Ct values and AUC Glucose (r_s= -0.042; p=0.9184). **B**. Indicates no significant correlation between CYP27B1Ct values AUC Insulin measurements (r_s= -.042; p = .91). **C**. Indicates no significant correlation between CYP27B1 Ct values and Percent Body Fat(r =-.05; p=.89).

Correlation of NHP Pancreas Calbindin Ct values and NHP Metabolic Markers

There are no significant strong correlation between calbindin Ct values and NHP AUC Glucose (r_s= 0.3212; p=0.3679), NHP AUC Insulin (r_s = 0.2121; p = 0.560), or Percent Body Fat (r_s=0.188; p=0.6073).

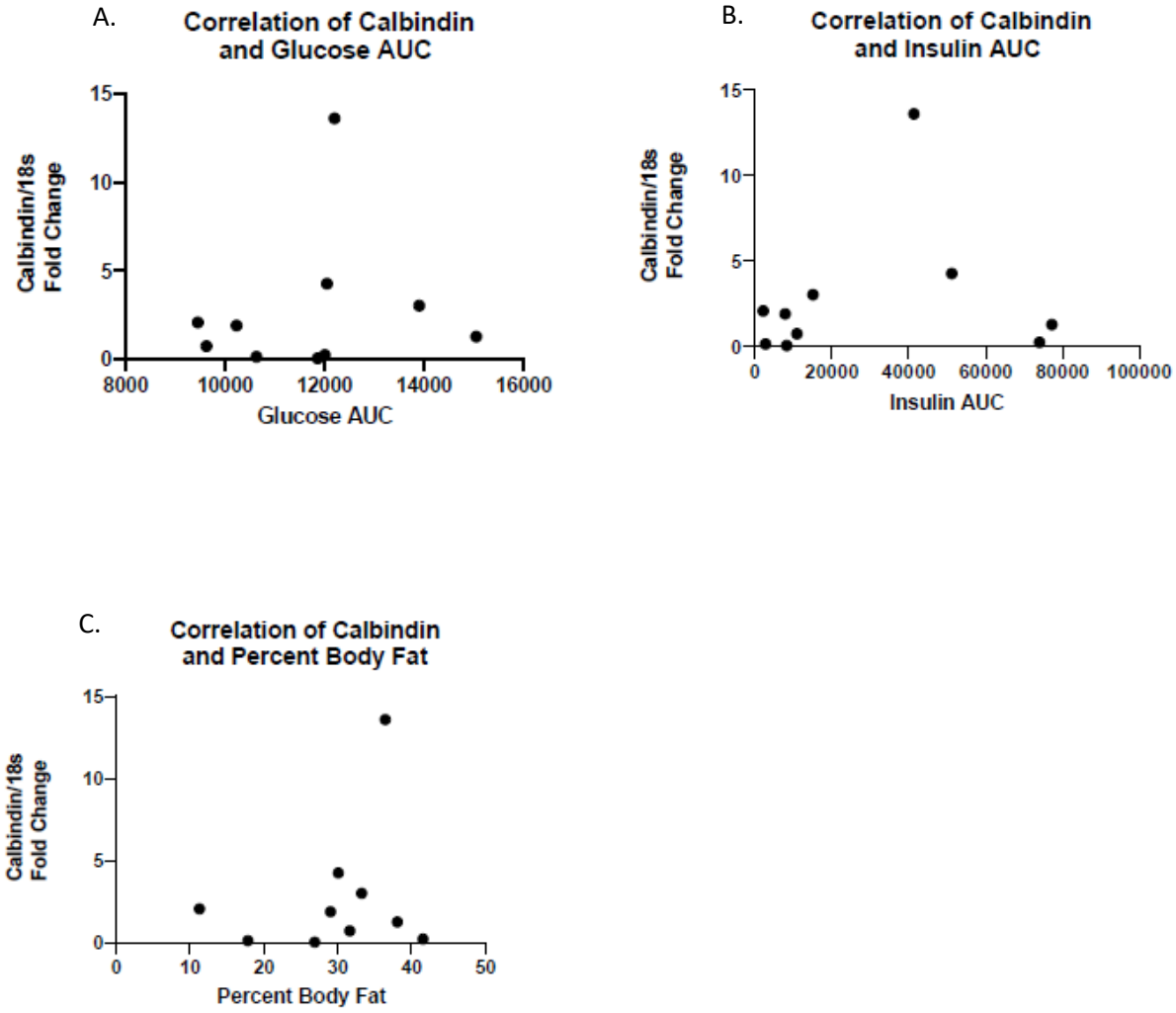

Figure 10. Correlation of NHP pancreas calbindin and NHP metabolic markers. **A**. Indicates no significant correlation between calbindin Ct values and AUC Glucose (r_s = 0.3212; p = 0.3679). **B.** Indicates no significant correlation between calbindin Ct values AUC Insulin measurements (r_s = 0.2121; p = 0.560). **C.** Indicates no significant correlation between calbindin Ct values and Percent Body Fat (r_s = 0.188; p = 0.6073)

Discussion

Major Findings and Differences

This is the first study to examine the expression of the vitamin D signaling pathway in the nonhuman primate pancreas. We found that VDR, CYP27B1, and calbindin are moderately

expressed in the NHP pancreas, but CPY24A1 was undetectable. We also observed increased VDR expression in obese NHP's fed a high fat diet compared to the NHP control group. VDR expression had a significant positive correlation to AUC blood glucose. Thus, higher VDR expression was associated with a higher glucose excursion after an IV glucose load. We were unable to proceed with planned immunohistochemistry experiments to verify changes in mRNA expression to changes to protein levels due to the COVID 19 pandemic, which caused mandated regulations that halted all laboratory experiments at the Oregon National Primate Center. Further validation of these preliminary findings are needed, including western blots or immunohistochemistry (IHC) to measure the amount of protein in the NHP pancreas.

We conducted this study because of previous published associations between glucose homeostasis and vitamin D, continued uncertainty about mechanistic functions of pancreatic VDR, and the lack of vitamin D studies in the NHP pancreas [9-12 24 42-44]. Similar to our hypothesis, qPCR analysis determined moderate expression of the VDR, calbindin, and CYP27B1 in the NHP pancreas. Opposite of our hypothesis, qPCR results showed undetected expression of CYP24A1 in the NHP pancreas which is an important point of discussion. The primary function of CYP24A1 is the degradation of active vitamin D, which prevents toxic levels of 1,25(OH)D_3. It has been shown that the make-up and location of CYP24A1 can vary between species, and its regulation may be cell-specific [5]. CYP24A1 expression is challenging to detect in the human pancreas. Yet, some human studies have shown that CYP42A1 may be upregulated in some disease conditions and during inflammation in the pancreas. Upregulation of CYP24A1 may increase the degradation of active vitamin D affecting the vitamin D regulation system and thus potentially affecting insulin secretion [57]. Human and rat pancreatic cells have shown the presence of both VDR and CYP27B1, possibly indicating local production and a regulating system that would involve the presence of CYP24A1 and a negative feedback system [56]. Our study did not detect

CYP24A1 in the NHP pancreas in either the control group or the obese group, leading to interesting questions about the local/autocrine regulation of vitamin D in the NHP pancreas and the apparent absence of a negative feedback system involving CYP24A1. These results may support the concept that the upregulation of CYP24A1 and negative feedback regulation of vitamin D differs between species and and/or tissue [5 57]. Although we did not detect CYP24A1 in either the control or obese group, it may be that the expression of CYP24A1 is observed only under certain metabolic conditions not included here. Undetected CYP24A1 in the NHP samples may also be due to the inconsistent expression of CYP24A1 in some, but not all, cells throughout the pancreas. The particular NHP samples selected for this experiment may have been taken from areas that did not express CYP24A1 which may have led to undetectable levels of transcripts in the qPCR results. Our data suggest the NHP pancreas does not express CYP24A1, similar to what is documented in the human atlas. The factors affecting expression as well as its effects on the vitamin D regulation system in the beta cell are not known and further studies to verify this finding are warranted.

Comparisons between the two NHP groups showed that the obese group had significantly higher body weight, % body fat, AUC glucose, AUC insulin, serum triglycerides and serum cholesterol than the control group. Although the groups had some significant metabolic differences, the obese group did not have glucose levels high enough to warrant insulin treatments. There was a significant difference in the upregulation of VDR expression in the obese group compared to the control group, which is consistent with our study hypothesis. In contrast to our hypothesis, our study did not show any significant difference in expression of these particular vitamin D regulating genes between the control versus obese groups. These results along with findings from other studies on vitamin D, obesity, and glucose homeostasis lead to interesting thoughts about the vitamin D pathway in the NHP pancreas. Obesity has

been associated with type 2 diabetes and insulin resistance, and it has also been suggested that the VDR is involved with insulin secretion [44]. This may suggest that when higher insulin concentrations are needed to maintain euglycemia, the body adapts by upregulating pancreatic VDR in order to produce more insulin. However, our study did not detect significant differences in the expression of downstream proteins involved in vitamin D homeostasis (CYP27B1, CYP24A1, and calbindin) between the control and obese groups. Why would the VDR be upregulated in the obese group but not the proteins involved in the vitamin D signaling cascade? The VDR may be upregulated in the pancreas of the obese group in response to produce more insulin as well as in response to the lack of available circulating serum vitamin D. The downstream enzymes may be less regulated by serum vitamin D levels than VDR itself. As adiposity rises, there is an increase in sequestered vitamin D causing a reduction in available serum vitamin D levels [53]. This may cause less conversion of 25(OH)D to active vitamin D, resulting in lower available active vitamin D thus upregulating the expression of the VDR to counter lower circulating 25(OH)D and assist in glycemic control. Cross-sectional and clinical studies also report findings of associations between serum vitamin D and glucose homeostasis. Associations of vitamin D and insulin measurements were often only noted in high glucose situations such as patients with prediabetes and diabetes but not in patients with normal fasting glucose levels [48-50]. We found a strong significant correlation between VDR expression and AUC glucose measurements. We observed a trend towards significance in AUC insulin measurements and VDR expression levels. This may suggests that differences in the action of vitamin D and VDR expression regulation may be associated with different metabolic states, such as glucose levels, adiposity, and insulin resistance.

Study Results and Nutrition

The results of clinical trials on vitamin D supplementation have been inconsistent, causing some debate over the therapeutic benefits and appropriate dose of vitamin D supplementation. Some clinical studies show the benefits of vitamin D supplementation in patients with obesity [30]. People with obesity have a higher vitamin D deficiency rate and need higher doses of vitamin D supplements to obtain sufficient serum vitamin D levels [53]. Our study results showed increased VDR expression in the NHP obese group and positive correlations with VDR and AUC glucose measurement. What is still unknown is the effect of vitamin D dietary intake and vitamin D serum levels on the regulation of VDR and how these affect the vitamin D cascade and insulin secretion. Vitamin D supplements are often prescribed to people based on their serum 25(OH)D levels. Therefore, understanding how these metabolic conditions affect an individual's ability to regulate the VDR and influence insulin secretion is critical to improving therapeutic recommendations of vitamin D supplements.

Study Limitations

This study had several limitations: suboptimal assay efficiency of the CYP27B1 primer-probe, differences in the vitamin D content between the control diet and the high fat diet, and research interruptions due to COVID 19 regulations and lab safety. The assay efficiency of CYP27B1 was calculated to be 73%, which is below what is considered good efficiency of 90-100%. The qPCR result showed a positive and moderate gene expression within the sample tissues, and there were no errors in the standard curve calculations. Although assay efficiency was low, the standard curve had a high r_2 value which suggests that fold changes were indicative of observed changes. Another study limitation was the difference in vitamin D content between the control diet and the high fat. The control diet had 6.6 IU/gram of vitamin D and the high-fat diet only

had 3.0 IU/gram of vitamin D. The differences in vitamin D content between diets could have affected study outcomes such as the expression levels of the target genes and measurements of the NHP metabolic markers. Unfortunately, serum levels of vitamin D for this cohort of NHP's were not available to make comparisons between serum vitamin D levels and gene expression. Lastly, the unforeseen event of COVID 19 caused requirements and regulations caused research laboratories to halt all experimental studies and did not allow us to complete the experiments on the target gene localization within the beta-cell immunohistochemistry in the NHP pancreas.

Future Directions

The 2nd International Conference of Vitamin D released the most recent and up to date Consensus Statement on Controversies in Vitamin D in March 2020. The statement reports there are still unsolved problems concerning vitamin D, although they did acknowledge that diabetes and obesity are associated with vitamin D deficiency. The committee has also recognized that vitamin D deficiency is associated decreased synthesis and secretion of insulin in animal studies. They also reported that there are demonstrated associations between type 2 diabetes, vitamin D, and parameters of insulin insensitivity and incidence of type 2 diabetes. There is still a continued need for research on vitamin D mechanisms and its relation to diabetes and obesity, the effects of vitamin D supplementation on the improvement of outcome measures of diabetes and obesity, and variation in the response to vitamin D supplementation [67].

This study reports the expression level of the VDR and several essential genes that regulate the vitamin D pathway in the NHP pancreas. The established obese NHP model can help us study the vitamin D pathway in the pancreas and establish more precise localization to improve our understanding of mechanistic functions. There are multiple future directions possible to study mechanistic functions of vitamin D and their associations with glucose homeostasis.

Determining localization in the NHP pancreas would be a great first step to understanding the relationship to insulin secretion of the vitamin D pathway in the NHP pancreas. In vivo studies using the NHP pancreas for GSIS (glucose stimulated insulin secretion) assays would be more physiologically relevant than other animal models to determine the VDR and vitamin D related enzymes' unsolved mechanistic question. Furthermore, determining the effects of both serum 25(OH)D, active vitamin D, and effects of vitamin D supplementation on the vitamin D pathway and feedback system in the beta-cell would be extremely valuable. These are just a few ideas for the next steps to advance the study of vitamin D, which has been shown to have complicated and tangled metabolic interactions. Although the study of vitamin D is like putting together a very complicated puzzle with some missing pieces, it also continues to show signs that it may be part of a solution to help improve chronic conditions that many people are burdened with on a daily basis.

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