

Document title:

Approved Clinical Study Protocol

Study Number/Cyprus National Bioethics Committee Approval Number:

EEBK/ΕΠ/2019/50

Review Bioethics Committee approving the Program:

Biomedical and Clinical Research Committee A

Study Title:

Open-Label, Single-Arm Pilot Study of the Effect of Cholecalciferol on Mucin-1 Levels in Individuals With Autosomal Dominant Tubulo-Interstitial Kidney Disease Due to MUC1 Mutations (ADTKD-MUC1)

Acronym:

CyCHOLEMUC1

Study Type:

Interventional

Date generated/Date submitted:

14 May 2019

Date of last change/Date of final approval:

01 July 2019

Sponsors:

University of Cyprus

Broad Institute of MIT and Harvard

Purpose:

This document describes the approved clinical study protocol implemented to evaluate the effects of a single high oral dose of cholecalciferol on wild-type MUC1 and frameshifted MUC1 (MUC1-fs) protein levels in urinary extracellular vesicles (uEVs) from patients with ADTKD-MUC1. It details the study rationale, objectives, design, eligibility criteria, clinical and laboratory procedures, outcome measures, planned statistical analyses, safety monitoring, and ethical considerations.

CLINICAL STUDY PROTOCOL

Open Label Study of the Effect of Cholecalciferol on Mucin-1 Levels in Individuals with Autosomal Dominant Tubulo-Interstitial Kidney Disease due to *MUC1* Mutations (ADTKD-*MUC1*)

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Protocol Summary

Name of Sponsor	SIGMA Project – Broad Institute
Study Title	Open-Label, Single-Arm Pilot Study of the Effect of Cholecalciferol on Mucin-1 Levels in Individuals with Autosomal Dominant Tubulo-Interstitial Kidney Disease due to <i>MUC1</i> Mutations (ADTKD- <i>MUC1</i>)
Study Type	Pilot Study: Open Label, Single Arm
Investigators and Study Centers	University of Cyprus, Nicosia, Cyprus The Broad Institute of MIT and Harvard, Cambridge, MA, USA
Number of patients	45 individuals with ADTKD- <i>MUC1</i>
Objective	To determine if oral cholecalciferol administration results in a decline in mucin-1 levels of urinary extracellular vesicles in individuals with ADTKD- <i>MUC1</i> .
Endpoints	<p>Primary endpoint: Mucin-1 levels in urinary extracellular vesicles measured before and after cholecalciferol administration, by iMRM.</p> <p>Secondary endpoints: Plasma mucin-1 levels measured before and after cholecalciferol administration by A. ELISA, B. iMRM</p>
Main Criteria for Inclusion/Exclusion	<p><u>Inclusion Criteria:</u></p> <ol style="list-style-type: none"> 1. Age at least 18 years and <75 years of age. 2. Prior established genetic diagnosis of ADTKD-<i>MUC1</i>. Participants must be genotyped positive for the C-insertion mutation in <i>MUC1</i>. 3. Must have estimated glomerular filtration rate >30 ml/min/1.73 m² (according to the modified MDRD formula¹) due to ADTKD-<i>MUC1</i>. 4. No other serious health conditions 5. If female and of childbearing potential (premenopausal and not surgically sterile) must have a negative urine pregnancy test at the screening visit (Day -20) and be willing to use contraception from the time of the screening visit to 2 weeks following study supplement administration. Acceptable methods of birth control include abstinence, barrier methods, hormones, or an intra-uterine device. 6. Able to understand and comply with requirements of the entire study and to communicate with the study team. 7. The patient has not taken cholecalciferol, ergocalciferol or other Vitamin D related supplements in the month prior to study initiation. 8. Participants agree NOT to take ergocalciferol or cholecalciferol (except for <500 IU that are contained

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	<p>in a daily multi-vitamin supplement) for one month after receiving the one-time dose of cholecalciferol.</p> <p>9. Written informed consent using a document that has been approved by the Cyprus National Bioethics Committee.</p> <p><u>Exclusion Criteria:</u></p> <ol style="list-style-type: none"> 1. Manifestation of a major systemic illness, other than chronic kidney disease. Specifically, history of granulomatous disorders (sarcoidosis, tuberculosis), hyperparathyroidism or other disorders of calcium metabolism. 2. A history of nephrolithiasis. 3. A history of osteoporosis or osteopenia. 4. A fracture or a fall that did not occur during exercise within the last six months. 5. Intolerance or known allergic reaction to cholecalciferol or Vitamin D in general. 6. Estimated glomerular filtration rate < 30 ml/min/1.73 m² or s/p kidney transplantation 7. 25 hydroxy Vitamin D level > upper limit of normal. 8. Pregnancy. 9. Lactating. 10. Hepatic disease. 11. Receiving glucocorticoids.
Product, Dose, and Mode of Administration	<p>Cholecalciferol is a vitamin that is available both over-the-counter or by prescription.</p> <p>Patients will receive one oral dose of 200,000 IU (4 capsules of 50,000 IU each)</p>
Randomization	Open label, single arm without randomization
Duration of treatment	One oral dose
Statistics	The primary endpoint will be change from baseline (days-10 and 0) in wild type and mutant mucin-1 levels found in urinary extracellular vesicles, to days 3, 7, 10, 14 and 30 after supplement administration.

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1. BACKGROUND AND RATIONALE

1.1 ADTKD-*MUC1*: Clinical Characteristics:

Autosomal dominant tubulo-interstitial kidney disease due to *MUC1* mutations (ADTKD-*MUC1*) is caused by mutations in the *MUC1* gene encoding the protein mucin-1³. The most frequent mutation recorded is a C-insertion causing a frameshift and coding for a neo-peptide (fsMUC1). The primary characteristic of this condition is slowly progressive chronic kidney disease⁴. There is a variable rate of progression, with individuals reaching end-stage kidney disease (ESRD) requiring dialysis or kidney transplant between 21 and >70 years, with a median age of approximately 45 years⁵. The reason for this variation in age of onset of ESRD is unknown. Even though mucin-1 is expressed by many epithelial cells in the body (including sebaceous glands, the mucosal lining of the respiratory tract, the breasts, and the gastro-intestinal tract)³, there are no other associated symptoms besides the development of slowly progressive chronic kidney disease. In Pafos, Cyprus resides the largest cohort of ADTKD-*MUC1* patients in the world, as 1 in every 580 individuals have the C-insertion in *MUC1*. The first indication of the genomic region at chromosome 1q22 associated with the disease was identified by our group in Cyprus, while the Cypriot cohort has been well described and has been followed for a substantial amount of time⁶⁻⁹.

1.2 Mucin-1

Mucin-1 is a type I transmembrane heterodimer composed of two subunits¹⁰. The larger alpha subunit is extracellular and consists of the N-terminus (104 amino acids), a 20 amino acid variable number of tandem repeat (VNTR) segment, which repeats 25-125 times, and a C-terminus of 170 amino acids. The alpha subunit is non-covalently bound to the beta transmembrane unit, which consists of a small extracellular region of 58 amino acids, a transmembrane region of 28 amino acids, and a cytoplasmic tail of 72 amino acids. The VNTR units of the alpha subunit undergo extensive glycosylation, giving the protein its characteristic mucoid properties. Mucin-1 is expressed by sebaceous glands, ceruminous glands, epithelial cells lining the gastro-intestinal tract, respiratory tract, mammary glands, vaginal mucosa, and urinary bladder cells. In the kidney, mucin-1 is expressed in the thick ascending limb and distal convoluted tubules³. Mucin-1 is also expressed on leukocytes, T-cells, B-cells, monocytes, and dendritic cells¹⁰.

1.3 ADTKD-*MUC1*: Genetics:

Patients with ADTKD-*MUC1* have a mutation in one allele of the *MUC1* gene, while the other allele is normal. In all reported cases, the causative mutation occurs in one of the VNTR units of the *MUC1* gene and results in a single nucleotide frameshift that is self-terminating³. In over 90% of cases, the mutation involves a cytosine insertion in a heptanucleotide cytosine tract within the VNTR. At the protein level, the VNTR units prior to the insertion are normal, whereas after the insertion all repeats are mutated. The N-terminus and VNTR regions prior to the site of mutation will be translated normally, whereas the VNTR repeats after the frameshift have an 80% reduction in serine and threonine residues and the introduction of one cysteine and six basic amino acid residues per mutated VNTR unit³. The new MUC1 frameshift protein (fsMUC1) has a very high pI and is postulated to be toxic to renal tubular cells.

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Immunohistochemical studies have revealed that in individuals with ADTKD-MUC1, fsMUC1 is deposited within all cell types that normally produce MUC1¹¹. While deposition occurs within all cell types, clinical disease only occurs within the kidney³. It is believed that pathologic changes only occur in the kidney because the renal tubular epithelial cells have a very low cell turnover, and fsMUC1 retention over time gradually leads to apoptosis. Epithelial cells in the skin and gastrointestinal tract have a much higher cell turnover, and the slow gradual buildup of fsMUC1 does not occur prior to normal cell death.

We have postulated that the fsMUC1 protein is responsible for the pathogenesis that develops with ADTKD-MUC1. There are several lines of evidence that support this hypothesis: (a) There have been five mutations reported to date, and all of them have resulted in the creation of the same fsMUC1 protein¹¹. (b) No disease-causing mutations have been found that result in truncation or deletion of the *MUC1* gene. (c) The *MUC1* knockout mouse does not show any clinical abnormalities, including related to the kidney¹². (d) Laboratory studies have demonstrated the deposition of the abnormal fsMUC1 protein in the cytoplasm of tubular cells of affected individuals³. (e) Unpublished data by our collaborators (Kmoch S. – personal communication) have revealed that the fsMUC1 protein leads to accelerated apoptosis. Given the likelihood that fsMUC1 is responsible for the progression of kidney disease in ADTKD-MUC1, we postulate that by down-regulating the transcriptional rate of *MUC1* expression, both fsMUC1 and wtMUC1 production will decrease. Limited production of fsMUC1 would in turn benefit renal function rates.

1.4 Preliminary Data/Rationale: Cholecalciferol lowers mucin-1 (wt and fs) *in vitro*.

The Broad Institute of Harvard Medical School and the Massachusetts Institute of Technology developed a high content screen (HCS) using an immunofluorescence cell-based assay and screened the response of primary patient-derived cells to the Broad drug repurposing library, a set of 4500 compounds at different stages of preclinical and clinical development including all FDA-approved drugs and supplements¹³. For a primary screen, the Broad Institute used ADTKD-MUC1 patient-derived P cells, which express the mutant *MUC1* gene under its endogenous promoter, allowing for both transcriptional and post-transcriptional regulation. Cholecalciferol was identified as a compound that down-regulated production of both wtMUC1 and fsMUC1, as treated cells demonstrated effective reduction of both proteins in immunofluorescence experiments (Figure 1).

The promoter sequences of both the wild-type and mutant *MUC1* alleles in patients remain unaffected by the mutation. Therefore, we believe that both *MUC1* alleles may be regulated in the same way by cholecalciferol at the level of transcription thus affecting protein levels in cells. We are interested to study the effects of cholecalciferol on the expression of both wtMUC1 and fsMUC1 in ADTKD-MUC1 patients. Down-regulation of mucin-1 by cholecalciferol may have benefits in other conditions, such as pulmonary interstitial fibrosis¹⁴ and breast cancer¹⁵⁻¹⁸.

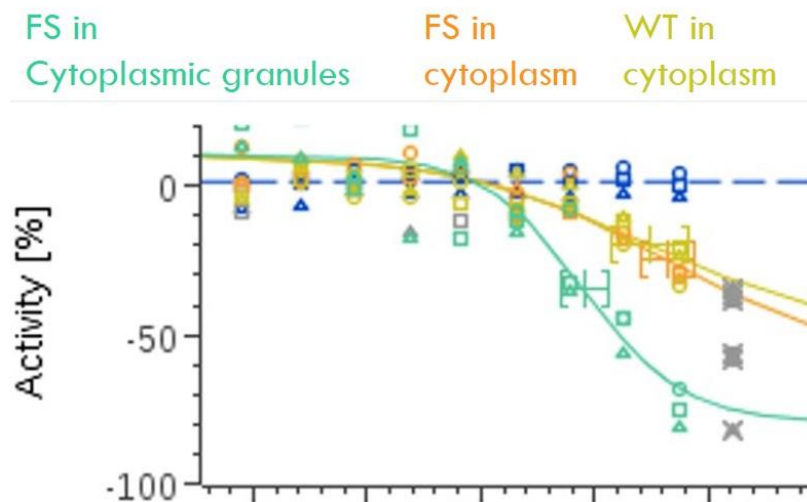


Figure 1: Response of patient-derived cells to Cholecalciferol treatment. Both wtMUC1 and fsMUC1 expression were reduced, while the specific localization of fsMUC1 in cytoplasmic granules was effectively diminished

1.5 Purpose of the study

As ADTKD-*MUC1* remains currently uncured, the purpose of this study is to identify a robust treatment for ADTKD-*MUC1*, by testing a single-high dose of Cholecalciferol in Cypriot patients. The primary read-out after administering this supplement will be the overall reduction of both wtMUC1 and fsMUC1 levels in urinary extracellular vesicles (EVs) of patients.

1.6 Hypothesis

A single dose of 200.000IU of cholecalciferol will result in a decrease of both wtMUC1 and fsMUC1 in urinary EVs from ADTKD-*MUC1* patients, 3 days after administration.

We believe that fsMUC1 is responsible for the pathogenesis of ADTKD-*MUC1*. In this context, by administering a single high dose of cholecalciferol in patients, the effect of fsMUC1 reduction observed *in vitro* will be amplified and easily recorded. Cholecalciferol or Vitamin D3 is a type of supplement used to treat Vitamin D deficiency. Hence, we sought to explore the effect of cholecalciferol in both wtMUC1 and fsMUC1 expression levels in the urine of Cypriot patients. The effect of ergocalciferol (Vitamin D2), that produced comparable results to cholecalciferol in *in vitro* experiments, will be investigated in parallel by our collaborators in the US (Prof. Anthony J. Bleyer).

2. DESCRIPTION OF STUDY SUPPLEMENT

Cholecalciferol is considered as a food supplement in Cyprus and the European Union. The European Food Safety Authority (EFSA) brought together a panel of experts to issue a scientific opinion on dietary

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reference values for vitamin D, which was adopted on the 29th of June 2016 (<https://doi.org/10.2903/j.efsa.2016.4547>). This panel considered that a serum concentration of 50nmol/L of 25(OH) Vitamin D is a suitable target value for all population groups, while the proposed daily intake of vitamin D is 15 µg/day. It is important to note that cholecalciferol supplements in Cyprus are usually administered after prescription and in many cases obtained over-the-counter.

Cholecalciferol (animal-derived) and ergocalciferol (plant-derived) are common medications used to treat hypovitaminosis D. Typical doses of cholecalciferol range from 400 µg orally each day to 50,000 units orally one time per month. A recommended starting dose of ergocalciferol in Vitamin D deficiency is 50,000 units orally once a week x 8 and then 2,000 - 10,000 units per day. For the treatment of hypoparathyroidism, 200,000 units of ergocalciferol are recommended daily. For Vitamin D resistant rickets, 500,000 units of oral vitamin D are recommended daily.

2.1 Vitamin D Metabolism

Both cholecalciferol and ergocalciferol are converted to calcidiols in the liver or stored in adipose tissue and are measured as circulating 25(OH)-Vitamin D. In the kidney, 25(OH)-Vitamin D is converted to 1,25(OH)-Vitamin D, which is considered as the active form and is highly regulated by parathyroid hormone and serum calcium levels (Figure 2).

2.2 Pharmacokinetic studies

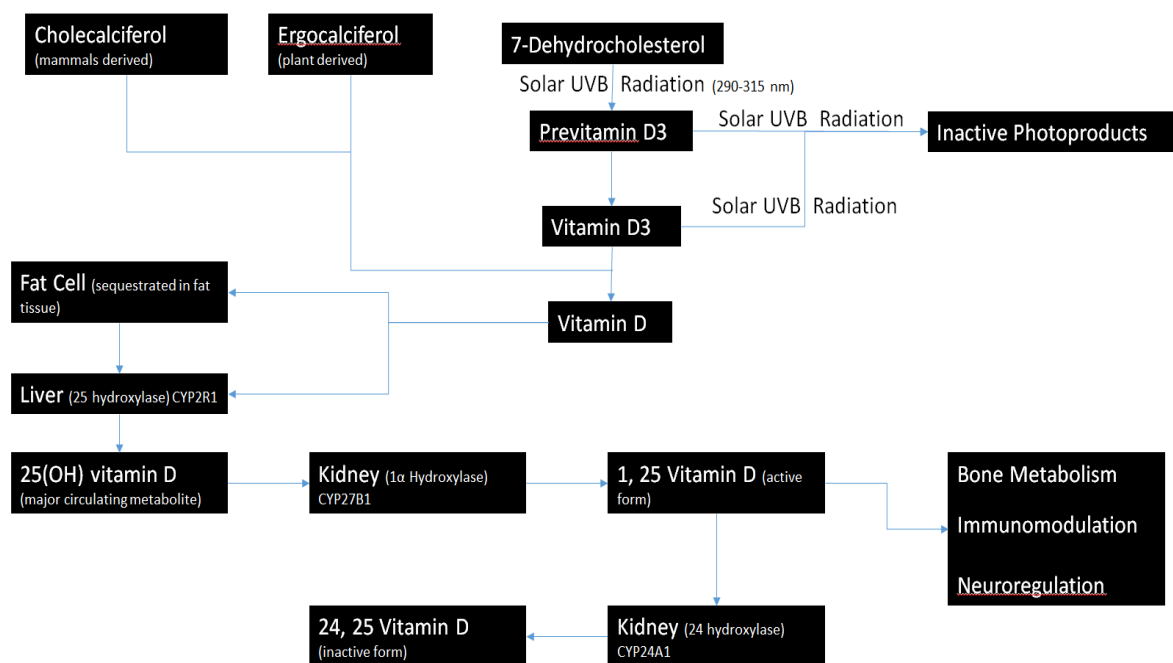


Figure 2: Vitamin D metabolism in humans.

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Administration of a large initial dose of cholecalciferol results in a rise in serum cholecalciferol levels over 1-2 days of administration. Very quickly over several days, cholecalciferol is redistributed within adipose tissues and cholecalciferol levels decrease. Serum 25(OH)-D levels rise modestly, peaking at 1-2 weeks. In a study by Heaney and colleagues, vitamin D₃ levels increased significantly after initial administration of 50,000 IU of cholecalciferol and ergocalciferol². A single large dose of cholecalciferol in Vitamin D (25-OH) deficient individuals resulted in increased serum calcidiol levels for up to 2 months after administration¹⁹. Following Vitamin D₃ serum presence after a single high dose, Heaney and colleagues demonstrated that cholecalciferol levels decreased significantly after initial administration (Figure 3)².

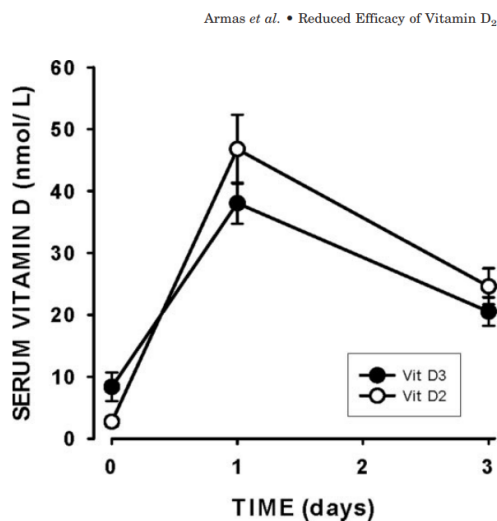


FIG. 1. Time course of serum concentrations of vitamin D after a single oral dose of 50,000 IU of cholecalciferol (vitamin D₃) or ergocalciferol (vitamin D₂) in healthy male subjects (n = 10 for each group). The error bars are 1 SEM. [Copyright Robert P. Heaney, 2004. Used with permission.]

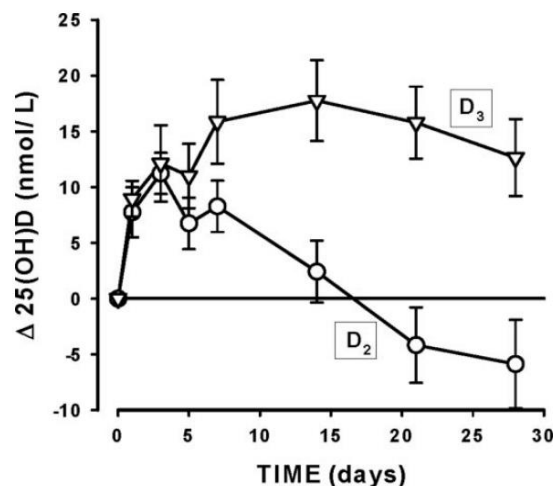


FIG. 2. Time course of the rise in serum 25(OH)D after a single oral dose of 50,000 IU of either cholecalciferol (vitamin D₃) or ergocalciferol (vitamin D₂) to two groups of 10 normal men each. Error bars are 1 SEM. The zero-change line incorporates a correction for the seasonal rise in 25(OH)D occurring at the time this study was performed. (To convert from nmol/liter to ng/ml, multiply by 0.4.) [Copyright Robert P. Heaney, 2004. Used with permission.]

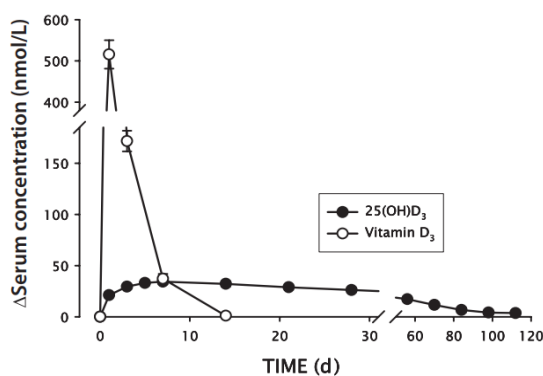


FIGURE 1. Time course of the rise from baseline of serum vitamin D₃ and 25-hydroxyvitamin D₃ for study A (23) after a single oral dose of 100 000 IU cholecalciferol to 30 healthy adults of both sexes. Baseline serum vitamin D₃ was 5.1 ± 1.3 nmol/L, and baseline 25-hydroxyvitamin D₃ was 67.6 ± 3.5 nmol/L.

Figure 3: In a study by Heaney and colleagues, vitamin D₃ levels decreased significantly after initial administration²

2.3 Large single-dose administration of cholecalciferol: Safety and adverse events

Large, single-dose oral Vitamin D supplementation has been used as an alternative to lower daily doses, with a goal of improving compliance. Single Vitamin D3 doses greater than or equal to 300,000 IU are most effective at improving Vitamin D status and suppressing parathyroid hormone levels. In a study by Kearns et al.,²⁰ 28 healthy adults were given a one-time dose of 250,000 IU of cholecalciferol. Within 5-10 days, the plasma 25 hydroxy Vitamin D level increased to 41.27 ng/ml (optimal Vitamin D is >30 ng/ml), while the effect faded after 90 days. There were no signs of hypervitaminosis D. Serum calcium was not significantly different between controls and the placebo group. Latham et al.²¹ randomized 243 frail older adults to 300,000 IU of calciferol or placebo and assessed quadriceps strength. There was no effect of the intervention, and no safety issues were noted. Yu et al.²² administered 200,000 units of Vitamin D3 at 27 weeks gestation. While Vitamin D levels increased, only a small percentage of women and babies became Vitamin D sufficient. There was no significant difference between treated and control patients in gestational age at delivery, birth weight, or adverse effects on mother or child. One woman developed significant proteinuria and was diagnosed with nephritic syndrome; therefore, her vitamin D treatment was stopped. One woman had vomited her tablets 15 min after taking her starting dose. In the daily treatment, three women changed their minds and three women reported nausea and vomiting. Overall, the average percentage of tablets taken was 60% in the daily vitamin D regime.

Cholecalciferol also is more effective than ergocalciferol at increasing vitamin D levels.²³⁻²⁶ In a systematic review by Kearns²⁷, 30 studies were identified that administered at least 100,000 units of vitamin D. Peak vitamin D levels were measured at 3 days. Ergocalciferol was found to be less effective, with doses in the range of 200,000 to 300,000 IU consistently failing to achieve 30 ng/ml concentrations of 25(OH)-D²⁷. Romagnoli et al.²⁸ administered 300,000 units of ergocalciferol or cholecalciferol to 32 elderly individuals. Ergocalciferol was significantly less effective at increasing serum 25(OH)-D levels, with cholecalciferol almost twice as potent. No adverse events were reported.

Based on the pharmacokinetics presented above, it was postulated that administration of a large single-dose of cholecalciferol would improve compliance, restore 25(OH)-D levels to the normal range, and possibly prevent fractures over the winter months. Other investigators believed that these larger doses would aid in the treatment of infection and improve the response of vasculature. For this reason, small and then larger studies of single-dose ergocalciferol or cholecalciferol were carried out. We identified 17 studies^{20,28-39} that administered at least 200,000 units of ergocalciferol or cholecalciferol as a single dose (Table 1) to 6502 participants. These studies showed the following: (1) Little change on serum calcium, phosphorus, and PTH levels. Most studies did not measure these laboratory parameters. (2) Increases in 25 OH-D levels that were modest and were not close to toxic levels of 25 OH-D. (3) An increased risk of fractures in elderly participants given doses of at least 500,000 IU of cholecalciferol annually for 3-5 years.

In a very high dose study of individuals who were not pre-screened for 25(OH)-D levels, a dose of 500,000 IU of cholecalciferol was given annually for 3-5 years³⁵. The baseline 25(OH)-D level was 49 nmol/L (interquartile range 40-63; normal lower limit, > 50 nmol/L), while the median level was slightly more than

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120 nmol/L at one month, with 24% > 150 nmol/L. There was no evidence of vitamin D toxicity. Reportedly, Vitamin D doses >500,000 units have been associated with adverse events^{20,27}.

The most important study in terms of assessing safety was carried out by Sanders et al.³⁵ In a randomized prospective clinical trial that was published in JAMA, 2256 women >70 years of age, considered to be at high risk for fracture were randomly assigned to placebo or 500,000 IU of cholecalciferol annually for three to five years. Participants tolerated drug administration well. Adverse events were similar between the two groups, except for an increased fall and fracture risk in the cholecalciferol group, with a fall rate of 83.4 per 100 person-years in the cholecalciferol group and 72.7 per 100 person-years in the placebo group (relative risk 1.15 (1.02-1.3, p=0.03)). The fracture rate of the cholecalciferol group was 4.9 per 100 person-years vs. 3.9 in the placebo group (relative risk 1.26 (95% CI 1.00-1.59, p=0.047). In a sub-study from this trial of 75 participants receiving cholecalciferol, the serum 25(OH)-D level increased from a median baseline of 49 nmol/L to 120 nmol/L at 1 month, with a decline thereafter.

2.3.1 Summary of adverse events

The previously described studies show an increased fracture rate of borderline statistical significance in elderly women >70 years of age receiving 500,000 IU of oral cholecalciferol. No significant adverse events were noted in other studies. We will be using a dose of 200,000 units of cholecalciferol orally, substantially lower than the doses in the fracture studies and most of the other studies.

2.3.2 Vitamin D toxicity

Jones reviewed the literature on Vitamin D toxicity⁴⁰. Vitamin D toxicity results in hypercalcemia, with other signs and symptoms secondary to the hypercalcemia. Data suggested that “the biomarker plasma 25(OH)D concentration must rise above 750 nmol/L to produce vitamin D toxicity, the more prudent upper limit of 250 nmol/L might be retained to ensure a wide safety margin.”⁴⁰. In a review of cases of chronic overexposure to vitamin D through fortified milk and peanut oil, the author concluded “hypercalcemia only results when 25(OH)D3 concentrations have consistently been above 375-500 ng/ml”. In a review of vitamin D levels over a 16-year period at the University of Iowa, the lowest level of 25(OH)D where hypercalcemia and vitamin D toxicity occurred was at a level of 194 ng/ml⁴¹. This level was not reached in participants receiving 500,000 IU of oral cholecalciferol, significantly higher than our dose of 200,000 IU of oral cholecalciferol.

Based on the above literature review we concluded that our projected single dose of 200,000 IU is a safe dose for the purpose of the proposed study.

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Table 1: Adverse events from single dose cholecalciferol or ergocalciferol.

Study	n	Ergocalciferol or cholecalciferol	Dose	Route	Adverse events
Grossmann, et al. ²⁹	15	Cholecalciferol	250,000	Oral	None
Witham, et al. ³⁰	20	Cholecalciferol	200,000	Oral	Asymptomatic bradycardia (1)
Premaor, et al. ³¹	14	Cholecalciferol	300,000	Oral	Gastric intolerance (2)
Sakalli, et al. ³²	30	Cholecalciferol	300,000	IM	None reported
	30	Cholecalciferol	300,000	Oral	None reported
Stoll, et al. ³³	141	Cholecalciferol	300,000	Oral	none reported
Bacon, et al. ³⁴	19	Cholecalciferol	500,000	Oral	These were hospitalized patients. 10 withdrew for poor health, 5 died during the study. The authors do not state which groups these were in and do not mention adverse effects.
	22	Cholecalciferol	500,000+ 50,000 monthly	Oral	
Sanders, et al. ³⁵	1131	Cholecalciferol	500,000	Oral	Vit D group with more falls RR 1.15, p=0.03. The fracture rate of the cholecalciferol group was 4.9 per 100 person-years vs. 3.9 in the placebo group (relative risk 1.26 (95% CI 1.00-1.59, p=0.047). Similar number of participants in each group reported at least 1 adverse event (19.7 Vit D group and 17.8% placebo). Cardiovascular events 1.5% Vit D vs. 1.2% placebo (NS). 0.6% in vitamin D group developed cancer vs 0.9% in placebo. Serious adverse events (hospitalization or death) were 244 individuals vitamin D and 207 placebo (p=0.06). None of the serious adverse events were considered related to study medicine. 40 Vit D deaths vs 47 deaths placebo
Smith, et al. ³⁶	4727	Ergocalciferol	300,000	IM	There were 279 non-vertebral fractures in the placebo group and 306 in the vitamin D group, p=0.29. For hips there were 44 in the placebo group and 66 in the vitamin D group (1.49, 0.04). No increase in falls. No adverse events reported.
Amrein, et al. ³⁷	12	Cholecalciferol	540,000	Oral	No sig differences
Rossini, et al. ³⁸	12	Cholecalciferol	600,000	Oral	None noted
Tellioglu, et al. ³⁹	34	Cholecalciferol	600,000	IM	4 hypercalciuria
	32	Cholecalciferol	600,000	Oral	2 hypercalciuria
Romagnoli, et al. ²⁸	8	Cholecalciferol	300,000	Oral	None reported
	8	Cholecalciferol	300,000	IM	
	8	Ergocalciferol	300,000	Oral	
	8	Ergocalciferol	300,000	Oral	
Latham, et al. ²¹	121	Ergocalciferol	300,000	Oral	No increase in falls (157 vs. 156). No other adverse events noted
Yu, et al. ²²	60	Ergocalciferol	200,000	Oral	No difference in gestational age at delivery, birth weight. One woman vomited high dose
Leventis and Kiely ⁴²	50	Ergocalciferol	300,000	IM	None reported

3. STUDY OBJECTIVES

3.1 Primary objective

The primary objective of this study is to determine if a one-time oral administration of 200,000 units of cholecalciferol results in a statistically significant decrease in urinary EVs mucin-1 (fsMUC1 or wtMUC1) levels at days 3 and 7 after supplement administration in 45 patients with ADTKD-*MUC1*, compared to baseline levels of urine samples collected at day-10 and day 0 and samples and protein levels during the recovery period at days 10, 14 and 30 post administration.

3.2 Secondary objectives

1. To determine if 200,000 units of cholecalciferol result in a statistically significant decrease in wtMUC1 and fsMUC1 levels found in plasma EVs measured by iMRM proteomics at days 7 and 14, compared to baseline levels at day 0.
2. To determine if 200,000 units of cholecalciferol result in a statistically significant decrease in serum wtMUC1 levels measured by ELISA at days 7 and 14, compared to baseline levels at day 0.

3.3 Exploratory Objective

To collect and store plasma and urine for future exploratory research into plasma/urine biomarkers related to ADTKD disease or that may influence the response (ie safety, tolerability, and efficacy) of cholecalciferol. These data will not be part of this study report.

4. INVESTIGATIONAL PLAN

This is a pilot, open-labelled, single arm study to determine if oral administration of a single dose of cholecalciferol lowers mucin-1 levels in urinary EVs. The study population will include 45 individuals with ADTKD-*MUC1*. This study will be conducted at, and coordinated by, the University of Cyprus in collaboration with Dr Christoforos Stavrou at Evangelismos Hospital in Pafos. The research teams/individuals joining forces in this project are the following:

1. Project Coordinator: Prof. Constantinos Deltas, Molecular Medicine Research Center, University of Cyprus, Nicosia, Cyprus. The project coordinator is responsible for monitoring and supervising the project and provide safety measures to preserve data and sample protection through the biobank facility at the Molecular Medicine Research Center, University of Cyprus. At the biobank premises, a total of two research assistants will facilitate activities pertaining to this trial.
2. Clinical Coordinator: Dr Christoforos Stavrou, Evangelismos Private Hospital, Pafos, Cyprus. The clinical coordinator has the responsibility to execute the clinical trial protocol and work with patients to successfully complete all tasks set. He will contact patients and explain the protocol procedures, record all events related to the study including adverse effects and perform all clinical activities in close communication with the project and biobank coordinators. Through his professional indemnity insurance, the clinical coordinator will undertake claims filed by participating patients in response to the administered supplement.
3. Biobanking Coordinator: Dr Gregoris Papagregoriou, Molecular Medicine Research Center, University of Cyprus, Nicosia, Cyprus. The biobanking coordinator is responsible to monitor and supervise all activities pertaining to correct sample storage in the biobank, extraction of biological materials, timely preparation and processing of samples for the primary and secondary endpoints of the study, distribution of samples to collaborators and management of data to track the response of patients to the administered supplement.
4. External Collaborator: Dr Anna Greka, The Broad Institute of MIT and Harvard, Cambridge, MA, United States of America. The external collaborator will monitor and supervise the biomarker analyses to meet the primary and secondary endpoints set for the study at the Broad Institute and will be in close collaboration with the Cypriot group as an experienced scientist and clinician to offer guidance for the successful completion of the study.

Benefits resulting from the study: At the moment, there is a lack of a robust and specific treatment for the vast majority of renal diseases, including ADTKD-*MUC1*. For this purpose, patients have to manage other factors to control the deterioration of renal function, such as the control of blood pressure, avoid specific medications, reduce the intake of proteins etc. Identifying a robust treatment for ADTKD-*MUC1* will help patients increase the chances of not developing End-Stage Renal Disease, have a good quality of life and increase life expectancy. The largest cohort of ADTKD-*MUC1* patients resides in Cyprus and most patients originate or live in the Pafos region. As ADTKD-*MUC1* lacks precise pathognomonic criteria, confident diagnosis is challenging; hence, it is inevitable to assume that actual patient numbers at a worldwide scale are much higher.

Methodology: ADTKD-*MUC1* is considered a rare disease and patients are quite limited. In Pafos, Cyprus resides the largest cohort of patients with this disease in the world. We have invited patients to express their interest in participating at a prospective clinical trial in 2018. After this, 52 mutation carriers expressed interest and have been already enrolled and are under follow-up. In the past year, we performed four quarterly sample collections (urine, serum and plasma) paired with biochemical and clinical data from potential participants. Hence, patients are already pre-screened and ready to take part in this trial. The study commences 20 days prior to supplement administration, when patients are invited to sign consent forms and a pregnancy test to be performed in women of child-bearing age. At Day 0, patients will be administered 200,000 units of cholecalciferol. Urine samples will be collected on days -10, 0, 3, 7, 10, 14 and 30. Blood, plasma and serum will be collected on days 0, 7 and 14.

4.1 Study population

4.1.1 General considerations

We performed clinical assessment and invited 52 individuals to participate in this study. We aim at recruiting a total of 45 patients living in Pafos, Limassol and Nicosia. Invited individuals are members of extended families that have been followed by our group for more than 20 years and share a common haplotype that flanks *MUC1*. This fact suggests that these families originated from a common founder ancestor. Although related, mutation carriers in Cyprus have a highly variable disease progression; even siblings might manifest the disease with different progression rates. Hence, our group is more homogeneous compared to a random selection of patients. Their genetic background is more similar, while they have similar lifestyles. Moreover, the small size of Cyprus makes patients more accessible and sample logistics and processing faster and more reliable.

4.1.2 Recruitment – Inclusion Criteria

1. Ages between 18-75 years
2. Prior established genetic diagnosis of ADTKD-*MUC1*. Participants must be genotyped positive for the C-insertion mutation in *MUC1*.
3. Must have estimated glomerular filtration rate >30 ml/min/1.73 m² (according to the modified MDRD formula¹) due to ADTKD-*MUC1*.
4. No other serious health conditions

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5. If female and of childbearing potential (premenopausal and not surgically sterile) must have a negative urine pregnancy test at the screening visit (Day -20) and be willing to use contraception from the time of the screening visit to 2 weeks following study supplement administration. Acceptable methods of birth control include abstinence, barrier methods, hormones, or an intra-uterine device.
6. Able to understand and comply with requirements of the entire study and to communicate with the study team.
7. The patient has not taken cholecalciferol, ergocalciferol or other Vitamin D related supplements in the month prior to study initiation.
8. Participants agree NOT to take ergocalciferol or cholecalciferol (except for <500 IU that is contained in a daily multi-vitamin) for one month after receiving the one-time dose of cholecalciferol.
9. Written informed consent using a document that has been approved by the Cyprus National Bioethics Committee.

4.1.3 Recruitment - Exclusion Criteria

1. Manifestation of a major systemic illness, other than chronic kidney disease. Specifically, history of granulomatous disorders (sarcoidosis, tuberculosis), hyperparathyroidism or other disorders of calcium metabolism.
2. A history of nephrolithiasis.
3. A history of osteoporosis or osteopenia.
4. A fracture or a fall that did not occur during exercise within the last six months.
5. Intolerance or known allergic reaction to cholecalciferol or Vitamin D in general.
6. Estimated glomerular filtration rate < 30 ml/min/1.73 m² or s/p kidney transplantation
7. 25 hydroxy Vitamin D level > upper limit of normal.
8. Pregnancy.
9. Lactating.
10. Hepatic disease.
11. Receiving glucocorticoids.

4.2 Concurrent medications

Concomitant medications have already been reviewed at the preparatory phase but will be additionally recorded and reviewed at screening (Visit 1, Day -20).

4.3 Excluded medications

The participant cannot be receiving > 1000 mcg oral cholecalciferol. The participant cannot be receiving ergocalciferol, calcitriol, or other vitamin D analogs. The participant cannot be receiving any medications for the treatment of osteoporosis. Participants must refrain from taking cholecalciferol or ergocalciferol except for doses <500 IU included in daily vitamins, for one month after Day 0.

5. METHODOLOGY

The assessments to be performed during the study are outlined below. This program will have a total duration of 18 months. The scheduled presented below, will correspond to the first 4 months of the project, while the remaining 14 months will be used for sample and data analysis. The day a patient receives study supplement is considered as “Day 0”. See Table 2Table 2 for an outline.

4.1 Study Schedule

4.1.1 Screening: 1st appointment (Not longer than 20 days prior to Day 0: Day-20): Patients will be contacted by telephone or in person. Study objectives, protocol, risks, benefits, and alternatives to participation will be discussed. If patients are interested, they will be mailed/emailed/given a description of the study and a consent form.

4.1.2 Baseline Collection: 2nd appointment (Not longer than 10 days prior to Day 0: Day-10): All patients that wish to participate in the trial will sign consent forms approved by the Cyprus National Bioethics Committee before any protocol-specific screening procedures are conducted. After informed consent is obtained, the following will occur:

Clinical activities

1. Updates in medical history records.
2. Collection of concomitant medication information.
3. Urine pregnancy test

Biobanking and biomarker activities

1. Collection of 120ml Urine.
2. Analysis of wtMUC1 and fsMUC1 proteins expressed in urinary EVs

4.1.3 Supplement administration / Day 0

Clinical activities

1. Sample collection for biobanking and biomarker activities.
2. Biochemical and hematological tests.

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3. Administration of 200,000 units of cholecalciferol (4 capsules of 50,000 UI Cholecalciferol).

Biobanking and biomarker activities

1. Collection of 120ml Urine and five vacutainer tubes of 4.5ml Blood in EDTA, 3.5ml Serum and 10ml Serum in SSTII and 2x4.5ml of Plasma in 9NC tubes, prior to supplement administration.
2. Analysis of wtMUC1 and fsMUC1 proteins expressed in urinary EVs.

4.1.4 Response tracking / Day 3

Clinical activities

1. Adverse events reviewed
2. Sample collection for biobanking and biomarker activities.

Biobanking and biomarker activities

1. Collection of 120ml Urine
2. Analysis of wtMUC1 and fsMUC1 proteins expressed in urinary EVs.

4.1.5 Response tracking / Day 7

Clinical activities

1. Adverse events reviewed
2. Sample collection for biobanking and biomarker activities.
3. Biochemical and hematological tests.

Biobanking and biomarker activities

1. Collection of 120ml Urine and five vacutainer tubes of 4.5ml Blood in EDTA, 3.5ml Serum and 10ml Serum in SSTII and 2x4.5ml of Plasma in 9NC tubes.
2. Analysis of wtMUC1 and fsMUC1 proteins expressed in urinary EVs.

4.1.6 Response tracking / Day 10

Clinical activities

1. Adverse events reviewed
2. Sample collection for biobanking and biomarker activities.

Biobanking and biomarker activities

1. Collection of 120ml Urine

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2. Analysis of wtMUC1 and fsMUC1 proteins expressed in urinary EVs.

4.1.7 Response tracking / Day 14

Clinical activities

1. Adverse events reviewed
2. Sample collection for biobanking and biomarker activities.
3. Biochemical and hematological tests.

Biobanking and biomarker activities

1. Collection of 120ml Urine and five vacutainer tubes of 4.5ml Blood in EDTA, 3.5ml Serum and 10ml Serum in SSTII and 2x4.5ml of Plasma in 9NC tubes.
2. Analysis of wtMUC1 and fsMUC1 proteins expressed in urinary EVs.

4.1.8 Observational phase / Day 30

Clinical activities

1. Adverse events reviewed
2. Sample collection for biobanking and biomarker activities.

Biobanking and biomarker activities

1. Collection of 120ml Urine
2. Analysis of wtMUC1 and fsMUC1 proteins expressed in urinary EVs.

4.2 Sample collections, processing and clinical tests

4.2.1 Urine

Each urine sample will be transferred to the lab within 3 hours after collection. Prior to processing, a urine dipstick will be used to collect information on urine density and the presence of protein, albumin and hemoglobin. Urines will then be spun, aliquoted and stored in -80°C freezers at the MMRC Biobank facility. From every urine collection, extracellular vesicles will be isolated and lysed in an appropriate buffer and both wtMUC1 and fsMUC1 will be analyzed by iMRM proteomics.

4.2.2 Blood and serum samples submitted for clinical testing

Blood and serum samples collected at specific time-points (Day 0, 7 and 14) will be transferred to a clinical laboratory upon collection. Each sample will be de-identified by using a unique code name. The following tests will be performed:

1. Full blood count
2. Serum Urea, Serum Creatinine, Serum Uric Acid and Serum Alkaline Phosphatase

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3. Serum 25-OH Vitamin D
4. Serum Calcium, Phosphate, Magnesium, Potassium and Sodium
5. Serum PTH

Clinical laboratory values that are outside the normal limits are considered abnormal. Abnormal clinical laboratory values will be noted as clinically significant or non-clinically significant by Dr Christoforos Stavrou. Non-clinically significant is defined as those abnormalities unlikely to indicate a significant medical condition or are consistent with the participant's underlying medical condition and do not warrant any further investigation or treatment. All new clinically significant abnormalities not present at baseline will be reported as adverse events. Values obtained will be compared to previously recorded clinical laboratory measurements obtained at the preparatory phase already performed during 2018.

4.2.3 Plasma and serum samples submitted for biobanking and biomarker assessment

Plasma and serum samples will be stored at 4°C upon collection and will be transferred to the MMRC biobank for processing within 3 hours after collection. Blood will be let to coagulate, and vacutainer tubes will be spun, and samples will be aliquoted and stored in -80°C freezers at the MMRC Biobank. Plasma samples will be analyzed by ELISA to measure wtMUC1 expression. Moreover, plasma samples will be processed in further to isolate proteins in extracellular vesicles. EV protein preps will be analyzed by iMRM proteomics to define fsMUC1 and wtMUC1 expression.

Table 2: Study schedule. The preliminary phase (pre-screening of available patients) was performed during 2018.

Study Period	Preliminary phase	Screening / Baseline		On Study	Response Tracking				Observation
Timing (Day)	During 2018	-20	-10	0	3	7	10	14	30
Procedure ▼ Visit ►		1	2	3	4	5	6	7	8
Meet patients and inform them	X	X							
Informed Consent Form		X							
Inclusion/Exclusion Criteria	X	X							
Medications	X	X							
Medical History	X	X							
Treatment	X	X							
Urine Pregnancy Test ¹		X							
Administration of 200.000IU Cholecalciferol				X					
Collection of urine samples			X	X	X	X	X	X	X
Measurement of urinary mucin-1 in EVs			X	X	X	X	X	X	X
Collection of blood, serum & plasma				X		X		X	
Comprehensive metabolic panel ²				X		X		X	
Plasma 25-OH Vitamin D				X		X		X	
Plasma mucin-1 level				X		X		X	
Full Blood Count				X		X		X	
Review of adverse events					X	X	X	X	X

¹Urine pregnancy test is for women of childbearing potential who are not surgically sterile.

²Comprehensive metabolic panel includes the following safety labs: Serum creatinine, urea, uric acid, alkaline phosphatase, PTH, calcium, phosphate, sodium, potassium, magnesium.

4.2.4 Pregnancy Evaluation

For females of childbearing potential who are not surgically sterile, urine pregnancy tests will be performed at Day -20 with a dipstick pregnancy test by the study team. A positive pregnancy test would make the participant ineligible according to Inclusion Criteria.

6. OUTCOMES/ENDPOINTS

6.1 Primary endpoint

Measurement of mucin-1 expressed in urinary EVs, including mutant fsMUC1: Both wtMUC1 and fsMUC1 will be identified and quantified using urine specimens. MUC1 is found in urinary extracellular vesicles⁴³. Through collaborative efforts between the team in Cyprus and the Broad Institute, we have developed a robust way to detect both wtMUC1 and fsMUC1 specifically in urinary EVs of patients with the disease, using immuno-multiple reaction monitoring mass spectrometry iMRM-MS implemented by the use of stable isotope standards and capture by anti-peptide antibodies (SISCAPA)⁴⁴.

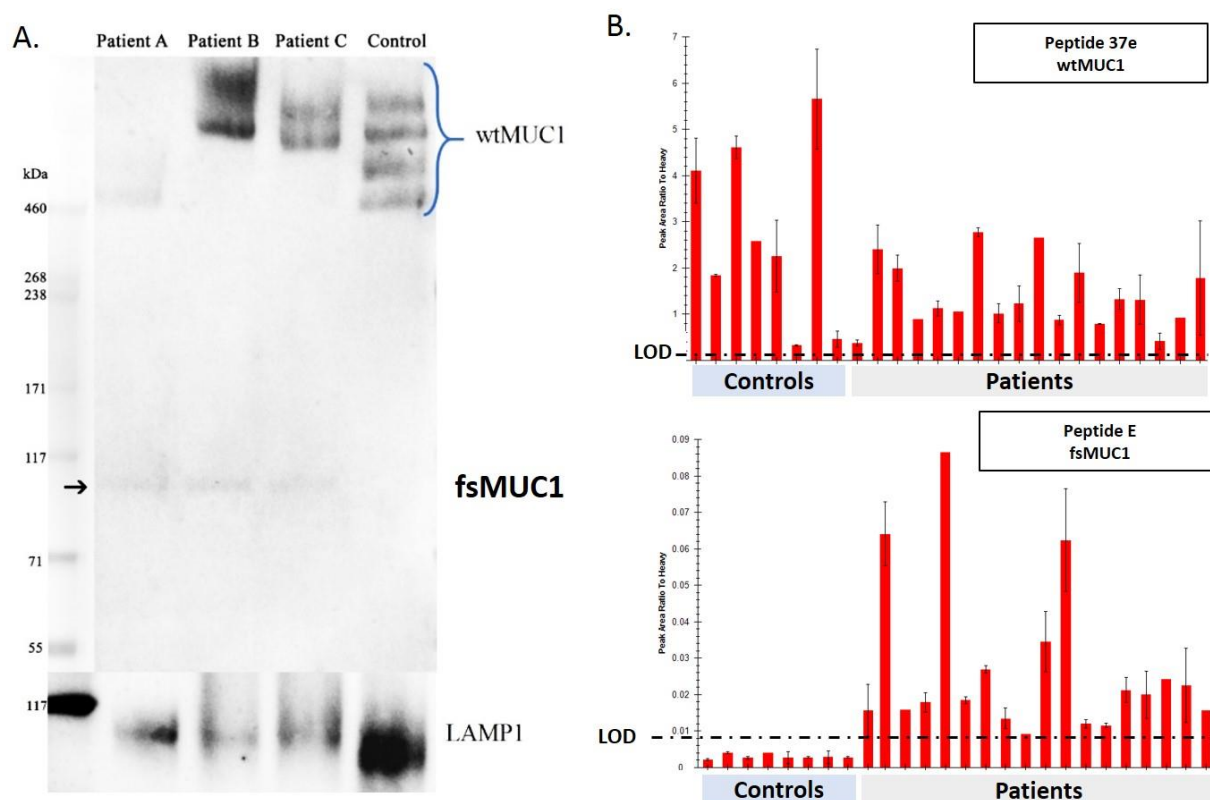


Figure 4: (A) Indicative image of western blot analysis of urinary EVs. Both wtMUC1 and fsMUC1 are strongly expressed in patient's samples. (B) iMRM-MS peak area ratio to heavy peptide (spike-in). Both wtMUC1 and fsMUC1 have been successfully measured in urinary EVs. In controls, fsMUC1 signal falls below the limit of detection (LOD).

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Urinary EVs will be extracted from patients' urine and lysed in an appropriate buffer on site in Cyprus and shipped to collaborators (*Figure 4A*). Samples will be deidentified and coded. iMRM-MS analysis of samples will be performed at the Broad Institute of MIT and Harvard, under the supervision of Dr Anna Greka. Results over different collection time points will be analyzed and plotted (*Figure 4B*). Baseline measurements will be compared to values obtained after supplement administration and during the observational phase.

6.2 Secondary endpoints

A. Plasma wtMUC1 measurement by ELISA: Mucin-1 is over-expressed in carcinomas and released from tumor cells into the plasma. There are a number of commercially available serum tests based on antibodies to mucin-1, including CA15-3. The immunoassay for CA15-3 is commercially available and uses the MUC1 variable number of tandem repeats (VNTR) Mab DF3 (epitope DTRPAP) as the catcher and radiolabeled anti-MUC1 carbohydrate Mab 115D8 (epitope STn) as the tracer. It is used routinely to monitor the response to breast cancer¹⁰.

Serum mucin-1 testing is performed routinely in Japan as a screening test for idiopathic pulmonary fibrosis. Investigators in Japan found that serum mucin-1 (which they refer to as KL-6 (Krebs von den Lungen)) levels are a useful biomarker for discriminating between idiopathic pulmonary fibrosis and other pulmonary diseases. Because of this, serum KL-6 levels are determined in over 1.8 million individuals each year in Japan⁴⁵. Our collaborators at Wake Forest Medical School (Prof. Anthony J. Bleyer) have performed plasma wtMUC1 measurements in coordination with the Broad Institute by ELISA (Table 3). In 22 healthy individuals, the mean plasma mucin-1 level was 14.1 ± 7.3 U/ml, with 15.1 ± 5.7 U/ml for samples from 15 men and 11.8 ± 10.1 U/ml in 7 women (p= not significant).

As a secondary endpoint, we will use a commercially available ELISA kit to measure Mucin-1 fluctuations in patients' samples. Since Cypriot patients share the same mutant allele, it is postulated that the insertion mutation is located at the same position within the VNTR of *MUC1*. Unpublished results indicate an extensive number of wt VNTR repeats before the C-insertion mutation occurs. Hence, antibodies against wtMUC1 VNTR will also have affinity for the fsMUC1 protein.

Table 3: Measurement of plasma mucin-1 levels in ADTKD-*MUC1* patients.

	Control			ADTKD- <i>MUC1</i>		
	N	mean	Stdev	n	mean	stdev
total	22	14.07	7.30	42	7.09	3.37
male	15	15.12	5.70	15	7.67	4.22
female	7	11.84	10.11	27	6.77	2.83

P values

total control v ADTKD- <i>MUC1</i>	0.000001
Control male v ADTKD- <i>MUC1</i> females	0.169136
ADTKD- <i>MUC1</i> males v females	0.207661
Control female v ADTKD- <i>MUC1</i> females	0.012393
Control male v ADTKD- <i>MUC1</i> males	0.000176

B. Measurement of Mucin-1 expressed in plasma EVs by iMRM-MS: As with urinary EVs (Primary endpoint), both wtMUC1 and fsMUC1 expressed in plasma EVs will be measured by iMRM-SISCAPA. Plasma EVs will be extracted from patients' samples and will be lysed in an appropriate buffer on site in Cyprus and shipped to collaborators. The same assay used for urine will also be implemented for plasma EVs, offering great specificity for each version of the protein. Plasma EVs will be isolated from samples collected at days 0 (baseline), day 7 and day 14 (response tracking phase).

6.3 Statistical analyses

Sample size calculation/justification of sample size: Based on plasma mucin-1 determinations at the Broad Institute, the mean plasma mucin-1 level was 14.1 ± 7.3 for 22 individuals. If we consider both genders together, to obtain 80% power to identify a difference in plasma mucin-1 levels of 50%, we would require 35 participants. The same calculations are applied for mucin-1 levels in urinary EVs.

Statistical analysis will include a paired T-test, comparing baseline mucin-1 levels in urinary EVs (average of values from visits on days -10 to 0) to values obtained at days +3, +7 as the primary endpoints. Further testing will be performed to compare mucin-1 levels in urinary EVs obtained at 7 and 10 days after administration of cholecalciferol. Continuous variables will be summarized using descriptive statistics including the number of observations, mean, standard deviation, minimum, median, and maximum values. Categorical values will be summarized using number of observations and percentages. All statistical tests will be performed at the two-sided, 5% significance level, unless otherwise specified.

7. INVESTIGATIONAL THERAPEUTIC AGENT

7.1 Identity of Investigational supplement

The investigational supplement is cholecalciferol. Cholecalciferol 1250µg (50,000IU Vitamin D3), supplied by HealthAid (HealthAid Vitamin D3 KDLF1311) and distributed in Cyprus by Louanfarm LTD, PO Box 26871, 1648 Nicosia. Cholecalciferol is considered as a food supplement in Cyprus and the European Union.

7.2 Formulation, Packaging, and Labeling

Cholecalciferol will be supplied as green, oval capsules (shelled by hydroxypropyl methylcellulose) containing 50,000 IU of cholecalciferol and include white rice powder as bulking agent and plant magnesium stearate and silicon dioxide as anticoagulating agents. It is available in bottles of 30 capsules.

7.3 Supplement Administration

7.3.1 Dose Administered

Investigational supplement will be administered to eligible participants in a single dose of 200,000 IU.

7.3.2 Dose Dispensation, Packaging, Labeling, and Storage

Four cholecalciferol capsules (50,000IU Vitamin D3 each) will be provided by the clinical coordinator to eligible participants at Day 0. Participants will take all four tablets with water.

7.4 Supplement Accountability

The clinical coordinator of the project will ensure that the study supplement is used only in accordance with this protocol. In collaboration with the research team at the MMRC, University of Cyprus, he will be responsible to maintain accurate records of the receipt of all study drug, including dates, quantities and participant unique identifiers.

8. ADVERSE EVENTS

8.1 Definition of adverse event

Although cholecalciferol is considered a food supplement by the EFNA, it will be considered as a putative therapeutic agent in this study. Hence, any adverse events emerging by the dosage will be reported. An adverse event (AE) means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an investigational supplement, whether or not considered related to the investigational supplement. This includes any newly occurring event or a previous condition that has increased in severity or frequency since the administration of the investigational supplement.

8.2 Recording of adverse events and observation period

The Investigator should question the participant about AEs and intercurrent illnesses since his/her last visit and record the information. The presence or absence of specific AEs should not be solicited from participants. The onset and end dates, severity, and relationship to study drug must be recorded for each AE. When possible, signs and symptoms indicating a common underlying pathology should be noted as one comprehensive event or diagnosis.

AEs will be recorded between administration of the study drug on Day 0 and Day 30. Medical events that occur between the signing of the informed consent and study drug administration will be considered part of the participant's medical history and recorded accordingly. Participants who experience AEs will be monitored with relevant clinical assessments and laboratory tests, as determined by the Investigator.

All AEs that are not resolved by the end of the study, or that were not resolved upon discontinuation of the participant's participation in the study, are to be followed until one of the following occurs: the event resolves, the participant's condition stabilizes or is fully characterized, or the event returns to baseline value (if a baseline value is available).

8.3 Serious adverse events

8.3.1 Serious adverse event definition

- **Serious or life-threatening adverse events are not expected in this study testing the effect of a single dose of vitamin D.**

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A serious adverse event (SAE) means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered related to the administered supplement, that results in one or more of the following outcomes:

1. In-patient hospitalization or prolongation of existing hospitalization. Hospitalization admissions and/or surgical operations scheduled to occur during the study period but planned prior to study entry are not considered AEs if the illness or disease existed before the participant was enrolled in the study, provided that it did not deteriorate in an unexpected manner during the study (e.g., surgery performed earlier than planned).
2. A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.
3. A congenital anomaly/birth defect.
4. A life-threatening AE. Life-threatening means that the participant was at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that, had it occurred in a more severe form, might have caused death.
5. Death.
6. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the participant or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in in-patient hospitalization, or the development of drug dependency or drug abuse.

8.3.2 Serious adverse event collection timeframe

The clinical coordinator will review each SAE and further evaluate the relationship of the AE to study drug and to the participant's underlying disease. Based on the assessment of the AE, a decision will be made concerning the need for further action. The primary consideration governing further action is whether new findings affect the safety of other participants participating in the clinical study. All SAEs, regardless of relationship to the investigational product, will be reported between study drug administration and Day 30.

8.3.3 Severity

For both AEs and SAEs, the clinical coordinator must determine the severity of the event using the following definitions:

- Mild: The event does not interfere in a significant manner with the participant's normal functioning level. It may be an annoyance but does not cause any limitation in usual activity.
- Moderate: The event produces some impairment of functioning but is not hazardous to health. It is uncomfortable or an embarrassment and may cause some limitation in usual activity.
- Severe: The event produces significant impairment or incapacitation and is a definite hazard to the participant's health.
- Life-threatening: The event places the participant, in the view of the Investigator, at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that had it occurred in a more severe form, might have caused death.

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The term “severe” is used to describe the intensity (severity) of a specific event. Even though the event itself may be of relatively minor medical significance (such as a severe headache), this is not the same as “serious,” which is based on participant/event outcome or action criteria as described above and is usually associated with events that pose a threat to a participant’s life or functioning. A severe AE is not necessarily serious. Seriousness (not severity) serves as a guide for defining reports on AE.

8.3.4 Causality

Association of AEs and SAEs to the study drug will be made using the following definitions:

- Unrelated: An AE that is not related to the use of the study drug.
- Unlikely: An AE that is unlikely to be due to the use of the study drug. An alternative explanation, e.g., concomitant drug(s), concomitant disease(s), is plausible. A relationship to the study drug is improbable but not impossible.
- Possible: An AE that conceivably could be due to the use of the study drug. An alternative explanation, e.g., concomitant drug(s), concomitant disease(s), is not conclusive. The relationship of the event in time follows a plausible temporal sequence from administration of the study drug so that a causal relationship cannot be excluded but could have been produced by the participant’s clinical condition or other therapy.
- Probable: An AE that might be due to the use of the study drug. An alternative explanation is less likely, e.g., concomitant drug(s), concomitant disease(s). The relationship in time is suggestive and follows a reasonable temporal association with study drug administration. The reaction cannot be reasonably explained by the known characteristics of the participant’s clinical state or other modes of therapy administered to the participant.
- Related: An AE that is almost certainly related to the use of the study drug. The AE cannot be reasonably explained by an alternative explanation, e.g., concomitant drug(s), concomitant disease(s).

8.3.5 Deaths and life-threatening events

An event leading to death from any cause or any life-threatening event will be reported as an SAE within 24 hours of the event, regardless of whether or not related to the study drug.

9. ETHICAL CONSIDERATIONS/PERSONAL DATA OF PARTICIPANTS

9.1 Participant informed consent and administration

The study will be conducted in accordance with ethical principles that have their origin in the Declaration of Helsinki. The Cyprus National Bioethics Committee will review all appropriate study documentation in order to safeguard the rights, safety, and well-being of the participants. Prior to entry in the study, the Clinical Coordinator must explain to potential study participants the study and the implications of participation. Participants will be told that their participation is voluntary, and they may withdraw consent to participate at any time. Participants will be told that their records may be accessed by authorized investigative bodies without violating the confidentiality of the participant, to the extent permitted by the applicable law(s) and/or regulations. Participants who are unable to comprehend the information provided cannot be enrolled. Therefore, participants will be asked to read and sign a consent form

pertaining to this project, which must be approved by the Cyprus National Bioethics Committee.

9.2 Participant confidentiality and personal data

Due to strict EU regulations (GDPR directive), all personal and demographic data, including but not limited to name, date of birth, ID numbers, full clinical records, telephone numbers, emails and postal addresses, will not be revealed under any circumstances. Records and data will be kept at the CY-Biobank database at the Molecular Medicine Research Center, University of Cyprus, Nicosia, Cyprus. Data will be kept anonymized. Collaborators will have access to biofluids obtained by patients (under given consent) for analysis under code numbering corresponding to each participant.

9.2.1 Rationale for the use of genetic data

Patients have donated genetic material (DNA) under given consent in the past and have been genotyped as having the C-insertion mutation in *MUC1*. Due to the variable progression of the disease, patients need to be genotyped in order to be included to the study. Chronic renal failure is a disease where patients might have a variable, but declining, degree of renal function, hence clinical data are not adequate in many cases to support disease pathology. This information is readily available, and patients are aware of their genotype status. In case of a study dissemination event, the genetic status of the participants will be stated as a general reference.

9.2.2 Clinical, personal and demographic data accompanying the sample

Each sample must be accompanied by relevant clinical data to assist his/her enrolment to the study. Clinical data include eGFR staging through serum creatinine measurements, biochemical data on vitamin D 25-OH serum levels, serum uric acid and urea levels, Na, K, Ca, Mg and P serum levels, investigation for anemia through a full blood count and urine creatinine levels. Other data include the patient's weight, ultrasonography of the kidney if available, blood pressure, allergies, major medical history and concurrent medication. The pregnancy status of female participants is also required for their enrollment in the study. Personal data required are the patient's name and surname and personal identification number (ID). Demographic data required are the participant's biological sex, date of birth, address, postal code, telephone number and email. Personal and demographic data are required for communicating with patients, defining clinical data requiring the age of the participant (eGFR calculations), investigate the association of treatment to sex, age or status of renal function.

9.2.3 Securing and storing participant's data and samples

The collection and storage of data are subject to given consent by the patient. When granted access to clinical, personal and demographic data, the Biobank facility at the Molecular Medicine Research Center, is responsible for storing and de-identifying all data. Data de-identification is implemented by assigning a specific and unique identifier having with both letters and numbers. This sample identifier does not give out information on the patient's sex, clinical status, demography or personal data. Access to the core data

is limited to the principal coordinator, the clinical coordinator and the biobank coordinator, and are stored in secure servers with password only access. Hard-copy or printed data that might occur, will be stored safely in the premises of the Molecular Medicine Research Center and access will be limited only to authorized personnel. Biosamples will be stored de-identified at -80°C freezers in the Biobank and secured with a lock. Participants will also give informed consent for shipping de-identified biosamples to collaborators abroad and service offering entities such as local clinical labs, for analyses concerning the endpoints of this study. All information exported to collaborators or local clinical labs from the Molecular Medicine Research Center will be de-identified (coded) and results will be returned by collaborators in the same form. In the case of result dissemination, any reference to specific sample will ensure anonymity and de-identification. Data and biosamples will be stored in the Biobank facility for a total of 20 years and then destroyed – electronic data will be deleted, hard-copy data will be shredded and discarded and biosamples will be incinerated. If a patient requires access to his/her biochemical analyses done during the study, access will be granted after the clinical coordinator contacts the patient personally. Hence, the clinical coordinator is responsible to interpret and inform patients of any results emerging from the study.

9.3 Accepting complaints/Indemnification procedure/Compensation of participants

Complaints from participants can be officially documented by the Director of the Research Support Service, Dr Marios Demetriades, at the University of Cyprus, tel. 22-894286. Complaints may concern the participation in the study, documented or undocumented adverse effects of the administrated supplement, management of personal data or any other complaint related to the study. The project coordinator and the clinical coordinator are responsible to communicate with participants to respond to reported complaints.

In case a participant suffers from a documented or an undocumented adverse effect caused by the administered supplement, Altius Insurance, who provides insurance in the form of professional indemnity for Dr Christoforos Stavrou the clinical coordinator of the study, will examine and respond to claims. This pipeline ensures that the identity and medical records of participants will not be exposed to any private insurance companies, thus eliminating any risk of stigmatization for such chronic kidney disease patients. Altius Insurance will examine each case as per complaints made on documented or undocumented adverse effects due to the supplement under study and will act in accordance to internal procedures.

Other than the above, the participants will not be compensated for participating in this study. As blood drawings are invasive clinical procedures, it is possible that participants might experience transient bruising, for which patients will not be compensated.

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