RESEARCH PROTOCOL

Pilot study to assess the effects of discontinuation of vitamin K antagonists on the rate of elastin degradation

(August 2017)

PROTOCOL TITLE

The effects of discontinuation of vitamin K antagonists on the rate of elastin degradation

Protocol ID	
Short title	VKAs and elastolysis
EudraCT number	NA
Version	4.0
Date	August 21 2017
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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

AE	Adverse Event
AR	Adverse Reaction
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
COPD	Chronic Obstructive Pulmonary Disease
DOAC	Direct oral anticoagulant
DSMB	Data Safety Monitoring Board
Dp-uc MGP	Dephosphorylated uncarboxylized Matrix Gla Protein
EudraCT	European drug regulatory affairs Clinical Trials
IC	Informed Consent
IMP	Investigational Medicinal Product
IMPD	Investigational Medicinal Product Dossier
INR	International normalized ratio
METC	Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)
MGP	Matrix Gla Protein
pDES	Plasma Desmosine
SAE	Serious Adverse Event
SD	Standard deviation
SNP	Single nucleotide polymorphism
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
SUSAR	Suspected Unexpected Serious Adverse Reaction
VKA	Vitamin K antagonist
VKORC1	Vitamin K 2,3-epoxide reductase complex 1
WBP	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale:
Elastin is a unique protein providing elasticity, resilience and deformability to dynamic tissues, such as lungs and vasculature. Elastin fibers are characterized by their high affinity for calcium. However, calcified elastin is more prone to the degrading effects of proteases and, in turn, partially degraded elastin has an even higher affinity for calcium. A disturbed balance between proteases and anti-proteases is a major underlying mechanism in the development of chronic obstructive pulmonary disease (COPD). Virtually the only protein that can protect elastin from calcification is matrix Gla-protein (MGP), which needs vitamin K for its activation. In COPD patients, a lower vitamin K status is found when compared to control subjects and an inverse association exists between vitamin K status and elastin degradation. In addition, vitamin K status is lower and elastin degradation is accelerated in Vitamin K antagonist (VKA) users.
VKAs are widely used. Nowadays, an increasing number of patients uses direct oral anticoagulants (DOACs), which do not influence vitamin K status. We hypothesize that discontinuation of VKAs results in an improved vitamin K status and deceleration of elastin degradation. In order to test this hypothesis, we want to conduct an observational pilot study in which the change in elastin degradation– quantified by plasma desmosine concentrations – in patients who discontinue use of VKAs will be used as primary endpoint.
Objective: To evaluate whether discontinuation of VKAs results in a higher vitamin K status and deceleration of the rate of mature cross-linked elastin degradation.
Study design: Observational study.
Study population: A total of 30 VKA users who will discontinue the use of VKAs.
Main study parameters/endpoints: The primary endpoint is the change in the rate of elastin degradation quantified by the plasma desmosine assay. Secondary endpoints are the change in vitamin K status quantified by measuring plasma levels of dephosphorylated uncarboxylated, (i.e. dp-uc, inactive) Matrix Gla Protein (MGP), the relation between desmosine and dp-ucMGP and differences of desmosine and dp-ucMGP levels among subjects with different polymorphisms of the vitamin K 2,3-epoxide reductase complex 1 (VKORC1) gene.
Nature and extent of the burden and risks associated with participation, benefit and group relatedness: We will draw extra blood collection tubes at two moments. The first time is during one of the last regular INR testing at the anticoagulation clinic, therefore no additional venipuncture has to be performed. At this moment two additional blood collection tubes will be drawn. The second moment is approximately 6 months after discontinuation of VKAs. At this point the patient will undergo an additional venipuncture. Patients are asked to

fill in a questionnaire concerning age, gender, length, height, indication for VKA use, duration of VKA use, smoking status and history, presence of COPD and other pulmonary disease, whether patients will switch to DOACs or will completely stop the use of anticoagulants, vitamin D supplementation, use of multivitamins and use of medications. Furthermore, we will record the INR at baseline. Participants may experience some discomfort during blood sampling. Participating in the study has negligible risks.

1. INTRODUCTION AND RATIONALE

Elastin is a unique protein providing elasticity, resilience and deformability to dynamic tissues, such as lungs and vasculature.[1] Elastin fibers are almost exclusively developed before birth and in the first period of life after which the synthesis of elastin is suppressed at a post-transcriptional level.[1] Elastin can be degraded by certain proteases, also referred to as elastases.[1] Elastin has high affinity for calcium,[1] and the calcium content of elastin fibers therefore increases during aging.[2] Calcium changes the conformation of elastin, which gives elastases access to elastin fibers leading to degradation.[3,4] In turn, partially degraded elastin has a higher affinity for calcium due to increased polarity.[4] Therefore, elastin calcification and elastin degradation are two pathologic processes that stimulate each other. When elastin is degraded, desmosine, a component of mature elastin, can be detected in the bloodstream. Since desmosine is only found in mature elastin, its plasma levels are a reflection of the rate of systemic elastin degradation.[5]

Chronic obstructive pulmonary disease (COPD) is characterized by a disturbed balance between proteases and anti-proteases, resulting in an accelerated degradation of elastin in the lungs, but also in blood vessels.[6] Besides elastin degradation, calcification of elastin, i.e. in coronary arteries, is also accelerated in patients with COPD.[7]

Matrix Gla protein (MGP) is a potent inhibitor of elastin calcification and needs vitamin K to become activated. In an observational epidemiological study, higher vitamin K2 intake was associated with less vascular calcification.[8] In addition, three years of vitamin K2 supplementation in postmenopausal women resulted in a decrease of arterial stiffness, while an increase was observed in the placebo group.[9] The dephophorylated uncarboxylated (i.e. inactive) form of MGP, dp-ucMGP, can be measured in plasma and its levels are negatively associated with vitamin K status. Therefore, dp-ucMGP levels are used as a measure for vitamin K status.

Earlier, we found a reduced vitamin K status in patients with COPD compared with patients without COPD and an inverse association between vitamin K status (quantified by dp-ucMGP levels) and elastin degradation (quantified by desmosine levels) among COPD patients (abstract ATS 2016). Recently, in 106 VKA users we found higher desmosine and dp-ucMGP levels when compared with patients who do not use VKAs. In VKA users, we also found an inverse association between vitamin K status and elastin degradation (quantified by plasma dp-ucMGP and desmosine levels, respectively).

During activation of vitamin K dependent proteins such as MGP, vitamin K is oxidated from vitamin K 2,3-epoxide to vitamin K hydroquinone. Subsequently, vitamin K 2,3-epoxide needs to be recycled to the quinone form by an enzyme called vitamin K 2,3-epoxide reductase complex 1 (VKORC1). Different single nucleotide polymorphisms of this gene exist, each resulting in a different level of expression of the enzyme. Since the VKORC1 enzyme is the direct pharmacologic target of VKAs, this gene is for an important part responsible for the interindividual dose variability of VKAs. The rs9923231 SNP (g.-1639G.A) is the main genetic determinant of VKA dose requirement and can alone explain between 25% to 30% of the dose variance among patients.[10]

Nowadays, oral anticoagulants exist which do not influence vitamin K status, the so-called direct oral anticoagulants (DOAC). An increasingly number of patients is switching from VKAs to DOACs.

We hypothesize that discontinuation of VKAs results in an improved vitamin K status and a deceleration of elastin degradation. In order to test this hypothesis, we want to conduct an observational pilot study in which the change in elastin degradation– quantified by plasma desmosine concentrations – in patients who discontinue use of VKAs will be used as primary endpoint.

2. OBJECTIVES

We hypothesize that in persons who use VKAs, discontinuation of vitamin K antagonists leads to a deceleration of elastin degradation.

Primary Objective:
To assess the change in the rate of elastin degradation after discontinuation of vitamin K antagonists in patients who use VKAs. Quantification of the elastin degradation will be done

by measuring plasma desmosine concentrations at baseline and at 6 months after discontinuation.

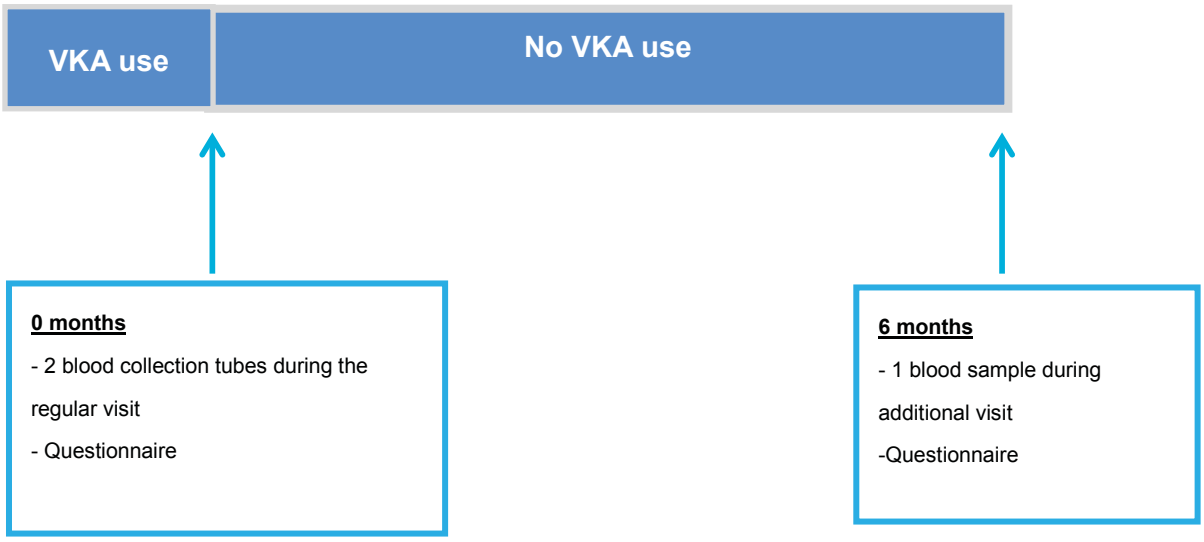
Secondary Objective(s):
To evaluate the possible effects of discontinuation of VKAs on dp-ucMGP (inversely correlated with vitamin K-status).
To evaluate if different polymorphisms of the VKORC1 gene are associated with desmosine and dp-ucMGP levels and the change of both parameters after discontinuation of VKAs.

3. STUDY DESIGN

The study is designed as a non-interventional study. The follow up per patient comprises 6 months. We will include 30 VKA users who will stop the use of VKAs. At baseline, patients are asked to fill in a questionnaire and two additional samples of blood are taken during the regular testing of the international normalized ratio (INR). One blood sample is needed for determination of the VKORC1 polymorphisms and one for desmosine and dp-ucMGP levels. After 6 months, patients are asked to draw one additional blood sample for determination of desmosine and dp-ucMGP. At baseline and 6 months blood can be drawn in the Canisius Wilhelmina Hospital or one of the outposts of the anticoagulation clinic. Since the blood samples have to be stored and frozen in the laboratory in the Canisius Wilhelmina hospital within 2 hours after drawing, patients can visit the outposts within one hour before the blood samples are transported to the laboratory, i.e. within one hour before closing the outpost. If both visiting the hospital or outpost is not possible, the patient can contact the investigator to make an appointment for drawing the blood samples at home.

An overview of is given in Figure 1.

Figure 1. Overview of study and time schedule



4. STUDY POPULATION

4.1 Population (base)

We will include 30 VKA users who are going to discontinue the use of VKAs at short time

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Use of VKAs for at least 3 months
- Stop VKAs at short time
- Written informed consent
- Age ≥18 years
- Ability to comply with all study requirements

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Active malignancy or cured malignancy <12 months prior to enrollment
- Use of maintenance dose oral corticosteroids
- Serious mental impairment
- Life expectation of less than 6 months on the basis of concurrent disease

4.4 Sample size calculation

It concerns a pilot study and therefore sample size calculation was not performed.

5. METHODS

5.1 Study parameters/endpoints

5.1.1 Main study parameter/endpoint

The primary endpoint is the difference in elastin degradation rate, quantified by the change in plasma desmosine levels, measured at baseline and 6 months after discontinuation of VKAs.

5.1.2 Secondary study parameters/endpoints

Secondary endpoints are:

- The difference in vitamin K status, quantified by the change in dp-ucMGP, measured at baseline and 6 months after discontinuation of VKAs.
- The association between desmosine and dp-ucMGP in patients who use VKAs and do not use VKAs.
- Differences in desmosine and dp-ucMGP levels between different VKORC polymorphisms.

5.1.3 Other study parameters

In addition, the following parameters will be registered, because of their possible confounding capacity:

- Gender
- Age
- INR
- Indication for VKA use
- Duration of VKA use
- BMI
- Smoking status and history
- Presence of COPD
- Presence of other pulmonary disease
- Medication
- Vitamin D supplementation
- Use of multivitamins
- Switch to DOACs or complete discontinuation of anticoagulant use

5.2 Study procedures

All participating subjects are asked to fill in a questionnaire at home and will be invited to visit the hospital at baseline and approximately 6 months after discontinuation of VKA use for taking blood samples. The first blood sample will be drawn during (one of) the latest regular control of their INR at the coagulation clinic, so this will not involve an extra visit or venipuncture. Patients will be invited to draw blood approximately 6 months after discontinuation of VKAs. This concerns an additional visit besides the regular care and patients will undergo an extra venipuncture.

Questionnaire

Patients are asked to fill in a questionnaire at home, concerning gender, age, indication for VKA use, length, height, smoking status and history presence of COPD, presence of other pulmonary disease, vitamin D supplementation, use of multivitamins and medication use, whether subjects switch to DOACs or completely discontinue the use of anticoagulants.

Vena punction:

Two non-fasting blood samples of 15mL (EDTA-plasma) and 10mL (EDTA-plasma) will be drawn at baseline. Since patients have regular INR determination at this moment, no extra venipunction has to take place. After six months one non-fasting blood sample of 15mL (EDTA-plasma) will be drawn. At this moment patients will have an additional vena punction. All samples will be collected in the Canisius Wilhelmina Hospital. EDTA-plasma will be centrifuged, divided in two portions of ~1 ml and frozen at -80°C within 2 hours after drawing. One portion will be used for quantification of vitamin K status and one for quantification of the rate of elastin degradation. DNA will be isolated from the samples of 10mL, which will be divided in two portions and frozen at -80°C.

Measurement of plasma desmosine levels:

The rate of elastin degradation will be quantified by measuring plasma (p)DES levels. Subjects with the highest pDES are assumed to have the highest rates of elastin degradation. Isodesmosine and desmosine fractions are measured separately by liquid chromatography-tandem mass spectrometry as previously described using deuterium-labelled desmosine as internal standard ({ HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed/?term=17494786" }; { HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed/?term=23727558" }).

Measurement of plasma dp-ucMGP levels:

Plasma dp-ucMGP will be measured using a dual-antibody test based on the previously described sandwich ELISA developed by VitaK (Maastricht, The Netherlands) ({ HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed/?term=18401181" }). The intra- and inter-assay variations of this ELISA are 7.6% and 6.8%, respectively, and its sensitivity is 50 pmol/L. Dp-ucMGP levels are very stable for up to fifteen years when frozen at -80°C. The within subject variability of dp-ucMGP is also very limited; in a cohort study little change in dp-ucMGP was observed after 5-6 years.

Determination of VKORC1 polymorphisms

High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) will be used for isolation of DNA, according to the manufacturer's instructions. For genotyping the

C1173T (rs 9934438) and G-1639A (rs 9923231) single nucleotide polymorphisms of the VKORC1 gene, real-time PCR Fluorescence Resonance Energy Transfer (FRET) analyses will be performed. FRET LightMix® assays (cat.-no 40-0302-16, TIB MOLBIOL, Berlin, Germany) on the LightCycler® (Roche Diagnostics) will be used, according tot he manufacturer's protocols. The assay consists of a duplex reaction measuring the melting curves of the used specific fluorescent probes in two different channels, each with a distinct wavelenght. With each run positive (heterozygote, provided with te kit) and negative controles will be determined.

5.3 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

General criteria for withdrawal of individual subjects

- Patients should be withdrawn if VKAs are restarted before all blood samples are drawn.
- Patients should also be withdrawn at any time if the investigator concludes that it would be in the patient's best interest for any reason.
- Patients may voluntarily withdraw from the trial for any reason at any time. They may be considered withdrawn if they state an intention to withdraw, fail to return visits, become lost to follow up for any other reason, or if any of the following occurs: discovery of failure of randomization; discovery of patient ineligibility; errors in treatment compliance; missed/unscheduled/off-schedule/incomplete/incorrect assessments.

If premature withdrawal occurs for any reason, the investigator must determine the primary reason for the patient's premature withdrawal from the trial and record this information in the CRF.

5.4 Replacement of individual subjects after withdrawal

Up to a maximum of 5 subjects who withdraw from the study will be replaced.

5.5 Follow-up of subjects withdrawn from treatment

Patients who prematurely withdraw from the study will be scheduled for an 'end-of-study visit' as soon as possible. At the end of study visit the exact date and the primary reason for withdrawal will be noted in the CRF.

5.6 Premature termination of the study

If failure to enroll sufficient patients occur, premature termination of the study might be warranted. No special actions are needed in case of premature termination of the study. Premature termination of the study will not have any negative implications for the study participants.

6. SAFETY REPORTING

6.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

6.2 AEs, SAEs and SUSARs

6.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

Participants may experience some discomfort during blood sampling.

6.2.2 Serious adverse events (SAEs)

Since this is an observational study, serious adverse events are not expected and will therefore not be reported.

6.3 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

7. STATISTICAL ANALYSIS

7.1 Primary study parameter(s)

The primary endpoint is the change in plasma desmosine levels. Plasma desmosine levels will be determined at baseline and 6 months after discontinuation of VKAs. Univariate analysis (ANCOVA) will be used for comparing plasma desmosine levels before and after discontinuation of VKAs. Results will be corrected for the following covariates (if statistical significant): age, gender, INR, indication for VKA use, duration of VKA use, BMI, smoking status and history, presence of COPD or other pulmonary disease, vitamin D supplementation, use of multivitamins and use of medication. A $P<0.05$ will be considered statistically significant. Statistics will be performed using SPSS (SPSS Inc., Chicago, Illinois, USA).
Both desmosine and dp-ucMGP levels will be log-transformed before performing statistical analysis.

7.2 Secondary study parameter(s)

The change in plasma dephosphorylated uncarboxylated matrix Gla-protein at baseline and 6 months after discontinuation of VKAs will be a secondary study parameter. Univariate analysis (ANCOVA) will be used, corrected for the following covariates (if statistical significant): age, gender, INR, indication for VKA use, duration of VKA use, BMI, smoking status and history, presence of COPD or other pulmonary disease, vitamin D supplementation, use of multivitamins and use of medication. Univariate analysis will be used to assess the relationships between dp-ucMGP and desmosine and Pearson's rho will be calculated. To assess the differences of both pDES and d-ucMGP levels between the various VKORC1 polymorphisms, univariate analysis will also be used, corrected for the same covariates as listed above.
Both desmosine and dp-ucMGP levels will be log-transformed before performing statistical analysis.

8. ETHICAL CONSIDERATIONS

8.1 Regulation statement

This Study will be conducted according to the principles of the Declaration of Helsinki, 64th WMA General Assembly, Fortaleza, Brazil, October 2013, and in accordance with the 'Medical Research Involving Human Subjects Act' (WMO).

8.2 Recruitment and consent

We will identify users of VKAs who are going to stop the use of VKAs from the anticoagulant clinic in the Canisius Wilhelmina Hospital. The anticoagulant clinic has an overview of patients who will discontinue the use of VKAs. We will not use medical records of patients before the patient has signed informed consent. Patients will receive an information letter and can contact the investigators for more information by email, mail or telephone.
The patient will get sufficient time to consider participation in this study to ask questions. If the patient wants to participate in the study, the patient can sign informed consent (two-fold) and thereafter initiate study participation.

8.3 Benefits and risks assessment, group relatedness

Participating in the study has no/marginal risks.

8.4 Compensation for injury

We wish to obtain dispensation from the statutory obligation to provide insurance, because participating in the study is without risks. We will make a reasoned request to the accredited METC.

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

8.5 Incentives

Participants will receive a reimbursement for their travelling expenses only for their second additional visit (€ 0.19/km).

9. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

9.1 Handling and storage of data and documents

Patient Information will be handled with care, taking into consideration the required confidentiality as stated by the Dutch 'Wet Bescherming Persoonsgegevens' ('Law for the Protection of Personal Information'). All patients will be coded by a subject number. A separate registry will involve the subject numbers and their corresponding data. Only the principal investigator is permitted access to this registry.

Research documents, from which patient identity can be deduced will only be accessible for third parties (for example METC or inspection by competent authorities)

9.2 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

9.3 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

9.4 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

9.5 Public disclosure and publication policy

After analysis, data will be used for scientific presentations and for writing manuscripts which will be submitted to international peer-reviewed scientific journals for publication. All data will be reported anonymously. There are no special arrangements made between the sponsor and the investigator concerning the public disclosure and publication of the research data.

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