Cover Letter

Title:

Efficacy of Vitamin D Supplementation in Females with Polycystic Ovary Syndrome: A Randomized Open-Label Delayed-Start Design

Clinical Trial Unit,

I am writing to submit my study, titled "Efficacy of Vitamin D Supplementation in Females with Polycystic Ovary Syndrome: A Randomized Open-Label Delayed-Start Design," for consideration in the Clinical Trial Unit. I am excited to present this research project for potential inclusion in your esteemed program.

Polycystic Ovary Syndrome (PCOS) is a prevalent and complex endocrine disorder that affects many women worldwide. The condition is characterized by a range of symptoms, including irregular menstrual cycles, hyperandrogenism, and polycystic ovarian morphology. There is a growing body of evidence suggesting a link between vitamin D deficiency and PCOS, as vitamin D plays a crucial role in various aspects of reproductive health and endocrine regulation.

Our study aims to investigate the efficacy of vitamin D supplementation as a potential therapeutic intervention for females with PCOS. The randomized open-label delayed-start design allows for a comprehensive examination of the long-term effects of vitamin D supplementation on PCOS symptoms and associated comorbidities. By comparing the outcomes between immediate and delayed-start groups, we hope to gain insights into the optimal timing of vitamin D intervention and its impact on the progression of PCOS.

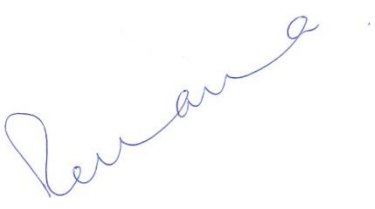
The significance of this study lies not only in its potential to improve the quality of life for individuals with PCOS but also in its contribution to the broader field of reproductive medicine. If our findings support the efficacy of vitamin D supplementation, it could lead to the development of cost-effective and accessible treatments for a condition that affects millions of women worldwide.

I believe that the Clinical Trial Unit is the ideal platform for conducting this research due to its commitment to advancing clinical science and improving patient care. Our study adheres to rigorous ethical and methodological standards, and we are confident that it aligns with the objectives and values of your organization.

I kindly request that you consider our study for inclusion in the Clinical Trial Unit and appreciate the opportunity to discuss this research further. The date of ERC approval is 7th December, 2021

Thank you for your time and consideration.

Best Regards,



Title:

Efficacy of Vitamin D Supplementation in Females with Polycystic Ovary Syndrome: A Randomized Open-Label Delayed-Start Design

Methodology

Study Design: This will be a randomized open label, delayed-start design trial conducted at Aga Khan University at Departments of: Biological & Biomedical Sciences, Pathology and Laboratory Medicine, Medicine (Endocrinology) and Family Medicine for a period of 2 years following approval from Clinical Trials Unit and Ethical Review Committee of Aga Khan University

Sample Size calculation; The sample size was calculated by PASS 11 software. In an equivalence test of means using two one-sided tests on data from two-periods, the minimum sample size that we will require is 142, 71 in each group with an inflation of 20% for loss to follow-up to achieve 80% power at a 5% significance level when the true difference between the means is 0.4, the standard deviation of the paired differences is 2.60.

Eligibility Criteria: Patients recently diagnosed with PCOS with presence of at least 2 of these 3 elements: clinical or biochemical signs of hyperandrogenism, chronic anovulation and polycystic ovaries (1) (from reports of available routine TVS), with VDD serum levels VD <20 ng/ml(10), age range 18- 45 years, from all ethnic background will be included. We will exclude patients who will be pregnant, with Hypercalcemia (plasma calcium concentrations> 2.65 mmol/L), Tuberculosis or other granulomatous disorders, Chronic liver disease or alanine transaminase (ALT) level 3 times higher than the normal limit, chronic, Kidney disease or serum creatinine >2.0 mg/dL, on drrug Therapies i.e participants who had received VD injection in the last 3 months prior to recruitment in the study, oral contraceptives, hormonal replacement therapy, glucocorticoids, calcium supplementation, insulin-sensitizing drugs (incretin mimetic drugs, thiazolidinedione, sulfonylurea), lipid-lowering drugs or other drugs affecting insulin sensitivity or serum androgens (e.g., niacin, corticosteroids, beta-blockers, calcium channel blockers, thiazide diuretics), anti-epileptics, anti-retroviral, cholestyramine, anti-fungal, statins, H2 blockers, immunosuppressant, chemotherapeutic agents, antimicrobials (Rifampicin, Isoniazid, Hydroxychloroquine) or any other drug modifying lipid metabolism in the previous 3 months prior to study , patients suffering from congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumors, type 2 Diabetes Mellitus, renal, hepatic or thyroid disorders, hyperparathyroidism, malabsorption syndromes, Chronic Kidney Disease, Hepatic failure, cystic fibrosis, vaginal bleeding of unknown etiology or /and suffering from COVID-19 (within 3 months).

Recruitment of Subjects: All diagnosed PCOS subjects as per the Rotterdam criteria visiting Endocrine, Family Medicine and/or Gynae Obs clinic qualifying the inclusion criteria with VD levels <20 ng/ml (7, 9, 10) and age range 18 to 45 years will be invited to participate in the study after acquiring written informed consent.

Randomization: Subjects will be brought to Clinical Trial Unit (CTU) of AKU which has full-fledged clinical space for consultation, trial and laboratory support. Randomization will be

done using the permuted block randomization technique generating blocks with equal representation of all arms in a 1:1 ratio. The randomization list will be generated using the AKUH developed randomization software (http://www.randomizer). The randomization list is confidential and will be kept within the premises of CTU under restricted access. Subjects will be randomized into Group A (Intervention) and B (Control) and will receive the treatment protocol

Intervention Group (Table 2) for the initial 12 weeks will receive: VD supplementation (600,000 IU I/M) once during the study period and elemental calcium 1000 mg/day . However from 12-24 weeks they will receive standard PCOS treatment, lucophage XR 750 mg once at dinner for 15 days then twice daily, capsule Progeffik 100 mg once at night every 3 weeks, then 1 week off and calcium 1000 mg/day.

Control Group for the initial-- 12 weeks the control group will receive the standard treatment for PCOS , Glucophage XR 750 mg once at dinner for 15 days then twice daily and Capsule Progeffik 100 mg once at night every 3 weeks, then 1 week off

However, from 12- 24 weeks they will receive VD supplementation (600,000 IUI/M) once during the study period , calcium 1000 mg/day and standard PCOS treatment will be continued

	Group A (intervention)	Group B (Control)
Initial 12 weeks	**Vitamin D:** 1. VD supplementation (600,000 IU I/M once) 2. Calcium 1000 mg/day	**Standard PCOS;** 1. Glucophage XR 750 mg once at dinner for 15 days then twice daily 2. Capsule Progeffik 100 mg once at night every 3 weeks, then 1 week off
At 12 weeks after consultation and biochemical tests treatment protocol will be changed as mentioned • Vitamin D & Calcium will be given to Group B with continuation of standard PCOS treatment (point of care) • Standard PCOS care for Group A with continuation of calcium (point of care)		
After 12 weeks	**Standard PCOS;** 1. Glucophage XR 750 mg once at dinner for 15 days then twice daily 2. Capsule Progeffik 100 mg once at night every 3 weeks, then 1 week off **3. Calcium 1000 mg/day (contd.)**	**Vitamin D:** 1. VD supplementation (600,000 IU I/M) 2. Calcium 1000 mg/day **3. Standard PCOS Contd**

Data Collection: Randomization and drug dispensing will be followed by detailed history, physical examination, biophysical and biochemical parameters estimation by research team. The data will be collected on the following variables ; Body Mass index. ^{2}, Waist circumference (WC)Waist -Hip Ratio, Biochemical estimation (results of following routine tests are available (Human Sex Hormone-Binding Globulin (SHBG), Total Testosterone (TT), Human Insulin and Glucose) to be included in the profile. For

estimation of Total Antioxidant Capacity (TAC), serum calcium and phosphorous levels, phlebotomist will collect blood samples (3 cc) by vein puncture).

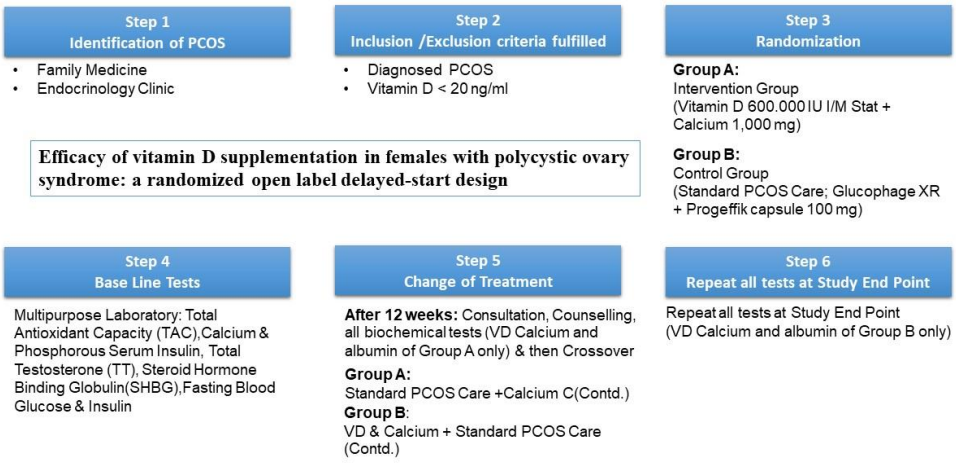
Calculations;

i. HOMA-IR : HOMA- IR index, a commonly used marker of IR, will be calculated using the formula: HOMA-IR = fasting glucose levels [mmol/L] × fasting insulin levels [μU/mL]/22.5 (34).

ii. Free Androgen Index will be derived from TT and SHBG, normally measured in nanomoles per liter. FAI =100 (TT /SHBG)

Outcome : Follow- up visits at 12 weeks (Study Mid Pont): At 12 weeks follow up, compliance to supplementation and occurrence of any adverse effect will be assessed by research team allocated members in the respective clinics. Moreover, drug for 12 weeks (as per group allocation) will be dispensed and any other queries of participants will be responded. Fasting blood sample (10 ml) will be collected for estimation of SHBG, TT, Human Insulin, Glucose, serum calcium and phosphorous and TAC in both groups and VD and serum albumin estimation in Group A. At this point of time, **treatment protocol of both groups will be changed** (Table 2).

1. **Follow- up visits at 24 weeks (study End Point):** After completion of 24 weeks study exposure, subjects will be informed about completion of study. Blood samples in fasting state will be collected for repeat analysis in Multi Disciplinary Lab (MDL) for all the biochemical parameters (FAI, HOMA IR and TAC) and VD and serum albumin estimation in Group B. Figure 6: Complete cycle of each patient in control and intervention group for a period of 24 weeks



Biohazardous Samples: According to the WHO the serum samples have low viral concentration. However, the centrifugation is a risk for aerosol generation and COVID exposure. To minimize the risk, we will ; Train the research staff about the Basics of Biosafety, Biosecurity & Bio risk Management approach for collection & processing of samples. All samples will be treated as potentially infectious, use of proper PPE will be mandatory while handling samples. After centrifugation, staff will wait for 10 minutes before opening the centrifuge. In case of Breakage the already defined laboratory protocols (available in safety manual) will be followed. For liquid waste disposal, will use 5% Hypochlorite.

Potential Risks: The potential risks likely to occur in this study are; Loss of patients during movement in respective clinics (Lost to follow up) Noncompliance of subjects, Biosafety Issues, At the time of collection of samples, unexpected delays in the processing of samples; during bench work in MDL lab and Drug Overdose

Risk management Plan for Potential Risks:

i. Movement of Subjects: We have hired 2 Research Assistants (RA) who will be in connection with the PI and Co-PIs (Gynecologists and Endocrinologists) to guide the included patients from clinics to CTU, blood collection point and follow up sessions at 12 and 24 weeks.

ii. Compliance: PI students and RA will be in communication with the subjects on emails, phone calls and WhatsApp group, will send them reminders to take their medicine, come for follow up visits and inform if any side effects occur

iii. Biosafety Issues: Collection of Samples: For the entire research procedure the risks of having blood drawn include temporary pain from the needle stick, bruising, and rarely, infection. Any gross reaction will be reported and informed to the concerned investigator. Unexpected load: Due to pandemic situation, there is an unexpected load on the centrifuge machine in MDL. We have therefore requested our mentor Dr Aysha Habib; Head of Chemical Pathology and Laboratory Medicine to allow centrifuge of our samples in the clinical lab of AKUH (if required). Bench work in MDL lab as per AKU COVID-19 **Safety Rules**: The research team will follow all the standard revised operating procedures (SOPs) required for biosafety of lab staff during the pandemic in their BSL2 facility. They include; judicious cleaning of labs, minimal 6 feet distance, cleaning of bench with 70% Isopropyl alcohol or 80% ethanol and follow all the other SOPs that are already implemented in the research areas from before the Covid-19 pandemic.

iv. Drug Safety: The vitamin doses that are planned for use in this study are based upon scientific evidence and recommendations from international societies and are unlikely to cause hypervitaminosis D. However, **if Hypervitaminosis D** occur in a rare situation, it can lead to **hypercalcemia** which can manifest as polyuria, polydipsia, nausea, vomiting, or constipation, muscle weakness, bone pain, weight loss or poor appetite, confusion or disorientation. We will warn all our subjects to inform us in case if any of these symptoms appear and RA will be in touch with them. We will stop the treatment and report to the respective consultant. We will also assess VD status along with calcium and albumin levels after they have received VD and calcium supplementation. All study related adverse effects will be managed and reported to the ERC, Clinical affairs and pharmacy as per institutional policy.

Investigator and study coordinator will follow up any adverse events and serious adverse events.

Plan of analysis: Data will be analyzed using STATA version 15. The main analysis for each outcome will be performed at the subject level using the **intention-to-treat (ITT) principle**, meaning that all participants with a recorded outcome will be included in the analysis and will be analyzed according to the treatment group to which they were randomized. Results will be presented as mean and standard error/ median (IQR) for the outcomes. Pretreatment, 12 weeks and 24 weeks post treatment levels of the outcomes will be assessed by repeated measure ANOVA/ Friedman test as appropriate. Categorical variables will be reported as frequency and percentages and will be assessed by chi-square/ fisher exact test. Unadjusted and adjusted beta coefficient with 95% CI will be reported by using Generalized estimating equation (GEE) to determine the association of various independent factors with the outcomes. We will adjust for the independent variables and determine the association of factors causing a greater decline in outcomes by multivariable GEE. Plausible interactions and confounders will also be assessed. A p value of < 0.05 will be considered as significant throughout the study

Ethical Considerations: In this research, we will adhere to ethical principles outlined in the Helsinki Declaration for medical research involving human subjects. We will seek approval from the Aga Khan Ethical Review Committee. The participants will be informed about the study's purpose and the possibility of being part of the intervention group based on computer randomization. We will obtain verbal and written consent from eligible participant. To ensure confidentiality, the entire process from participant enrollment to testing and outcome assessment will be handled discreetly. Consent forms and completed questionnaires will be assigned unique codes and securely stored in locked cabinets accessible only to the Principal Investigator. Participant identifiers will be removed, and only codes will be used in subsequent steps. Serum samples will be collected and coded without mentioning names. These samples will be processed at MDL-AKU and disposed of following AKU clinical lab's institutional policies for biological material disposal, which involves sealing them in red bags and incineration. Hard copies questionnaires will be stored and archived for a minimum of 7 years in line with AKU policy. Afterward, information will be deleted from computers, and the forms and questionnaires will be securely shredded. Results and data will be appropriately coded, decoded, and documented.

Strength & Limitations: The delayed-start design has **some methodological issues** in comparison with standard parallel-group study design of RCT. Nonetheless the effects of VD develop over time and we do not want to deprive VD deficient PCOS from VD supplementation in both groups, therefore we have selected this study design; **all subjects will be given same dose of VD either before or after standard PCOS care.** To the best of our knowledge this is the **first clinical trial** which will confirm not only the

impact of VD but also **predict the time of its administration**. Novelty of the study is that in addition to the routine parameters of IR and hyperandrogenism, this study will also estimate and document the **improvement in OS** as a result of the replacement therapy.