PROTOCOL TITLE: ***'Evaluation of biomarkers in VTE study; the EVA study.***

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PROTOCOL SIGNATURE SHEET

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LIST OF ABBREVIATIONS

CI		Confidence interval
CDR		Clinical Decision Rule
CRP	-	C-reactive protein
CTPA	-	Computed tomography pulmonary angiography
DVT	-	Deep venous thrombosis
FU	-	Follow-up
GP	-	General Practitioner
METC	-	Medical Ethics Committee (medical ethics review board)
PE	-	Pulmonary embolism
POC	-	Point-of-Care
TAT	-	Thrombin AntiThrombin complex
VTE	-	Venous thromboembolism

SUMMARY

Rationale: Venous thrombo-embolism (VTE), i.e. deep vein thrombosis (DVT) or pulmonary embolism (PE), poses a major diagnostic challenge for the general practitioner (GP) because signs and symptoms can be non-specific and even often quite minimal. The diagnostic work-up starts with scoring a clinical decision rule (CDR). If the CDR yields a low score (low VTE probability) a negative D-dimer test result can safely rule-out VTE without referral for imaging. However, the usability of this diagnostic approach is hampered in two clinical situations. First, D-dimer levels increase with increasing age (more false positives) and recently an age adjusted cut-off level for D-dimer test results was proposed to increase the diagnostic yield of D-dimer (i.e. better rule-out VTE) in elderly patients. Second, the most important differential diagnosis of VTE is an infectious disease (community-acquired pneumonia in the case of a primary suspicion of PE, or erysipelas in the case of a primary suspicion of DVT). In these cases, due to inflammation, D-dimer levels are also increased, in the absence of VTE, again decreasing the diagnostic yield of D-dimer.

Objectives: The primary objective of this study is to perform a clinical and analytical validation of novel point-of-care (POC) D-dimer assays, in particular regarding their ability to rule-out VTE using an age-adjusted D-dimer cut-of. Secondary objectives are evaluating the added diagnostic information as obtained from inflammatory biomarkers (C-reactive protein and procalcitonin). Finally, we want to evaluate a novel biomarker for coagulation that has recently been developed (e.g. thrombin-anti-thrombin complex; TAT). We hypothesize that TAT-levels more accurately predict actual coagulation, and thus likely suffer less from false positive findings due to ageing or concurrent infectious diseases.

Study design: Prospective cohort study in patients suspected of VTE in a primary care setting, in whom the GP considers ruling-out VTE. All patients routinely undergo the CDR followed by a D-dimer testing in case of a low CDR-score. The experimental intervention under study is limited to the sampling of blood additional to a routine-care blood sample in order to perform POC D-dimer, POC inflammatory biomarkers, and TAT-levels. Next, the samples are stored centrally at the UMC Utrecht for potential future analyses.

Study population: Consecutive patients suspected of VTE with a low CDR value, sent by the GP to a primary care laboratory for a diagnostic D-dimer test.

Number of participants: 500 patients with a low score on the CDR.

Time frame: 6-9 months inclusion and 3 months follow-up (total study duration 12 months).

Main outcomes: The main purpose of the study is to validate (for ruling-out VTE) novel commercially available POC D-dimer tests. Secondary outcomes are the evaluation of the added diagnostic information from inflammatory or coagulation biomarkers.

Protocol of the Evaluation of biomarkers in VTE study; the EVA study.

1. INTRODUCTION AND RATIONALE

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are two expressions of Venous Thrombo-Embolism (VTE). They pose a major diagnostic challenge for the general practitioner (GP) because signs and symptoms can be non-specific and even often quite minimal, e.g. mimicking a simple respiratory infection. Consequently, only 10-15% of the patients who are referred to the hospital to undergo diagnostic imaging procedures are ultimately diagnosed with the condition.

Therefore, the use of a Clinical Decision Rule (CDR) score in combination with a D-dimer test in primary care is recommended.[1] Referral for imaging in a hospital setting is only recommended for those with a high score on the CDR, and those with a positive D-dimer test (regardless of the CDR-score). This approach is validated as a safe diagnostic strategy in suspected patients to refrain from referring nearly half of the patients and is recommended in current guidelines.[2-5]

However, there are two important issues to resolve. First, the D-dimer test that is currently applied for use in the GP office is a qualitative Point-of-Care (POC) D-dimer test with a fixed cut-off value. In elderly patients, though, D-dimer levels are often raised as a mere consequence of ageing, thus in the absence of VTE (i.e. false-positive D-dimer elevation). For ruling-out VTE it is therefore more efficient to use a quantitative POC D-dimer test so that this ageing effect can be incorporated, e.g. by using age adjusted D-dimer cut-off values.[6] The use of such an age-dependent cut-off has – until now – been only evaluated retrospectively and in one prospective cohort study in a secondary care setting.[7] Its use has not been tested in primary care patients, and certainly not with a POC quantitative D-dimer test. This is needed prior to widespread implementation in daily primary care, given that differences in case-mix between primary and secondary care may interfere with the transferability of study findings between both settings of care.[8] Second, a large proportion of patients in whom PE cannot be ruled-out because of an elevated D-dimer level, have a lower respiratory tract infection and similarly most patients suspected of having DVT turn out to have erysipelas instead.[9, 10] Analogue as what is observed with ageing, D-dimer levels also increase in the presence of infectious diseases, thus in the absence of VTE. Hence, in both situations – ageing and infectious diseases – the use of a fixed cut-off value largely diminishes the diagnostic value of D-dimer testing due to false-positive findings and thus the usability of the diagnostic approach as a whole.

Therefore the diagnostic algorithm to rule out VTE needs refinement by including an age-dependent use of D-dimer testing plus other inflammatory biomarkers to the procedure to better categorize patients. If such an updated algorithm is proven safe, the number of referrals for compression ultrasound and CT pulmonary angiography (CTPA) can be lowered. This is especially important for elderly patients suspected of VTE to raise the

efficiency of excluding VTE and lower the burden of referring frail patients and diminishing the risk of side effects such as contrast nephropathy due to CTPA. But in terms of societal impact and cost-conscious use of healthcare budget, the importance of improving the diagnostic strategy is important for other patient groups as well.
Thus, **the primary objective** of this study is improving the diagnostic approach in patients suspected of VTE in primary care by validating novel quantitative POC D-dimer tests to allow for an aged-dependent interpretation of D-dimer values. Secondary objectives are i) evaluating the additional diagnostic information obtained from inflammatory POC biomarkers such as C-reactive protein (CRP) and procalcitonin (PCT) into the diagnostic (regression) model, and ii) evaluation of a novel coagulation biomarker that in the future may improve the diagnostic strategy even further, namely thrombin-anti-thrombin complex (TAT).
Hereto, we propose a **prospective cohort study** of 500 patients suspected of VTE in primary care: the *EVAluation of biomarkers study in VTE,* **EVA-study**. Only patients with a low score on the CDR are included, as in those patients the GP may potentially enable a safe exclusion of VTE without referral. As explained earlier, all patients with a high score on the CDR are referred for imaging, regardless of D-dimer testing (or results of any biomarker), given that the pre-test probability in these patients is too high to enable a safe exclusion in the GP office using biomarkers. The only experimental intervention under study will be taking an additional blood sample (40 milliliter) to i) validate and analyze novel quantitative POC D-dimer tests (i.e., for use of age-dependent D-dimer testing in primary care), and ii) validate other biomarkers for inflammation and coagulation. Additional blood will be stored centrally at the "biobank" of the UMC Utrecht for future research of new available tests and for other cardiovascular research. Patient management in this study will be guided by routine laboratory based D-dimer testing, and will be completely based on current guideline recommendations. Within this cohort, we aim to answer **the following research questions**:

1. What is the analytical and clinical diagnostic performance of novel commercially available quantitative point-of-care D-dimer tests in patients suspected of VTE in primary care, in particular regarding the use of an age-adjusted cut-of value?
2. What is the additional diagnostic information as obtained from a clinical decision rule of point-of-care biomarkers of inflammation, such as C-reactive protein (CRP) and procalcitonin (PCT)?
3. What is the additional diagnostic information as obtained from a clinical decision rule of a novel biomarker of coagulation, namely thrombin-anti-thrombin complex?

2. OBJECTIVES

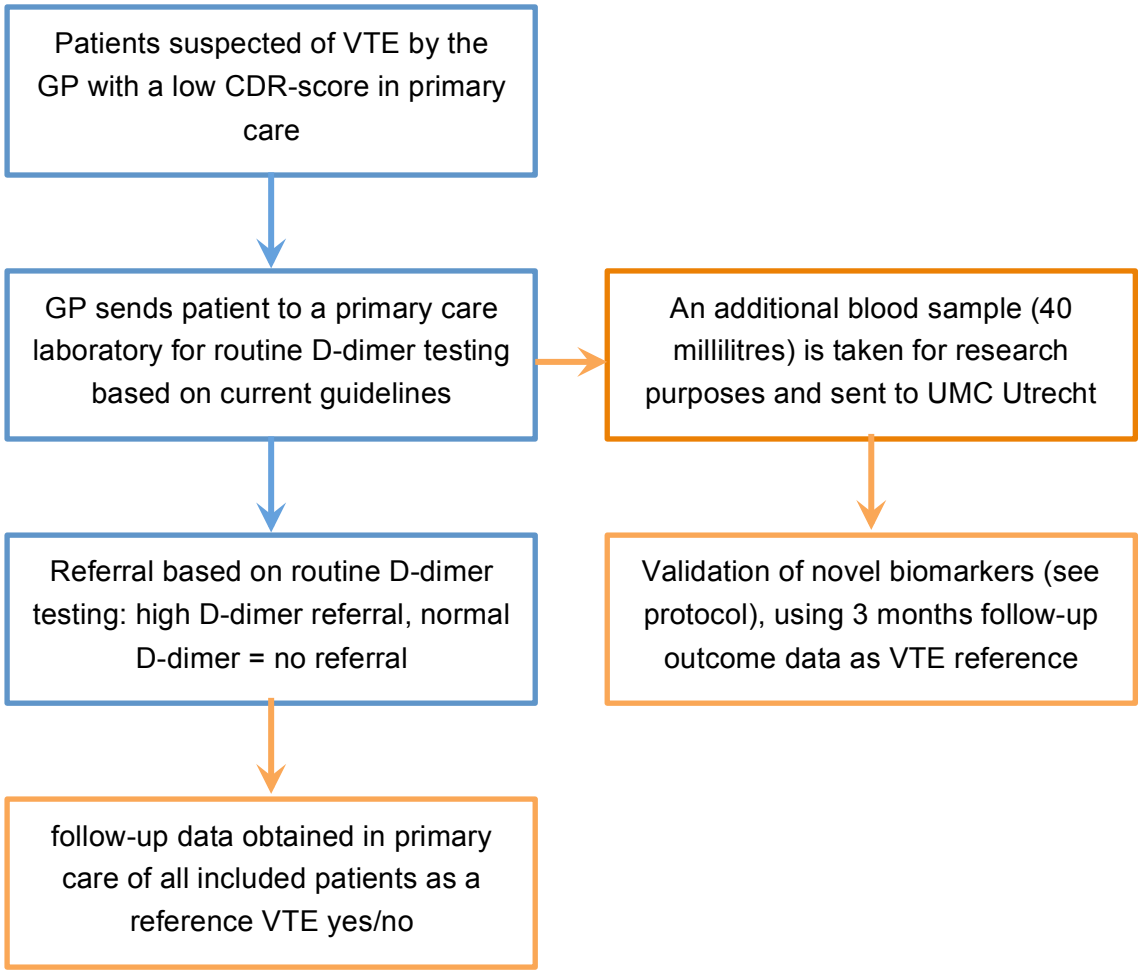
The objectives of this study are twofold:

- First, we want to evaluate the analytical and diagnostic performance of novel quantitative POC D-dimer tests, in particular regarding the use of an age-adjusted cut-of value for D-dimer testing in primary care patients suspected of VTE.
- Second, we want to evaluate novel other biomarkers for inflammation and coagulation on their added diagnostic value for ruling-out VTE in primary care.

3. STUDY DESIGN

This is a prospective cohort study in patients suspected to have DVT and/or PE with a low score on the CDR in primary care with 3 months of follow-up (VTE reference). See flowchart.

Flowchart of study design; boxes in blue are routine care procedures; boxes in oranges are study procedures.



4. STUDY POPULATION

4.1 Population

All subjects with a suspicion of having DVT or PE are assessed by the GP for risk estimation to rule-out VTE. Only patients with a low score of the CDR are sent to the laboratory for D-dimer testing and are the target patients for the study. Patients with a high score on the CDR will be referred directly for imaging to the hospital, and these patients will not be included into this study given that (any) biomarker testing will not enable a safe exclusion of VTE in primary care. All patients receive written information regarding study purposes. Given the minimal risk involved with participating in this study (only sampling of 40 milliliters of blood from the same venipuncture as used for the routine D-dimer test), as well as the complex logistics regarding additional blood sampling for an acute condition (VTE) by a very large group of GPs, we propose a modified informed consent procedure, which we will describe in more detail in section 9.

4.2 Inclusion criteria

Patients are eligible for the study if:

- The GP has a suspicion of DVT or PE, i.e. unexplained pain, swelling, and/or redness of the leg in case of DVT, or unexplained shortness of breath and pain when breathing in case of PE.
- Patients have a low score on the CDR, and thus the GP aims to rule-out VTE (if possible) using routine care D-dimer testing.

4.3 Exclusion criteria

Exclusion criteria are:

- Age below 18;
- Already using anticoagulant treatment with vitamin K antagonists, Non vitamin K Oral Anti Coagulants (NOAC) and/or low molecular-weight heparin (LMWH) for other reasons than VTE;
- Life expectancy less than 3 months.
- Unwilling to participate with this study (see section 9).

4.4 Sample size calculations

In our previous studies, the risk of having DVT in patients with a low score on the CDR and with a negative results on the D-dimer test (this proportion of missed VTE cases is often referred to as the failure rate of the diagnostic strategy) was 1.4% (95% CI: 0.6% to 2.9%) in our AMUSE-1 study on DVT [11] and the failure rate for having pulmonary embolism (PE) was 1.5% (95% CI: 0.4% to 3.7%) in the PE study (AMUSE-2).[2]

Our primary research aim is to generate evidence that the new POC D-dimer test at least has a similar failure rate as in the previous DVT study, so the expected percentage is 1.4%. An upper margin of the 95% CI around this failure rate of 3.0% is internationally deemed as an acceptable safety margin for ruling out VTE. Using this upper limit of 3.0% of a one-sided confidence interval (CI), we need to include 300 patients with a low score on the CDR and a negative result on the new POC D-dimer test. Hence, we need to include at least 500 suspected patients with a low score on the CDR, as the expected proportion of patients that will have a negative D-dimer in this group is 60%, based on our previous studies and meta-analysis.[3]

5.TREATMENT OF SUBJECTS

Based on the CDR score and D-dimer test results of the regional laboratory (usual care), the GP will refer patients or not for further diagnostic procedures, following standard procedures and in accordance with guideline recommendations. As such, no experimental treatment or intervention is evaluated in this study, other than taking an additional blood sample for laboratory validation purposes, as explained in this proposal.

6.PATIENTS AND METHODS

6.1 Patients

The GP determines the pre-test probability for DVT or PE by filling out a case-record-form including information on the CDR to calculate the score, as recommended by current guidelines, also for primary care.

Patients with a low CDR score are routinely sent to a primary care (clinical) laboratory for blood collection and D-dimer testing. With the routine-care D-dimer test result, the GP decides to refer or not to refer the patient for further objective diagnostic (i.e. imaging) procedures, again as based on current guidelines.

For study purpose, an extra blood sample is taken from the same vena-puncture in all these study patients with a low CDR score to determine additional POC D-dimer and inflammation or coagulation marker levels in a central laboratory.

Besides the clinical information regarding the CDR-score, this case-record-form only includes gender, age and information regarding the GP self (name, address). The GP gives a code to the patient administration and the case-record-form for retrieval of the patient. Thus, the case-record-form does not include the name, date of birth or address of the patient, as the above-described information is sufficient to obtain follow-up information (see section 6.7).

With this case-record-form, the patient will visit the primary care laboratory where – if willing to participate with this study – he/she presents the (signed) study form to the laboratory

worker. Here, next to the routine D-dimer test, additional blood is sampled for this biomarker study. Subsequent patient management is based on the result of the routine-care D-dimer test. All case-record forms are collected by each participating laboratory and ultimately are sent together with the blood samples to the investigators at the UMC Utrecht. After three months and one year follow up, the GP is asked to provide the investigators of the information if the clinical diagnosis of (recurrent) DVT and/or PE was present (see section 6.7).

6.2 Blood collection

Venous whole blood samples will be drawn from the anterior cubital vein into four 10 ml standard tubes for obtaining neutral, citrate (3.2%), EDTA and Li-heparin plasma after centrifugation at 2500 x *g* for 10 minutes at 21^{o}C within 4 hours after collection, aliquoted, and stored at -80^{o}C until analysis. The aliquots are labeled with unique number, and an extra label with this number is attached to the case-record-form. The frozen plasma specimens are sent at regular intervals to the central research laboratory (UMC Utrecht). After thawing, POC D-dimer, POC inflammation and coagulation markers are determined in the central research laboratory, together with a reference D-dimer and inflammation marker.
The remaining blood samples are stored in the Central Biobank of the University Medical Center Utrecht for the purpose of testing future available POC D-dimer and POC inflammation markers and for medical research of yet not defined cardiovascular or thrombosis research, following the UMC Utrecht Biobank protocols.

6.3 Point of Care study assays

The POC D-dimer assays are performed in the plasma specimens according to the manufacturer's instructions by technicians who ware unaware of the results of routine D-dimer testing and CDR score by the GP. The following POC D-dimer assays are evaluated:

- Nano-Check™ D-dimer; Nano-Ditech Corporation Cranbury NJ 08512 USA
- Cobas h 232 D-dimer; Roche Diagnostics Ltd Rotkreuz Switzerland
- AQT90 D-dimer; Radiometer Benelux BV Zoetermeer The Netherlands

In addition, the following POC inflammatory biomarkers will be evaluated:

- Nano-Check™ CRP Nano-Ditech Corporation Cranbury NJ 08512 USA
- Nano-Check™ PCT Nano-Ditech Corporation Cranbury NJ 08512 USA
- AQT90 PCT Radiometer Benelux BV Zoetermeer The Netherlands

6.4 Evaluation of new biomarkers

In this study, we will measure a novel biomarker of active coagulation, namely complexes of the enzyme thrombin and its primary inhibitor anti-thrombin (TAT complex). Thrombin is the final enzyme of the coagulation reaction and is responsible for the cleavage of fibrinogen, resulting in fibrin formation. During the formation of a fibrin clot, new thrombin is continuously produced and subsequently inhibited by the serine protease inhibitor anti-thrombin. An elevated concentration of TAT-complexes reflects an active coagulation process. The department of clinical chemistry and hematology of the UMC has developed a nanobody that specifically captures these complexes. We will use a test based on this nanobody to determine TAT-levels. We hypothesize that TAT-levels more accurately predict the actual coagulation status, and thus suffer less from false positive interferences due to ageing or inflammation (as is the case with routine-care D-dimer testing).

6.5 Blinding

The GP decides on the result of CDR and the routine lab D-dimer result to refer or not to refer the patient to imaging. The laboratory worker who performs the POC tests is blinded for referral and for the (routine-care) lab test results.

6.6 Withdrawal of individual subjects

Patients objecting to the use of their blood sample for study purpose will not provide the (signed) case-record-form to the laboratory personnel. As such, extra blood samples will therefore not be taken and the patient will not take part in the study. Patients willing to withdraw their consent can do so at any time. In those cases, the additionally sampled blood will be destroyed.

6.7 Follow up

Three months after the blood taken, the GP is asked to provide the information about the patient if DVT and/or PE were diagnosed during this follow up period, similar as done in previous studies, including our own.[2, 11] This is done by sending a follow-up form to each GP that included a patient for this study. This form includes the gender, age and the code given by the GP of the included patient. Based on previous studies, we know that this information is sufficient and safe to make a unique match with a patient enlisted with the corresponding GP practice, that thus enables the GP to provide us with outcome data on VTE presence (yes/no) without revealing privacy information (name, address, etc.) to the researchers.

7.SAFETY REPORTING

There will be no safety reporting for this laboratory research. The treatment of patients will be based upon care as usual, and the associated risk for the patients is only minimal.

8.STATISTICAL ANALYSIS

8.1 Analytical validation of POC D-dimer and inflammatory biomarkers

For analytic comparison, Passing Bablok linear regression analysis will be used and the Pearson product-moment correlation coefficient r will be calculated in the clinically relevant range up to twice the highest age-adjusted cut-off value (i.e., 1600 µg/L). The association between the various (POC) D-dimer assays will be calculated with the Spearman's correlation coefficient. Next, for the POC D-dimer and inflammation marker assay, the within-day and between-day coefficients of variation (CVs) will be measured using the clinical and laboratory standards EP5 and EP9 guidelines. The routine lab procedure will be used as the analytical reference method.

8.2 Diagnostic validation of POC D-dimer assays

To quantify the diagnostic power to (safely) rule out VTE, we will calculate the failure rate, plus corresponding 95% CI (using Fischer's exact test) of each POC D-dimer assay, both for using a fixed cut-of value of 500 ng/ml and for using an age-dependent cut-of, which is age x 10 when age above 50 years of age.[6] The failure rate is defined as the proportion of patients diagnosed with VTE during 3 months of follow-up in those with D-dimer below the chosen threshold in our study patients (i.e. those with a low score on the CDR). A point estimate for this failure rate below 2% with an upper margin for the corresponding 95% of maximally 3.5% is deemed as a safe tool for ruling-out VTE.

8.3 Added diagnostic information as obtained from inflammatory or coagulation biomarkers.

The added diagnostic information from inflammatory or coagulation biomarkers will be quantified by using multivariable logistic regression analysis, with VTE presence as the binary outcome of the model. Various logistic models will be constructed. In a first basic model, (inflammatory and/or coagulation) biomarkers will be added to a model including all items from the CDR plus the results from D-dimer testing. Next, this model will be expanded using interaction terms for biomarker results with D-dimer testing, CDR score, gender and/or age. Biomarker results will be added as continuous variables, after checking linearity assumptions.

For all analyses, IBM SPSS version 20 will be used, and statistical significance will be tested two-sided and defined as a p-value <0.05.

9.ETHICAL CONSIDERATIONS

9.1 Regulation statement

The study will be conducted according to the principles of the Declaration of Helsinki (latest version as adopted by the 59th WMA assembly, Seoul October 2008) and in accordance with the Medical Research Involving Human Subjects Act (WMO).

9.2 Recruitment and consent

Patients will be recruited from GPs affiliated to primary care laboratories that are informed on the study procedures for taken extra blood samples. Patients are referred to the laboratory for routine-care D-dimer measurements for ruling-out VTE (thus the group of patients with a low score on the CDR). The GP will inform the patients that participation in the study will mean donation of an extra blood sample, and provide them with a short leaflet explaining the study. The GP stresses that participation will be completely voluntarily and by no means will interfere with routine care patient management. If – after reading the leaflet containing study information and purpose – the patient has additional questions, the GPs refers to the researchers, who are available by mobile phone during the phase of patient recruitment of this study. Instead of a full informed consent procedure in all study patients, we propose a modified procedure for a few reasons:

- There is a minimal burden and risk for patients participating in the study, as the only intervention is to take an additional blood sample of 40 ml from the same vena-puncture for a regular D-dimer test. Patient management will be completely guided by current guidelines.
- Clinical follow-up information is retrieved in an encrypted way (see section 6, and in particular section 6.7) from the GP. No privacy sensitive information, such as name, date of birth or address, is collected or available for the researchers.
- The study is of great social value, as it will enable a better way to rule-out the diagnosis of VTE in primary care and refrain more patients (especially elderly) from referring for imaging procedures in a hospital setting.
- When VTE is suspected, immediate D-dimer testing (with additional blood taken for study purpose) is essential. The GP has not an independent relation with the patient and thus is not in the position to ask informed consent, given the longstanding relationship between the GP and his/her patient. Moreover, in the daily workflow of laboratory workers asking for a written informed consent is not feasible, in particular also regarding the educational background of these laboratory workers. The logistics

in this (semi) acute-care setting, as well as the large number of GPs willing to participate in this study (nation-wide), prevents us as researchers to carry out a full informed consent procedure in all study patients.

Therefore, given these arguments, we propose a modified method to inform patients. All patients will receive a leaflet explaining the purpose of the study. If, after reading the information on the study, the patient decides he/she does not want provide an extra blood sample for study purposes, he/she gives only the standard laboratory form and not the additional (signed) case-record-form to the laboratory personnel. Additional blood sample will only be collected in those presenting this signed study case-record-form to laboratory workers. As such, the patient only participates in the study when he/she is willing to do so, as patients not presenting this signed case-record-form will not be included in this study.

10.ADMINISTRATIVE ASPECTS AND PUBLICATIONS

10.1 Handling and storage of data and documents:

All data will be stored by the data management department of the Julius Center for Health Sciences and Primary Care (UMC Utrecht) and kept for 15 years. Data will be stored anonymously as data regarding patient identification will be replaced by a patient study ID number. Data will (only) be analysed by the investigators of the study.

10.2 Monitoring and Quality Assurance

There will be no monitoring of the study. After consultation of the Quality staff, we decided to refrain from a monitor plan because of:

- The minimal and single intervention with negligible or no risk and burden for the patient.
- No privacy risk as the GP codes already the patient information.
- Monitoring of patient data is only possible with decoded information from the GP, which is not available to the researchers.
- Laboratory examination itself is usual technique and not subject of research.

10.3 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.

10.4 Annual progress report:

There will be no annual report considering the short study duration (maximum of 12 months).

10.5 End of study report:

There will be only a final study report considering the short study duration.

10.6 Public disclosure and publication policy:

The researchers will seek publication of their results in international peer reviewed journals. There have been no restrictions placed upon publication by the sponsors (Nano-ditech and Roche Diagnostics) of this study.

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