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Running title: Circulating Vitamin K1 and glucose metabolism in T2D

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Key words: Type 2 Diabetes; Insulin resistance; Hyperglycemia; Vitamin K1; SIRT1/AMPK; Hepatic glucose metabolism

The abbreviations used are: AMPK, AMP activated protein kinase; CPT1A, carnitine palmitoyltransferase 1A; GK, glucokinase; G6P, glucose 6 phosphate; G6Pase, glucose 6 phosphatase; GGCX, gamma glutamyl carboxylase; GHb, glycated hemoglobin; GLUT2, glucose transporter 2; IL-6, interleukin 6, MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor kappa B; PI3K, phosphoinositide 3-kinase; PPAR\textalpha, peroxisome proliferator-activated receptor alpha; PTEN, phosphatase and tensin homolog; SIRT1, sirtuin 1; ucMGP, uncarboxylated matrix gla protein; VK1, vitamin K1; VKOR, vitamin K epoxide reductase
ABSTRACT

There is no previous study in the literature that has examined the relationship between circulating vitamin K1 (VK1) with glycemic status in type 2 diabetes (T2D). Moreover, scientific explanation for the beneficial role of VK1 supplementation in lowering glycemia in diabetes is yet to be determined. This study for the first time demonstrated that circulating VK1 was significantly lower in T2D patients compared to age-matched control subjects, and VK1 levels in T2D were significantly and inversely associated with fasting glucose and insulin resistance (HOMA-IR), which suggest that boosting plasma VK1 may reduce the fasting glucose and insulin resistance in T2D patients. Using high fat diet-fed T2D animal model this study further investigated the positive effect of VK1 supplementation on glucose metabolism and examined the underlying molecular mechanism. Results showed that VK1 supplementation (1, 3, 5 µg/kg BW, 8 weeks) dose dependently improved the glucose tolerance, decreased body weight gain, fasting glucose and insulin, glycated hemoglobin, HOMA-IR, and cytokine secretion (MCP-1 and IL-6), and regulated the signaling pathway of hepatic glucose metabolism (SIRT1/AMPK/PI3K/PTEN/GLUT2/GK/G6Pase), lipid oxidation (PPARα/CPT1A), and inflammation (NF-κB) in T2D mice. Comparative signal silencing studies also depicted the role of SIRT1/AMPK in mediating the effect of VK1 on glucose metabolism, lipid oxidation, and inflammation in high glucose-treated cultured hepatocytes. In conclusion, this study demonstrates that circulating VK1 has a positive effect on lowering fasting glucose and insulin resistance in T2D via regulating SIRT1/AMPK signaling pathway.
1. Introduction

Vitamin K (VK) is an essential micronutrient known for its beneficial role in blood coagulation [1]. Recently, however, coagulation-unrelated functions of VK have attracted scientific attention, such as stimulating glucose metabolism, modulating vascular inflammation, and improving bone mineralisation [1,2]. Various studies have demonstrated that increasing dietary VK intake is associated with improved glycemic status, reduced risk of type 2 diabetes (T2D), and lowered the prevalence of metabolic syndrome among normal subjects including both young and elderly volunteers [3-7]. Studies with VK supplementation have also demonstrated a decrease in insulin resistance and fasting glucose and an increase in insulin sensitivity in normal subjects [8-11]. All of these studies suggest a novel role for VK in improving insulin sensitivity and regulating glucose homeostasis among normal subjects [2]; however, there is no information about the relationship between plasma VK and glycemic status in individuals with T2D. In addition, the molecular mechanisms underlying the beneficial role of VK in improving glucose metabolism are not known.

The liver plays an important role in the regulation of whole body glucose homeostasis. Hepatic insulin resistance is one of the risk factors that play an important role in the development of metabolic syndrome and its related abnormalities including hyperglycemia, dyslipidemia, and increased inflammatory factors [12,13]. Defects in the insulin signaling cascade and development of hepatic insulin resistance cause an increase in hepatic glucose production via glycogenolysis and gluconeogenesis pathway [12]. AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) are emerging as important signaling molecules in the regulation of energy metabolism, inflammation, and metabolic syndrome associated chronic diseases [14]. Activation of AMPK has been found to inhibit gluconeogenesis and lower glucose levels and is useful for the regulation of glucose homeostasis [15]. SIRT1-mediated metabolic modulation has also been found to be beneficial against hepatic fat deposition in high fat diet (HFD)-fed mice and genetically-induced obese mice [16]. Furthermore, activation of SIRT1 also attenuated the hepatic inflammation by decreasing the NF-κB-induced expression of pro-inflammatory cytokines [17]. There is no reported study in the literature examining whether the beneficial effect of VK on glucose metabolism can be mediated via the activation of SIRT1/AMPK signaling pathway.

Phylloquinone (VK1) and Menaquinones (VK2) are the two naturally occurring dietary forms of VK. Among them, VK1 is the major dietary source and primary circulating form of VK and has been measured successfully in both population and clinical-based studies to rank individuals’ VK status [18]. This study for the first time examined the plasma VK1 levels in T2D patients and age-matched healthy control subjects and investigated the relationship between circulating VK1 levels and glycemic status in T2D patients. Furthermore, the role of VK1 supplementation on glucose metabolism in diabetes has been examined using HFD-fed animal
model of T2D and the molecular mechanism has been investigated using the liver tissues from experimental animals. Comparative signal silencing studies with hepatocyte cell culture model using both SIRT1 and AMPK siRNAs this study also dissected the molecular mechanism underlying the beneficial role of VK1 on glucose metabolism in diabetes.

2. Materials and Methods

2.1 Materials

Anti-AMPK, anti-phosphorylated AMPK (Thr 172), and anti-PI3K (p85α) were purchased from Cell Signaling Technology (Beverly, MA). Anti-CPT1A, anti-G6Pase, anti-GGCX, anti-GK, anti-GLUT2, anti-PPARα, anti-PTEN, anti-phosphorylated NF-κB (p65) (Ser 276), anti-SIRT1, and anti-VKOR primary antibodies were purchased from Abcam, Inc. (Cambridge, MA). Anti-NF-κB (p65) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise mentioned.

2.2 Study enrolment and blood collection from T2D patients and normal subjects

Informed written consent was obtained from all patients and control subjects according to the protocol approved by the Institutional Ethics committee for Human Experimentation. All subjects included in this study were T2D patients (n=25) attending clinics at North East Institute of Science and Technology (NEIST). Volunteers for age-matched controls (n=20) were enrolled from a group including siblings of patients or workers at NEIST. T2D patients and healthy controls were excluded if they had any history of cardiovascular disease, sickle cell disease, smoking habits, uncontrolled hypertension, hypothyroidism, hyperthyroidism, and treatment with anticoagulant or insulin as evident from the records maintained at clinical center. Subjects were also excluded if they showed signs of coagulation complications, defined as a prothrombin time greater than 15 sec, hepatic dysfunction, defined as any underlying chronic liver disease or liver function tests greater than 1.5 times the upper limit of normal or renal dysfunction, defined as a serum creatinine value greater than 1.5 mg%. Women with a positive pregnancy test or those nursing infants were also excluded. Subjects taking any supplemental vitamins or herbal products were excluded. All patients and normal subjects who gave written informed consent were invited to return to have blood drawn after fasting overnight (8 h). Serum tubes containing blood for chemistry profiles, sodium citrate (3.2%) vials for Prothrombin Time test, and EDTA-blood tubes for HbA1c were promptly delivered to the NEIST clinical laboratory. Additional EDTA-blood was brought to the research laboratory, plasma were separated via centrifugation at 3000 rpm for 15 min, then transferred to cryovials, and stored at -80°C for further analysis.
2.3 Estimation of plasma vitamin K1 levels

Plasma VK1 levels in T2D patients and normal subjects were measured following the method described previously [19]. Samples, deproteinized with absolute alcohol, were purified on polymeric RP-SPE cartridges, eluted with acetonitrile: isopropanol: dichloromethane 70:10:20 (by volume), evaporated under nitrogen and stored at -20°C for no more than 15 days. Before analysis the residue was dissolved with 50 µL of the HPLC mobile phase (95% eluent A comprised of 994.5 mL methanol plus 5.5mL of a solution 2M ZnCl2, 1M CH3COONa, and 1M CH3COOH, and 5% eluent B containing absolute ethanol) and injected into HPLC (Waters) equipped with a C30 column (Accucore, 3x100 mm, 2.6 µm) followed by a post-column on-line zinc metal reactor and fluorometric detector (λex 248 and λex 430). A proprietary VK1 derivative was used as an internal standard (Immundiagnostik AG, Wiesenstrasse, Bensheim, Germany, KC2400is).

2.4. Animal studies

2.4.1. Animals - Male Swiss albino mice (5 weeks old, 25-30 g) were obtained from Bose Institute animal research facility. All animals were kept in the animal care facility where ambient environmental conditions (12:12-h light-dark cycle, 22–24°C) were maintained. Experiments were carried out according to the guidelines of the Institutional Animal Ethical Committee (IAEC), Bose Institute, Kolkata. The study was also approved by both CPCSEA (Committee for the Purpose of Control & Supervision on Experiments on Animals), Ministry of Environment & Forests, New Delhi, India (1796/PO/Ere/S/14/CPCSEA) and IAEC.

2.4.2. Animal experimental design - Mice were divided into seven groups by computer-generated randomization, so that each group contained six animals. The mice were fasted overnight and then weighed and tested their blood glucose concentration. VK1 was dissolved in 0.1% olive oil (OO) and an aliquot of 0.1 mL of the stock solution was given per 100 g BW. The mice were treated as follows:

Normal - animals were fed a low fat diet (providing 10% calories as fat) for 16 weeks.

VK1-5 - animals were fed a low fat diet for 16 weeks and VK1 was administered at a dose of 5 µg/kg BW daily by oral gavage for last 8 weeks.

T2D - animals were fed a HFD (providing 45% calories as fat) for 16 weeks.

T2D+OO - animals were fed HFD for 16 weeks and OO was administered at a dose of 100 µL/100 g BW daily by oral gavage for last 8 weeks.

T2D+VK1-1, T2D+VK1-3, T2D+VK1-5 - animals were fed HFD for 16 weeks and VK1 was administered at a dose of 1, 3, and 5 µg/kg BW respectively daily by oral gavage for last 8 weeks.

The composition of the experimental diet has been given in Table I. The model of diet-induced
hepatic insulin resistance is well-recognized for creating both fasting hyperglycemia and hyperinsulinemia and is thus a reasonable model for T2D. The recommended adequate intake for VK1 is 1 µg/kg BW [18]; however, it is not known whether this adequate intake is sufficient to meet all the physiological needs, especially coagulation unrelated health outcome like diabetes. At present no known toxicity has been reported with higher doses of VK1 supplementation [2]. Thus, no tolerable upper intake limit has been set yet. Body weight was measured weekly and food intake was recorded daily. At the end of 16 weeks the animals were fasted overnight and then weighed and euthanized by exposure to isoflurane. Blood was collected via heart puncture with a 191/2 gauge needle into EDTA Vacutainer tubes. Plasma was isolated after centrifuging the blood in a 4°C centrifuge at 3000 rpm for 15 min. Liver tissues excised from the experimental animals were perfused with cold saline to remove leftover blood, weighed, and immediately stored at -80°C until further use.

2.5. Oral glucose tolerance test (OGTT) - After the mice were fasted for overnight, they were administered 50% glucose at a dose of 2 g/kg BW orally. Blood samples were collected from tail veins before (0 min) and at 15, 30, 60, and 120 min after glucose loading. Blood glucose level was measured by glucose-oxidase method using an Advantage Accu-Chek glucometer.

2.6. Measurement of basal glucose and insulin, glycosylated hemoglobin (GHb), insulin resistance, lipid profile, liver and kidney function tests, and secretion of pro-inflammatory cytokines in animals - Blood glucose levels, lipid profile (triglyceride and total cholesterol), and liver (aspartate aminotransferase and alanine aminotransferase) and kidney (creatinine) function tests were examined by using kits from Robonik Pvt. Ltd. (Mumbai, India). The levels of plasma insulin and pro-inflammatory cytokines [monocyte chemoattractant protein-1 (MCP-1) and interleukin 6 (IL-6)] were measured by ELISA kits from ALPCO (Salem, NH) and Sigma respectively. GHb was determined using glycohemoglobin kit from Teco Diagnostics (Anaheim, CA, USA). Insulin resistance was examined via homeostatic model assessment (HOMA) method using the formula as described earlier [(insulin (µU/mL) x glucose (mg/dL))/ 2430] [20]. All appropriate controls and standards as specified by each manufacturer’s kit were used.

2.7. Measurement of glucose-6-phosphatase (G6Pase) and glucokinase (GK) activities and levels glucose-6-phosphate (G6P) and NAD⁺/NADH in liver tissue - The enzymatic assay of G6Pase was performed following the method as mentioned earlier [21]. The activity was expressed as µmol min⁻¹ mg protein⁻¹. The activity of GK was determined following the method of Goward et al [22] and result was expressed as pmol min⁻¹ mg protein⁻¹. The level of glucose-6-phosphate and
NAD$^+$/NADH ratio were examined using the commercially available kits from Sigma.

2.8. Cell culture and treatment of hepatocytes with VK1 and high glucose (HG) - The FL83B murine hepatocyte cells, purchased from American Type Culture Collection (ATCC, Manassas, VA) were cultured and maintained in F12K complete medium. Cells were treated with high glucose (HG, 25 mM) with or without VK1. Different concentrations (1, 5, or 10 nM) of VK1 were supplemented for 2 h followed by HG exposure for the next 20 h. Mannitol was used as an osmolarity control. In the mannitol group, cells were exposed to 19.5 mM mannitol since the media contains 5.5 mM glucose. Doses of VK used in this study are in the physiological range [18].

2.9. Cell viability and glucose uptake assay with hepatocytes
The viability of treated cells was determined using the Alamar Blue reduction bioassay (Alamar Biosciences, Sacramento, CA). The glucose uptake assay was performed using 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (6-NBDG; Invitrogen), a fluorescent analogue of 2-deoxyglucose, following the method of Manna et al [23]. Briefly, after treatment, cells were incubated with serum-free low-glucose medium containing 6-NBDG (20 μM) for 30 min. After the incubation, cells were washed with PBS and then lysed with 70 μL PBS containing 1% TritonX-100 and kept at dark for 10 min. Then 30 μL DMSO was added in each sample, homogenized by pipetting up and down, and read the plate immediately using a microplate reader at an excitation/emission wave length of 466/540 nm. Results were expressed as percentage over control.

2.10. siRNA transient transfection studies – SIRT1, AMPK, and control siRNAs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Hepatocytes were transiently transfected with 100 nM siRNA complex using LipofectamineTM2000 transfection reagent (Invitrogen, Carlsbad, CA) following the method as described earlier [23,24].

2.11. Immunoblotting - For the immunoblotting studies, after treatment, tissues or cells were lysed in radio immune precipitation assay (RIPA) buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM EDTA, 10 mM NaF, and 1 mM NaVO$_4$). Lysates were cleared by centrifugation, and total protein concentrations were determined using a BCA assay kit (Pierce/Thermo Scientific, Rockford, IL). All samples contained approximately the same amount of protein (~20-30 μg) were used for immunoblotting with either anti-AMPK (1:1000) (#5831S), anti-phospho AMPK (1:1000) (Thr 172) (#2535S), anti-CPT1A (1:1000) (#ab128568), anti-GGCX (1:500) (#ab197982), anti-GLUT2 (1:1000) (#ab54460), anti-G6Pase
(1:1000) (#ab83690), anti-GK (1:1000) (#ab37796), anti-phosphorylated NF-κB(p65) (Ser 276) (1:1000) (#ab86299), anti-NF-κB (p65) (1:1000) (#sc109), anti-PI3K (p85α) (1:1000) (#4292S), anti-PTEN (1:1000) (#ab32199), anti-PPARα (1:1000) (#ab8934), anti-SIRT1 (1:1000) (#ab110304), or anti-VKOR (1:1000) (#ab176118) primary antibody and appropriate HRP conjugated secondary antibody (1:5000 dilution). The intensity of each immunoblotting band was measured using the histogram tool of Adobe Photoshop CS5.

2.12. Statistical analysis - Multiple linear regression analyses were used to determine whether dependent variable can be predicted from a linear combination of independent variables. Body weight was always used as an additional independent variable to determine regression and P-value. Y-axis parameter was used as a dependent, and x-axis and body weight were used as independent variables using Sigma Stat software (San Jose, CA, USA). Data from human, animal and cell culture studies were analysed statistically using one-way ANOVA with Sigma Stat statistical software (San Jose, CA, USA). When data passed a normality test, all groups were compared using the Student–Newman–Keuls method. A $p$ value of less than 0.05 was considered significant for a statistical test.

3. RESULTS
3.1 VK1, fasting glucose, and insulin resistance (HOMA-IR) levels in T2D patients and control subjects - Table II represents the similar distribution of age, male/female ratio, and BMI in both groups. Blood parameters, like fasting glucose, HbA1c, and HOMA-IR were significantly higher in T2D patients compared to those of control subjects. Plasma level of VK1 was significantly lower in T2D patients compared to those seen in control subjects. This difference was examined because VK is a lipid-soluble vitamin and it may be more appropriate to express its concentration after normalization with lipid [18]. All the studied subjects showed no signs of coagulation associated complication.

3.2 Relationship between circulating VK1, fasting glucose, and IR among T2D patients - Figures 1A-D show that among T2D patients there was a statistically significant inverse relationship between fasting glucose and blood levels of VK1, whether it is expressed as per volume of plasma (1A, 1B) or lipid level (1C, 1D). Similarly, Figures 2A-D illustrate that a significant negative correlation exist between blood levels of VK1 and those of IR. Although an inverse relationship was observed between VK1 and A1C, it was not significant ($r$=-0.221, $p$=0.346, data not given). This suggests that blood VK1 status has a positive effect on lowering fasting glucose.
and IR among T2D patient population. The regression analyses given here were determined while controlling for body weights using multiple linear regression analyses.

3.3. Effect of VK1 supplementation on OGTT, GHb, fasting glucose and insulin, IR, and lipid levels in T2D mice – To investigate the role of VK1 on glucose metabolism, first of all we have examined the effect of VK1 on glucose tolerance in overnight fasted normal and T2D mice by using OGTT assay (Figure 3). Results showed that VK1 supplementation in T2D mice caused a significant reduction in plasma glucose and AUC at a dose of 3 and 5 µg/kg BW compared to those seen in T2D and T2D+OO groups. The levels of body weight gain, fasting glucose and insulin, GHb, and IR were also significantly higher in T2D mice compared to normal (Table III). Supplementation with VK1 dose-dependently reduced the gain in body weight, basal glucose and insulin levels, percentage of GHb, and IR (HOMA) in T2D mice. Moreover, a significant increase in both triglyceride and total cholesterol has also been observed in T2D mice and that could be attenuated by the VK1 supplementation. OO supplementation did not cause any significant effect on body weight gain, fasting glucose, insulin, GHb, IR, and lipid profile compared to those seen in T2D mice. There was no significant difference in food intake among the groups. The blood levels of liver and kidney function tests did not differ significantly and all the studied animals showed no signs of coagulation associated complication (data not shown). This suggests that no negative side effects result from VK1 supplementation.

3.4. Effect of VK1 on the signaling pathway of hepatic glucose metabolism, lipid oxidation, and inflammation in T2D mice - The effects of VK1 supplementation on the levels of glucose-6-phosphate (G6P) and NAD+/NADH ratio, the activities of glucose-6-phosphatase (G6Pase) and glucokinase (GK), and the expression of different signaling molecules involved in hepatic glucose metabolism are shown in Figures 4 and 5. Results demonstrate that VK1 supplementation dose-dependently increased G6P, NAD+/NADH ratio, and GK activities and decreased the G6Pase activities in T2D mice compared to T2D and T2D+OO groups. Furthermore, VK1 supplementation also caused an increase in the protein expression of SIRT1, phospho AMPK, PI3K, and GK and a decrease in PTEN, GLUT2, and G6Pase in the liver tissue of T2D mice compared to those seen in T2D and T2D+OO groups.

The effects of VK1 on the secretion of pro-inflammatory cytokines and the protein expression of signaling molecules involved in hepatic lipid oxidation and inflammation have also been represented in Figures 6 and 7. Results show that HFD-feeding caused an increase in the protein expression of phospho NF-κB and the levels of MCP-1 and IL-6 and a decrease in PPARα and CPT1A protein expression in the liver tissue of T2D mice. Supplementation with olive oil did
not cause any effect on inflammation and lipid oxidation. However, VK1 supplementation significantly decreased the secretion of both MCP-1 and IL-6 and NF-κB phosphorylation and increased the protein expression of PPARα and CPT1A in the liver tissue of T2D mice. Treatment with VK1 did not cause any significant effect on hepatic glucose metabolism, lipid oxidation, and inflammation in Normal mice.

The enzymes, namely gamma glutamyl carboxylase (GGCX) and vitamin K epoxide reductase (VKOR) play an important role in maintaining the hepatic vitamin K status in our bodies. Measurement of the blood levels of uncarboxylated vitamin K dependent proteins, such as uncarboxylated matrix gla protein (ucMGP) is considered to be an indicator of circulating VK status. Figure 8 represents the hepatic protein expression of GGCX and VKOR and the plasma level of ucMGP in the experimental animals. Results show that there was no change in protein expression of both GGCX and VKOR in the liver tissue of all the animals. However, HFD-feeding caused an increase in the plasma levels of ucMGP compared to normal diet-fed mice suggesting a VK deficiency in T2D mice. Supplementation with VK1 dose dependently decreased the ucMGP level in the T2D mice compared to olive oil control group.

The outcome of the animal studies demonstrate that VK1 supplementation regulates the signaling pathway of glucose metabolism (SIRT1/AMPK/PI3K/PTEN/GLUT2/GK/G6Pase), lipid oxidation (PPARα/CPT1A), and inflammation (NF-κB) in the liver tissue and lowers insulin resistance, impaired glucose tolerance, basal glucose and insulin levels, GHb, lipid levels, and the secretion of pro-inflammatory cytokines in T2D mice.

3.5. Effect of VK1 on glucose uptake and its associated signaling pathways in both normal and SIRT1/AMPK-knockdown hepatocytes – In vitro studies has been carried out using murine hepatocyte cell culture model to dissect the molecular mechanism underlying the positive effect of VK1 on glucose uptake and its associated signaling pathways (Figure 9). Results show that VK1 supplementation dose dependently increased the glucose uptake and the protein expression of SIRT1, phospho AMPK, and PI3K and decreased GLUT2 in HG-treated cells. Interestingly, in SIRT1-knockdown cells, VK1 supplementation did not cause any increase in glucose uptake, AMPK phosphorylation, PI3K activation, and decrease in GLUT2 against HG exposure. However, in AMPK-knockdown cell, treatment with VK1 could increase SIRT1 but not upregulate glucose uptake and PI3K and decrease GLUT2 in HG-treated cell. The outcome of these knockdown studies suggest that SIRT1 mediated activation of AMPK signaling pathway plays an important role in regulating the positive effect of VK1 on glucose metabolism in HG-treated hepatocytes. Different treatments did not cause any change in cell viability level (data not given).
3.6. Effect of VK1 on lipid oxidation and inflammation in normal and SIRT1-knockdown hepatocytes – Figure 10 represent that effect of VK1 on the protein expression of signaling molecules involved in lipid oxidation (PPARα) and inflammation (NF-κB) and the secretion of pro-inflammatory cytokine (MCP-1) in normal and SIRT1-knockdown hepatocytes treated with high glucose. Supplementation with VK1 caused an increase in PPARα and a decrease in phospho NF-κB expression and MCP-1 secretion in HG-treated hepatocytes. However, in SIRT1-knockdown cells treatment VK1 did not increase PPARα and decrease phospho NF-κB and MCP-1 secretion against HG exposure, which suggest that the activation of SIRT1 signaling pathway positively regulates the beneficial effect of VK1 on lipid oxidation and inflammation in T2D.

4. DISCUSSION
Recent studies demonstrate a potentially significant role of dietary VK intake or VK supplementation in improving glycemic status and reducing the T2D risk factors and the prevalence of metabolic syndrome among normal subjects including both young and elderly volunteers [2]. Animal studies have also demonstrated the hypoglycemic action of VK supplementation in streptozotocin-induced diabetic rats [24-26]. However, the information on circulating VK level and its relationship with the glycemic status among T2D patients is lacking. In addition, the mechanism of action of VK on glucose metabolism is also not known.

The number of studies that have evaluated the circulating VK1 concentration in relation to chronic disease is relatively few compared to the studies that assessed the dietary VK1 intake and its health benefits. VK1 is the primary circulating form of VK and has been successfully measured in various population based and clinical-based studies worldwide to assess the circulating VK status [18]. This study for the first time demonstrates that plasma VK1 levels were significantly lower among T2D patients compared to age-matched normal subjects. In addition, the levels of fasting glucose, HbA1c, and HOMA-IR were also significantly higher in T2D patients compared to controls. Multiple linear regression analyses after adjustment of body weight showed that circulating levels of VK1 were significantly and inversely associated with fasting glucose and HOMA-IR among T2D patients. Earlier studies suggest that in circulation VK is transported on triglyceride-rich lipoproteins, with smaller fractions carried on HDL and LDL cholesterol [18]. Thus, it is appropriate to express the concentration of circulating VK1 after normalization with lipid and the same has also been argued for other lipid soluble vitamins [27,28]. Results demonstrate that even after normalization with total lipid, circulating VK1 is significantly and inversely correlated with fasting glucose and HOMA-IR among T2D populations. This suggests that boosting plasma VK1 may reduce the fasting glucose and insulin resistance in T2D patients. Using HFD-fed animal model of T2D the present study further demonstrates that supplementation with VK1 dose
dependently increased glucose tolerance, decreased body weight gain, fasting glucose and insulin, GHb, and HOMA-IR, and regulated lipid profile in T2D mice compared to olive oil control group. This study demonstrates that circulating VK1 has a positive effect on lowering fasting glucose and insulin resistance in T2D.

SIRT1 is a NAD$^+$ dependent histone/protein deacetylase that plays an important role in the regulation of energy metabolism, inflammation and mitochondrial function [29,30]. Recently, activation of SIRT1 has been implicated in the regulation of metabolic syndrome associated chronic diseases [29]. Pfluger and colleagues reported that overexpression of SIRT1 improves insulin sensitivity and glucose homeostasis via inhibiting hepatic gluconeogenesis in high fat diet-fed T2D mice [16]. Treatment with SIRT1 activator, SRT1720A decreased the hepatic gluconeogenesis in Zucker diabetic fatty rats [31]. Activation of AMPK has also been found to inhibit hepatic gluconeogenesis and lower glucose level [14,15]. Gluconeogenesis in the liver is regulated via the function of the enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase. This study demonstrates that VK1 supplementation dose-dependently increased the intracellular NAD$^+$/NADH ratio, SIRT1 protein expression, and AMPK phosphorylation and inhibited the enzyme activity and protein expression of G6Pase in the liver tissue of T2D mice thus positively influenced the hepatic insulin sensitivity in T2D.

The PI3K/PTEN pathway plays a central role in mediating insulin responses to the glucose transporters to regulate the equilibrium between intracellular and extracellular glucose concentration [32,33]. The present study shows that VK1 supplementation caused an increase in PI3K and a decrease in PTEN expression in the liver tissue of T2D mice. In liver, GLUT2 is the major isoform of glucose transporters, and GK is the key enzyme in the process of glucose phosphorylation [34]. This study demonstrates that treatment with VK1 at a dose of 3 and 5 µg/kg BW significantly downregulated GLUT2 and upregulated GK expression and activity and G6P level in the liver tissues of T2D mice compared to those seen in T2D+OO group suggesting a beneficial role of VK1 on glucose metabolism in T2D.

Besides lowering gluconeogenesis, SIRT1/AMPK signaling also regulates cellular lipid metabolism by stimulating the β-oxidation of fatty acids [35]. The enzyme, CPT1 catalyzes the rate limiting step of β-oxidation in the mitochondria [36], and the transcription of CPT1 is triggered by the transcription factor PPARα via binding to PPAR response elements [37]. This study shows that VK1 supplementation increased the protein expression of both PPARα and CPT1 in the liver tissue of T2D mice, which may play an important role in the improvement of lipid metabolism. The measurements of subclinical inflammation are also related to insulin resistance and predict the onset of T2D pathophysiology [38]. The activation of transcription factor NF-κB induces the secretion of various pro-inflammatory cytokines such as, TNF-α, MCP-1, and IL-6 [39]. This study shows that
VK1 supplementation dose dependently decreased the NF-κB phosphorylation and the secretion of both MCP-1 and IL-6 in T2D mice and thus inhibited the vascular inflammation in T2D.

*In vitro* studies have been carried out to validate the molecular mechanism underlying the positive effect of VK1 on hepatic glucose uptake and vascular inflammation. Supplementation with VK1 dose dependently increased glucose uptake and the protein expression of SIRT1, phospho AMPK, and PI3K and decreased GLUT2 in HG-treated hepatocytes. However, in SIRT1-knockdown cells, VK1 supplementation did not increase glucose uptake, AMPK phosphorylation, PI3K activation, and decrease GLUT2 against HG exposure. Moreover, in AMPK-knockdown cell, VK1 treatment increased SIRT1 but not upregulated glucose uptake and PI3K and decrease GLUT2 in HG-treated cell. These knockdown studies suggest that VK1-induced activation of SIRT1/AMPK/PI3K/GLUT2 signaling pathway plays an important role on hepatic glucose metabolism in T2D. Supplementation with VK1 also increased PPARα and decreased phospho NF-κB expression and MCP-1 secretion in HG-treated hepatocytes and that positive effect of VK1 was also inhibited in SIRT1-knockdown cells, which further suggests the role of SIRT1 in mediating the effect of VK1 on lipid oxidation and vascular inflammation in T2D.

In liver, VK is recycled between its oxidized and reduced form via the action of two enzymes, gamma glutamyl carboxylase (GGCX) and vitamin K epoxide reductase (VKOR) and thus maintain hepatic VK status in our bodies. The present study showed that there was no change in the protein expression of both GGCX and VKOR in the liver tissues of all the experimental animals. In addition, all the studied animals showed no signs of coagulation associated complication. This suggests that there is no clinical vitamin K deficiency among the experimental animals. Measurement of the blood levels of uncarboxylated vitamin K dependent proteins, such as uncarboxylated matrix gla protein (ucMGP) is considered to be an indicator of circulating VK status. Interestingly, ucMGP level was significantly higher in the plasma of T2D mice compared to Normal group, which suggests a sub-clinical VK deficiency in T2D. Supplementation with VK1 decreased the plasma levels of ucMGP in T2D mice.

In conclusion, this is the first report to demonstrate the novel observation that plasma VK1 levels were significantly lower among T2D patients and it is significantly and inversely associated with fasting glucose and HOMA-IR, which suggests a positive effect of VK1 on reducing fasting glucose and IR in T2D. Using HFD-fed T2D animal this study shows that supplementation with K1 regulates the SIRT1/AMPK/PI3K/PTEN/GLUT2/GK/G6Pase signaling cascade of hepatic glucose metabolism, PPARα/CPT1A pathway of lipid oxidation, and NF-κB–induced vascular inflammation in the liver tissue and lowers the body weight gain, impaired glucose tolerance, insulin resistance, fasting glucose and insulin, and the secretion of pro-inflammatory cytokines in T2D mice. Moreover, signal silencing studies with hepatocyte cell culture model also depicted the
role of SIRT1/AMPK pathway in mediating the positive effect of VK1 on hepatic glucose metabolism and vascular inflammation in T2D. Figure 5 demonstrates the proposed mechanism underlying the beneficial role of VK1 on glucose metabolism in T2D.

The discovery of this novel link between VK1, SIRT1/AMPK, and glucose metabolism will allow for better understanding of and care for the excess diabetes and cardiovascular disease associated with VK deficient population. At present the recommended adequate intake for VK1 is 1 µg/kg BW [40]; however, it is not known whether this adequate intake is sufficient to meet all the physiological needs, especially coagulation unrelated health outcome like diabetes. Moreover, the established threshold of plasma/serum VK1 that indicates insufficiency or deficiency is also missing. Interestingly, no known toxicity has been reported with higher doses of VK1 supplementation [2]. Thus, no tolerable upper intake limit has been set yet. Future clinical trials are needed to examine whether VK1 supplementation decreases glycemia and vascular inflammation in the T2D patient population.

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Conflict of interest
The authors have declared that no conflict of interest exists.

Author contributions
DO and PKB performed the clinical studies. AD and PM designed and performed the cell culture experiments, vitamin K analysis, biochemical analyses, and immunoblotting studies. SS, AS, PCS, and PM performed the animal experiment. PM compiled and analyzed the data. PM, AD, and JK wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.
5. References


FIGURE LEGENDS

FIGURE 1. The relationship between plasma levels of fasting glucose and vitamin K1 expressed per volume (nM) (1A and 1C) or per lipid [nM/μM triglycerides (TG) and cholesterol (TC)] (1B and 1D) in the plasma of T2D patients. Body weight was used as an independent variable to determine regression and p-value.

FIGURE 2. The relationship between insulin resistance (HOMA-IR) and VK1 expressed per volume (nM) (2A and 2C) or per lipid [nM/μM triglycerides (TG) and cholesterol (TC)] (2B and 2D) in the plasma of T2D patients. Body weight was used as an independent variable to determine regression and p-value.

FIGURE 3. Effect of VK1 on glucose tolerance (OGTT) (A) and AUC glucose at 120 min (B) in Normal and T2D animals. Normal mice were gavaged with VK1 at a dose of 5 μg/kg BW (VK5-1) and T2D-mice were gavaged with either olive oil (T2D+OO) or VK1 at a dose of 1 (T2D+VK1-1), 3 (T2D+VK1-3), or 5 (T2D+VK1-5) μg/kg BW daily for 8 wks. Data are expressed as mean±SE (n=6). **"*" denotes the significant difference from Normal (p*<0.05) and "#" denotes the significant difference from T2D (p<0.05).

FIGURE 4. Effect of VK1 supplementation on the protein expression of SIRT1 (A), phospho AMPK/AMPK (C), Glucose-6-phosphatase (G6Pase) (D), and NAD/NADH level (B) and enzyme activity of G6Pase (E) in Normal and T2D animals. The blots are representatives of three independent experiments. Normal mice were gavaged with VK1 at a dose of 5 μg/kg BW (VK5-1) and T2D-mice were gavaged with either olive oil (T2D+OO) or VK1 at a dose of 1 (T2D+VK1-1), 3 (T2D+VK1-3), or 5 (T2D+VK1-5) μg/kg BW daily for 8 wks, each group consisting of six mice. Data are expressed as mean±SE. **"*" denotes the significant difference from Normal (p*<0.05), "#" denotes the significant difference from T2D (p<0.05), and "@" denotes the significant difference from T2D (p<0.05).

FIGURE 5. Effect of VK1 supplementation on the protein expression of PI3K (A), PTEN (B), GLUT2 (C), Glucokinase (GK) (D) and GK enzyme activity (E) and Glucose 6 Phosphate (G6P) level (F) in Normal and T2D animals. The blots are representatives of three independent experiments. Normal mice were gavaged with VK1 at a dose of 5 μg/kg BW (VK5-1) and T2D-mice were gavaged with either olive oil (T2D+OO) or VK1 at a dose of 1 (T2D+VK1-1), 3 (T2D+VK1-3), or 5 (T2D+VK1-5) μg/kg BW daily for 8 wks, each group consisting of six mice. Data are expressed as mean±SE. **"*" denotes the significant difference from Normal (p*<0.05) and "#" denotes the significant difference from T2D (p<0.05).

FIGURE 6. Effect of VK1 supplementation on the protein expression of PPARα (A) and CPT1A (B) in Normal and T2D animals. The blots are representatives of three independent experiments. Normal mice were gavaged with VK1 at a dose of 5 μg/kg BW (VK5-1) and T2D-mice were gavaged with either olive oil (T2D+OO) or VK1 at a dose of 1 (T2D+VK1-1), 3 (T2D+VK1-3), or 5 (T2D+VK1-5) μg/kg BW daily for 8 wks, each group consisting of six mice. Data are expressed as mean±SE. **"*" denotes the significant difference from Normal (p*<0.05) and "#" denotes the significant difference from T2D (p<0.05).

FIGURE 7. Effect of VK1 supplementation on the protein expression of phospho NF-κB/ NF-κB (A) and levels of MCP-1 (B) and IL-6 (C) in Normal and T2D animals. The blots are representatives of three
independent experiments. Normal mice were gavaged with VK1 at a dose of 5 µg/kg BW (VK5-1) and T2D-mice were gavaged with either olive oil (T2D+OO) or VK1 at a dose of 1 (T2D+VK1-1), 3 (T2D+VK1-3), or 5 (T2D+VK1-5) µg/kg BW daily for 8 wks, each group consisting of six mice. Data are expressed as mean±SE. “*” denotes the significant difference from Normal (p<0.05) and “#” denotes the significant difference from T2D (p<0.05).

FIGURE 8. Effect of VK1 supplementation on the protein expression of GGCX (A), VKOR (B), and level of uncarboxylated matrix gla protein (ucMGP) (C) in Normal and T2D animals. The blots are representatives of three independent experiments. Normal mice were gavaged with VK1 at a dose of 5 µg/kg BW (VK5-1) and T2D-mice were gavaged with either olive oil (T2D+OO) or VK1 at a dose of 1 (T2D+VK1-1), 3 (T2D+VK1-3), or 5 (T2D+VK1-5) µg/kg BW daily for 8 wks, each group consisting of six mice. Data are expressed as mean±SE. “*” denotes the significant difference from Normal (p<0.05) and “#” denotes the significant difference from T2D (p<0.05).

FIGURE 9. Effect of VK1 on the signaling cascade of glucose uptake (SIRT1/AMPK/PI3K/GLUT2) either in normal (left panel) or SIRT1 (middle panel) and AMPK (right panel) knockdown hepatocytes exposed to high glucose (HG). Cells were pre-treated with VK1 (1, 5, or 10 nM) for 2 h followed by HG (25 mM) exposure for the next 20 h. Values are mean±SE (n=3). “*” denotes the significant difference from untreated group (p<0.05), “#” denotes the significant difference from HG alone-treated group (p<0.05), and “@” denotes the significant difference from untreated group transfected with either SIRT1 or AMPK siRNA (p<0.05).

FIGURE 10. Effect of VK1 on the protein expression of signaling molecules involved in lipid oxidation (PPARα) and inflammation (NF-κB) and the secretion of pro-inflammatory cytokine (MCP-1) either in normal (left panel) or SIRT1 (right panel) knockdown hepatocytes exposed to high glucose (HG). Cells were pre-treated with VK1 (1, 5, or 10 nM) for 2 h followed by HG (25 mM) exposure for the next 20 h. Values are mean±SE (n=3). “*” denotes the significant difference from untreated group (p<0.05), “#” denotes the significant difference from HG alone-treated group (p<0.05), and “@” denotes the significant difference from untreated group transfected with SIRT1 siRNA (p<0.05).

FIGURE 11. Schematic diagram of proposed mechanism underlying the beneficial role of vitamin K1 on glucose metabolism and vascular inflammation in T2D.
Table 1: Composition of experimental animal diet

<table>
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<tr>
<th>Selected nutrient information</th>
<th>Normal Diet</th>
<th>High Fat Diet (HFD)</th>
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<tbody>
<tr>
<td></td>
<td>gm  Kcal (%)</td>
<td>gm  Kcal (%)</td>
</tr>
<tr>
<td>Protein</td>
<td>19  20</td>
<td>24  20</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>67  70</td>
<td>41  35</td>
</tr>
<tr>
<td>Fat</td>
<td>4  10</td>
<td>24  45</td>
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<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm</th>
<th>Kcal</th>
<th>gm</th>
<th>Kcal</th>
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<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>800</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>12</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Corn Starch</td>
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<td>Maltodextrin</td>
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<tr>
<td>Fructose</td>
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<td>0</td>
<td>345.6</td>
<td>1382</td>
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<tr>
<td>Cellulose</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>0</td>
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<tr>
<td>Soybean Oil</td>
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<td>225</td>
<td>25</td>
<td>225</td>
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<td>Beef Tallow</td>
<td>20</td>
<td>180</td>
<td>177.5</td>
<td>1598</td>
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<tr>
<td>Mineral Mix</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
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<tr>
<td>Calcium Carbonate</td>
<td>5.5</td>
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<td>5.5</td>
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</tr>
<tr>
<td>Potassium Citrate</td>
<td>16.5</td>
<td>0</td>
<td>16.5</td>
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<tr>
<td>DiCalcium Phosphate</td>
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<tr>
<td>Vitamin Mix</td>
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<td>40</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
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<td>Total</td>
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<td>4057</td>
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<tr>
<td>Kcal/gm</td>
<td>3.8</td>
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Table II: Age, M/F ratio, BMI, fasting glucose, HbA1c, HOMA-IR, and vitamin K1 levels in healthy control and patients with type 2 diabetes (T2D). Values are mean±SE. “*” denotes the significant difference from healthy control ($p$*<0.05)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Control</th>
<th>T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52±1.4</td>
<td>54.6±1.6</td>
</tr>
<tr>
<td>Male/Female</td>
<td>12/8</td>
<td>15/10</td>
</tr>
<tr>
<td>Body weight (Kg)</td>
<td>22.71±0.77</td>
<td>54.72±2.22</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>22.71±0.77</td>
<td>21.53±0.92</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>72.13±8.53</td>
<td>173.08±8.95*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.63±0.09</td>
<td>7.4±0.25*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.8±0.08</td>
<td>1.72±0.11*</td>
</tr>
<tr>
<td>Vitamin K1 (nM)</td>
<td>5.28±1.76</td>
<td>1.93±0.50*</td>
</tr>
</tbody>
</table>
Table III. Body weight gain, basal plasma glucose and insulin, glycosylated hemoglobin (GHb), insulin resistance (HOMA-IR), triglyceride (TG), and total cholesterol (TC) levels in the experimental animals. Normal mice were gavaged with VK1 at a dose of 5 µg/kg BW (VK5-1) and T2D-mice were gavaged with either olive oil (T2D+OO) or VK1 at a dose of 1 (T2D+VK1-1), 3 (T2D+VK1-3), or 5 (T2D+VK1-5) µg/kg BW daily for 8 wks. Values are mean±SE. “*” denotes the significant difference from Normal (p*<0.05) and “#” denotes the significant difference from T2D (p#<0.05).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>VK1-5</th>
<th>T2D +OO</th>
<th>T2D +VK1-1</th>
<th>T2D +VK1-3</th>
<th>T2D +VK1-5</th>
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</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>1.55±0.29</td>
<td>1.6±0.25</td>
<td>3.36±0.24*</td>
<td>3.12±0.35</td>
<td>2.88±0.33</td>
<td>2.28±0.26#</td>
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<tr>
<td>Plasma Glucose (mM)</td>
<td>6.4±0.95</td>
<td>5.84±0.57</td>
<td>10.47±0.58*</td>
<td>9.32±1.11</td>
<td>7.95±0.55</td>
<td>6.64±1.04#</td>
</tr>
<tr>
<td>Plasma Insulin (ng/mL)</td>
<td>0.63±0.03</td>
<td>0.73±0.08</td>
<td>1.26±0.2*</td>
<td>1.16±0.04</td>
<td>1.07±0.07</td>
<td>0.95±0.09</td>
</tr>
<tr>
<td>GHb (%)</td>
<td>6.26±0.23</td>
<td>6.29±0.44</td>
<td>7.65±0.07*</td>
<td>7.42±0.14</td>
<td>7.21±0.27</td>
<td>6.3±0.54#</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.85±0.14</td>
<td>0.89±0.11</td>
<td>2.85±0.504*</td>
<td>2.39±0.511</td>
<td>1.91±0.417</td>
<td>1.23±0.215#</td>
</tr>
<tr>
<td>Triglyceride (mM)</td>
<td>0.58±0.012</td>
<td>0.65±0.03</td>
<td>1.66±0.052*</td>
<td>1.87±0.19</td>
<td>1.51±0.082</td>
<td>1.22±0.066#</td>
</tr>
<tr>
<td>Total Cholesterol (mM)</td>
<td>0.29±0.017</td>
<td>0.32±0.043</td>
<td>0.53±0.071*</td>
<td>0.46±0.054</td>
<td>0.4±0.016</td>
<td>0.34±0.049#</td>
</tr>
<tr>
<td>Food intake (g/mouse/day)</td>
<td>2.54±0.027</td>
<td>2.53±0.016</td>
<td>2.48±0.008</td>
<td>2.49±0.012</td>
<td>2.51±0.010</td>
<td>2.52±0.004</td>
</tr>
</tbody>
</table>
Figure 1

**Figure 1A**

- Scatter plot showing the correlation between Fasting Glucose (mg/dL) and Vitamin K1 (nM).
- Correlation coefficient: $r = -0.526, p = 0.01, n=21$ (T2D).

**Figure 1B**

- Scatter plot showing the correlation between Fasting Glucose (mg/dL) and Log (VK1) (nM)/ TG+TC (mM).
- Correlation coefficient: $r = -0.591, p = 0.004, n=21$ (T2D).

**Figure 1C**

- Scatter plot showing the correlation between Fasting Glucose (mg/dL) and Log (Vitamin K1) (nM).
- Correlation coefficient: $r = -0.591, p = 0.004, n=21$ (T2D).

**Figure 1D**

- Scatter plot showing the correlation between Fasting Glucose (mg/dL) and Log (VK1) (nM)/ TG+TC (mM).
- Correlation coefficient: $r = -0.627, p = 0.002, n=21$ (T2D).

Figure 2

**Figure 2A**

- Scatter plot showing the correlation between HOMA-IR and Vitamin K1 (nM).
- Correlation coefficient: $r = -0.445, p = 0.04, n=21$ (T2D).

**Figure 2B**

- Scatter plot showing the correlation between HOMA-IR and VK1 (nM)/ TG+TC (mM).
- Correlation coefficient: $r = -0.428, p = 0.05, n=21$ (T2D).

**Figure 2C**

- Scatter plot showing the correlation between HOMA-IR and Log (Vitamin K1) (nM).
- Correlation coefficient: $r = -0.457, p = 0.03, n=21$ (T2D).

**Figure 2D**

- Scatter plot showing the correlation between HOMA-IR and Log (VK1) (nM)/ TG+TC (mM).
- Correlation coefficient: $r = -0.477, p = 0.02, n=21$ (T2D).
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

A

GGCX

β-actin

0.4

Liver

GGCX/Actin (ratio)

0.3

0.2

0.1

0.0

B

VKOR

β-actin

0.4

Liver

VKOR/Actin (ratio)

0.3

0.2

0.1

0.0

C

Plasma

ueMGP (ng/mL)

Normal

VKI-5

T2D

T2D+OO

T2D+VKI-1

T2D+VKI-3

T2D+VKI-5

* #

0

1

2

3

4

5
Figure 9
Figure 10

[Diagram showing protein expression levels and MCP-1 levels under different conditions.

- Normal Hepatocytes
  - PPARα
  - β-actin
  - pNF-κB
  - NF-κB

- Control siRNA
  - PPARα
  - β-actin
  - pNF-κB
  - NF-κB

- SIRT1 siRNA
  - PPARα
  - β-actin
  - pNF-κB
  - NF-κB

HG (25 mM) and VK1 (nM) concentrations:
- HG (25 mM): - - + + +
- VK1 (nM): 10 1 5 10

MCP-1 levels (pg/mL):
- HG (25 mM) and VK1 (nM) concentrations:
  - HG (25 mM): - - 10 - 10
  - VK1 (nM): - 1 5 10

Statistical symbols:
- *: Significant difference
- #: No significant difference
- @: Significant difference
Figure 11