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Complete List of Authors:	Dehnen, Dorothea; University of Duisburg-Essen Faculty of Medicine Neuberger, Elmo; Johannes Gutenberg Universitat Mainz, Department of Sports Medicine, Rehabilitation and Disease Prevention in der Schmitten, Jürgen; University of Duisburg-Essen Faculty of Medicine Giagkou, Ekaterini; University of Duisburg-Essen Faculty of Medicine Simon, Perikles; Johannes Gutenberg Universitat Mainz, Department of Sports Medicine, Rehabilitation and Disease Prevention Botzenhardt, Suzan; University of Duisburg-Essen Faculty of Medicine,
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cfDNA as a predictive marker for the prognosis of severe courses in patients with influenza-like symptoms with and without SARS-CoV-2 infections in general practice: A prospective cohort study

Dorothea Dehnen[1], Elmo Neuberger[2], Jürgen in der Schmitten[1], Ekaterini Giagkou[1], Perikles Simon[2], Suzan Botzenhardt[1*]

[1]Institute of General Practice, Medical Faculty, University of Duisburg-Essen, Essen, Germany

[2]Department of Sports Medicine, Rehabilitation and Disease Prevention, Johannes Gutenberg University of Mainz, Mainz, Germany

***Corresponding author**

Suzan Botzenhardt, PhD

Institute for General Practice

University Hospital Essen

Hufelandstr. 55

45147 Essen / Germany

Tel: +49-201-723-8036, Fax: +49-201 723-8044

Email: suzan.botzenhardt@uk-essen.de

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Abstract

Introduction: The clinical course of patients with a SARS-CoV-2 infection varies widely, from symptom-free to severe courses that can lead to death. Laboratory values of SARS-CoV-2 patients such as lymphocyte counts or C-reactive protein (CRP) do not allow a prediction of the actual course of the disease. To identify a possible predictive marker for the differentiation and prognosis of illness with influenza-like symptoms with and without SARS-CoV-2 infections in general practice we will analyse the concentrations of cell-free DNA (cfDNA) levels, laboratory and clinical parameters, temperature, oxygen saturation, breathing rate and concomitant symptoms in patients with flu-like symptoms with and without a SARS-CoV-2 infection.

Methods and analysis: This is a single-centre, two-arm, parallel longitudinal cohort study with a total of 44 patients. 22 patients with flu-like symptoms without a SARS-CoV-2 infection and 22 patients with flu-like symptoms with a SARS-CoV-2 infection will be recruited by their general practitioners. The primary objective is to compare the concentrations of cfDNA levels in ambulatory patients in general practice with flu-like symptoms with and without a SARS-CoV-2 infection during the disease (day 7 and day 14). The secondary objective is to determine whether there is a correlation between cfDNA concentrations on the one hand, and laboratory and clinical parameters on the other hand. cfDNA, hsCRP and ESR will be measured in blood samples, concomitant symptoms will be surveyed via a self-assessment questionnaire, and oxygen saturation, breathing rate and examination of the lungs will be reported by treating physicians.

Ethics and dissemination: Ethical approval was issued on 2021/03/01 by the Ethics Committee Essen under the number 21-9916-BO. Informed consent will be obtained from all participants in this study. The study was registered in the German Clinical Trials Registry (DRKS) in March 2021 under the number DRKS00024722.

Article Summary

Strengths and limitations of this study

- Measurement of cfDNA is cost-effective and requires only minimal amounts of blood, which in the future can be collected in the primary care physician's office.
- Both clinical and serological parameters are collected in a setting (family practice) where patients with mild or moderate symptoms are predominantly treated, but where no suitable prognostic markers are available at this time.

- The success of the study should be ensured by the close follow-up and home visits of the patients by the general practitioner and the short duration of the study.
- cfDNA is already a well-established biomarker that is associated with various diseases and has been used in different research areas, such as oncology, non-invasive prenatal diagnosis, organ transplantation, autoimmune diseases, trauma, coronary heart disease and diabetes.
- Limitations of the study include that the patients with a SARS-CoV-2 infection could have a severe disease course requiring hospital treatment, which could eventually lead to a study drop-out.

Keywords: SARS-CoV-2 infection, cell-free DNA (cfDNA), flu/influenza, predictive marker, general practice.

Introduction

The current SARS-CoV-2 (severe acute respiratory syndrome coronavirus type 2, COVID-19) pandemic is confronting humanity with a new dimension of medical, economic and social problems [1]. Among humans, the virus spreads rapidly and causes varying degrees of severity of symptoms and illness in patients [2, 3]. Thus, the clinical course of patients with a SARS-CoV-2 infection can vary widely, from symptom-free courses to severe courses that can lead to death. The incubation period of the virus ranges from 1 to 14 days, the duration of viral excretion can last from 8 to 37 days, and the time from disease onset to discharge or death ranges from 15-25 days [4, 5]. Furthermore, it has been shown that mortality rates correlate with increasing age and pre-existing concomitant diseases, such as cardiovascular disease, diabetes, overweight and hypertension [2, 3, 6]. In the course of analysing laboratory values of SARS-CoV-2 patients, such as measurements of lymphocyte counts, C-reactive protein (CRP), as well as secondary bacterial infections it was found that the analysis and evaluation of these do not allow an assessment of the actual course of the disease [7, 8]. In order to be able to better assess the course of this disease, it would be important to find a predictive marker that could be used to determine the severity of the disease at the earliest possible stage.

One marker that could play an important role in this determination is cell-free DNA (cfDNA), which is usually released from cells by apoptosis, necrosis, NETosis, as well as actively [9, 10]. It comprises a high variability of fragmented molecules that contain valuable information about gene expression and the nucleosome pattern in relation to their tissue of origin [11–13]. Numerous studies have already demonstrated that cfDNA levels are associated with various diseases and have been used in various research areas, such as oncology, non-

invasive prenatal diagnosis, organ transplantation, autoimmune diseases, trauma, coronary heart disease and diabetes [11–16].

Recent studies have investigated the role of cfDNA as a potential marker for therapeutic targets of SARS-CoV-2 in order to develop new therapeutic strategies for the disease [17]. In their study, Chen et al. systematically analysed whole-genome sequencing (WGS) of cfDNA in patients with mild and severe SARS-CoV-2 courses to explore the clinical value of cfDNA in SARS-CoV-2. They showed that significantly different levels were measured between mild and severe SARS-CoV-2 courses in patients, which could indicate the involvement of potential genes, tissues and signalling pathways in disease progression and severity [18]. In a further study, Andargie et al. showed that cfDNA levels correlated positively with COVID-19 disease severity, C-reactive protein and D-dimer, and that the cfDNA profile at admission identified patients who subsequently required intensive care or died during hospitalization. They conclude that cfDNA could be used as a potential diagnostic biomarker to map sources of injury and as a prognostic biomarker to predict COVID-19 trajectory and outcome by providing mechanistic information about COVID-19-induced tissue injury [19]. However, in this study, cfDNA levels were not measured in patients before they presented at the hospital. Our study will instead focus on patients who visit their GP's office with mild flu-like symptoms. In addition to the studies on cfDNA levels and SARS-CoV-2, further studies have measured the impact of influenza on cfDNA levels. Again, it was shown that patients had significantly increased cfDNA levels [19, 20].

Methods

Design

This is a single-centre, two-arm, parallel cohort study with a 1:1 allocation ratio. 22 patients with flu-like symptoms without a SARS-CoV-2 infection and 22 patients with flu-like symptoms with a SARS-CoV-2 infection will be included (n=44, age over 18 years) (see Figure 1). Due to our study design, no randomisation is needed. Thus, no blinding will be performed.

insert Figure 1 here

Patients

Setting of the study and characteristics of participants

This single-centre study will be conducted at the University Hospital in Essen. Patients who visit their general practice in Mülheim an der Ruhr with flu-like symptoms will be asked to complete a baseline screening questionnaire prior to randomisation to assess their eligibility. If they meet the eligibility criteria, they will be included in the study. Written informed consent will be obtained by the principal investigator from all patients willing to participate in the study.

CfDNA concentrations will be determined at the Department of Sports Medicine, Prevention and Rehabilitation at the Johannes Gutenberg University Mainz using a self-established qPCR [20]. The material will be processed in an S2 laboratory.

Inclusion and exclusion criteria

All persons enrolled in the study must provide full written informed consent and are required to complete a baseline screening questionnaire prior to randomisation to assess their eligibility.

Inclusion criteria are:

1. Age 18-99 years,
2. Consent given by the patient or legal representative for blood draw, oropharyngeal or nasopharyngeal swab for a rapid SARS-CoV-2 antigen test and subsequent qPCR, if applicable,
3. Sufficient knowledge of the German language to understand the study content and instructions.

Exclusion criteria are:

1. Severe acute or chronic illness with known elevated cfDNA levels due to the underlying disease, e.g.:
    a. Tumour disease
    b. Severe renal insufficiency
    c. Severe/moderate inflammatory diseases
    d. Autoimmune diseases
    e. Rheumatological diseases

Intervention description

All patients included in this study will receive a point-of-care antigen rapid test for SARS-CoV-2 (Roche SARS-

CoV-2 Rapid Antigen Test) and a subsequent RT-PCR on the day of initial presentation. If the rapid antigen test result is negative, the swab will be tested by RT-PCR not only for SARS-CoV-2 but also for influenza in order to exclude both a SARS-CoV-2 infection and an influenza infection in the control group. Afterwards, patients will be assigned either to the group “SARS-CoV-2 positive with flu-like symptoms” or to the group “SARS-CoV-2 negative with flu-like symptoms” depending on the test results. All patients will have venous blood (22.4 mL) collected on three different visits to determine cfDNA concentrations as well as the inflammation markers hsCRP, ESR and a differential blood count (t0= day of recruitment, t1= after 7 days, t2= after 14 days). In addition, temperature, oxygen saturation and respiratory rate will be measured at all three visits and patients must complete a questionnaire about current symptoms [21].

Blood samples will be centrifuged directly at the general practitioner’s office at 1600 rpm to obtain the necessary blood plasma to avoid possible lysis of the cells. The samples will be stored at -20 °C until the required case numbers are reached and will thereafter be transported to Mainz. Once there, the samples will be stored in the freezer at -80 °C until the further analysis for the same purpose of the study objectives is performed. The cfDNA concentrations, including non-disease-specific qualitative aspects such as integrity of DNA, will be determined at the Department of Sports Medicine, Prevention and Rehabilitation at the Johannes Gutenberg University Mainz using a self-established qPCR [22]. The material will be processed in an S2 laboratory.

After three and six months, patients with a SARS-CoV-2 infection will be contacted again by telephone to inquire about possible long-term symptoms and to again determine cfDNA levels in patients who remain symptomatic.

All participants can discontinue their participation in the trial at any time for any reason without prejudice to current or future medical care. The investigator may discontinue patients’ participation in the trial for any reason for their safety or in their best interest. If participants express a desire to withdraw from the study, they will receive instructions to complete an “end-of-study” visit, which will also be voluntary.

All participants can receive any concomitant treatment at any time during the trial. However, participants must indicate at each study visit whether they are receiving any concomitant drug therapy.

In the context of this research project, the participants will be insured for potential damages of the biomaterial collection as well as for commuting accidents in accordance with § 2 Para. 1 No. 13b Social Security Code (SGB) VII. After the trial, the patients will receive their primary care by the general practitioner as usual. There is thus no need to provide any additional post-trial care.

Participant timeline

The participant timeline is presented in Table 1.

Table 1: Research timeline for each participant

Timepoint	T0	T1	T2
Consent collection	X		
Demographics, medical history, disease characteristics	X		
Point-of-care antigen rapid test for SARS-CoV-2	X		
Additional RT-qPCR	X		
cfDNA determinations	X	X	X
hsCRP, ESR, temperature, oxygen saturation and breathing rate	X	X	X
Differential blood count	X	X	X
Concomitant symptoms	X	X	X
Questionnaire	X	X	X

Outcomes

Main outcome measures

The primary outcome is the determination of cfDNA concentrations in patients with flu-like symptoms with and without a SARS-CoV-2 infection in general practice using a self-established qPCR from plasma and serum. These cfDNA concentrations will be determined at t_0 (day 0), t_1 (day 7) and t_2 (day 14) to investigate whether any changes in cfDNA concentrations occur during the course of the disease and whether there are differences in cfDNA levels between the two cohorts.

Secondary outcome measures

The secondary objective is to analyse whether there is a correlation between the cfDNA concentrations with symptoms/wellness and the severity of the disease. To this end, we will determine whether there is an

association between cfDNA concentrations and the variables mentioned below:

1. High-sensitivity CRP (hsCRP, collected at t_0, t_1 and t_2). hsCRP is a routine inflammatory biomarker and will be measured in the patients' blood samples.
2. Erythrocyte sedimentation rate (ESR, collected at t_0, t_1 and t_2) at day 0, day 7 and day 14. ESR is a routine inflammatory biomarker and will be measured in the patients' blood samples.
3. Differential blood count, especially regarding lymphocyte and neutrophil granulocytes. Lymphopenia and neutropenia appear to be associated with a severe COVID-19 course [23].
4. Temperature taken at day 0, day 7 and day 14. Several studies suggest that high fever increases the risk of ARDS and should be controlled accordingly at an early stage [24, 25].
5. Oxygen saturation measured at day 0, day 7 and day 14, because impairment of oxygen is associated with critical illness [26]. Thus, a target spO2 of 92-96 % is recommended [27].
6. Breathing rate measured at day 0, day 7 and day 14. It has long been known that determining the respiratory rate is a simple way to assess the prognosis in pneumonia or other lung diseases [28].
7. Concomitant symptoms (collected at t_0, t_1 and t_2). Concomitant symptoms will be queried via a self-assessment questionnaire [21] where the patients can report any current concomitant symptoms which then have to be rated on a Likert scale.

To assess the severity of dyspnoea, the American Thoracic Society's Dyspnea Scale will be used. The examiner will apply the WHO Clinical Progression Scale to assess the patient's general condition [29]. In addition, we want to compare cfDNA concentrations in blood plasma. Plasma is typically preferred over serum samples for cfDNA analysis, since the coagulation process further increases cfDNA values. After three and six months, the recruited patients who were SARS-CoV-2 positive will be contacted again by telephone and asked about their condition. In symptoms that could be attributed to a post-Covid-19 syndrome are present, the cfDNA concentration will be determined again in these patients.

Safety

Adverse event reporting and harms

The risk of the venous blood sampling required for the cfDNA determination can be considered minimal. As with any other venous blood draw, pain may occur during the blood draw. Bruising may also occur, especially if there is insufficient compression on the puncture site after blood collection. In very rare cases, the blood draw may result in infection of the puncture site (thrombophlebitis) or nerve injury. However, there are no

serious complications associated with blood sampling. The swab for the SARS-CoV-2 Ag rapid test and the subsequent RT-PCR will be performed by a general practitioner who is experienced in this procedure. Rarely, minor bleeding can occur during nasopharyngeal swabbing; serious injuries do not occur if the procedure is performed correctly. The investigator will assess the severity of each adverse event and will report all serious and non-serious adverse events in the electronic case report form. The investigator will also assess the causal relationship of the serious adverse events to the trial intervention. Termination criteria have not been defined, as it does not seem reasonable for the planned study with a short survey period.

Sample size calculation

With respect to the group with flu-like symptoms without a SARS-CoV-2 infection, we expect –with a relatively high variance– a twofold increase in cfDNA concentrations compared to healthy subjects. A group comparable to this can be found, for example, in the group of chronic inflammatory and currently non-acute diseases, such as systemic lupus erythematosus. In one study, we showed that lupus patients had a mean cfDNA level of 44.7 ng/mL with a standard deviation of 53.5 ng/mL [16]. We hypothesize that a SARS-CoV-2 infection will increase the levels by 100% once more compared with the cohort with influenza-like symptoms without a SARS-CoV-2 infection. Furthermore, we want to be sure enough to obtain group sizes of at least 22 participants in each intervention group (total n=44) and to adjust for any dropouts during the study. Sample size is planned by a two-sample t-test on a two-sided significance level of a=5 % to achieve a power of more than 80 %. We therefore plan to include a total of around 35 patients of comparable age and sex in both the group with a positive and the group with a negative test.

Recruitment will take place in the general practice in Mülheim an der Ruhr via the respective principal physician. We plan to include a total of 44 patients until February 2022.

Plans to promote participant retention and complete follow-up

Participants will benefit from the study as they will know immediately whether a SARS-CoV-2 infection is present via point-of-care diagnostics (Roche SARS-CoV-2 Rapid Antigen Test: sensitivity: 96.52 %, specificity: 99.68 %) which will be subsequently validated by RT-qPCR. Those with a negative result in the point-of-care diagnostic will be additionally tested for influenza. During the study, the patients will be cared for in their home environment by their general practitioner. If participants express a desire to withdraw from the study, they will be asked to complete an “end-of-study” visit. Data collected up to the time of withdrawal will remain in the trial

database and be included in data analysis, unless otherwise indicated by the participant.

Data management

Trial data will be collected in the electronic case report form by the principal investigator at the Institute of General Practice at the University Hospital in Essen. Source documents, defined as any original document or object making it possible to prove the existence or accuracy of data or facts recorded during the research, will be kept by the principal physician according to the regulations in force. All questionnaire data will be entered twice by two different persons to ensure the dual control principle. Using a software tool, a third person will check the agreement between the two datasets resulting from the double entry. In cases where entries deviate from one another, the third person will determine the correct entry by looking at the questionnaire. In cases where the questionnaire answers are ambiguous, two persons will decide what should be entered by discussion until a consensus is reached. All data concerning participant information will be stored in locked file cabinets accessible only by the principal investigator. All collected data will be pseudonymised and will therefore be traceable only by means of a code. All files containing names or other personal identifiers, such as the informed consent forms, will be stored separately from the data containing this code number.

Statistical methods

Statistical methods for primary and secondary outcomes

The statistical analysis of the data will be performed with the statistical program SPSS, R or SAS using a pseudonymised dataset. A correlation of the cfDNA concentration with the presence or absence of a SARS-CoV-2 infection, as well as a correlation of these results with the data obtained from the questionnaires will be determined. Descriptive statistics will summarise all study variables.

All data will be tested for normal distribution before and after log transformation using the Kolmogorov-Smirnov test with the Lilliefors correction. If the assumption of normally distributed data cannot be rejected, arithmetic group means ± standard deviations will be calculated. If the normality test fails in at least one of the study groups compared, all data will be expressed as group medians with data ranges given in parentheses. Between-group comparisons of primary and secondary outcomes will be performed at t_0 and at two consecutive time points independently (t_1, t_2) using unpaired Student's t-tests (comparing two groups, normally distributed) or the Mann-Whitney U test (comparing two groups, not normally distributed) or analysis of

variance (three groups) and chi-square tests for numerical and categorical data, respectively. P values will be considered statistically significant if $p \leq 0.05$. We plan to perform a subgroup analysis for the concomitant symptoms and the cfDNA levels at t_0, t_1 and t_2 in the event of differences within each arm.

Statistical analyses will be carried out according to the intention-to-treat approach and therefore will include all participants. The extent of missing data will be analysed. We will explore missing data patterns and determine the type of missing data. We will use multiple imputation to substitute missing values.

Patient and Public Involvement

No patients were involved in the development of the research questions. The results of the temperature measurement, the oxygen saturation and breathing rate of each individual patient will be communicated to the patient directly after the examination by the attending physician. The laboratory and clinical parameters and the levels of the cfDNA will be disseminated after they have been measured.

Declarations

Ethics and dissemination

Ethical approval was issued on 2021/03/01 by the Ethics Committee Essen under the number 21-9916-BO. Findings will be disseminated initially to the impacted community, then by publication in scientific journals and on international congresses.

Registration: The study is registered with DRKS under the number DRKS00024722. Informed consent will be obtained from all participants in this study.

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Author statement

DD is the principal investigator; she conceived the study, obtained funding, led the proposal and protocol development, and assisted in manuscript preparation and revision. EN contributed to the study concept and design and assisted in manuscript preparation and revision. JidS assisted in manuscript revision. EG contributed to the study concept and design and assisted in manuscript preparation. PS contributed to the acquisition and analysis of qualitative data and the development of the intervention and assisted in manuscript preparation and revision. SB contributed to the study concept and design and drafted the manuscript. All authors read and approved the final manuscript.

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Conflicts of interests

The authors declare that they have no conflicts of interests.

Consent for publication

Not required.

Data Statement

The study principal investigator and the co-investigators will have access to the full study data and materials. The authors will be willing to share the individual-level study data after completion and publication of primary and secondary analyses.

Figure Legends

Figure 1: Flowchart of the study design.

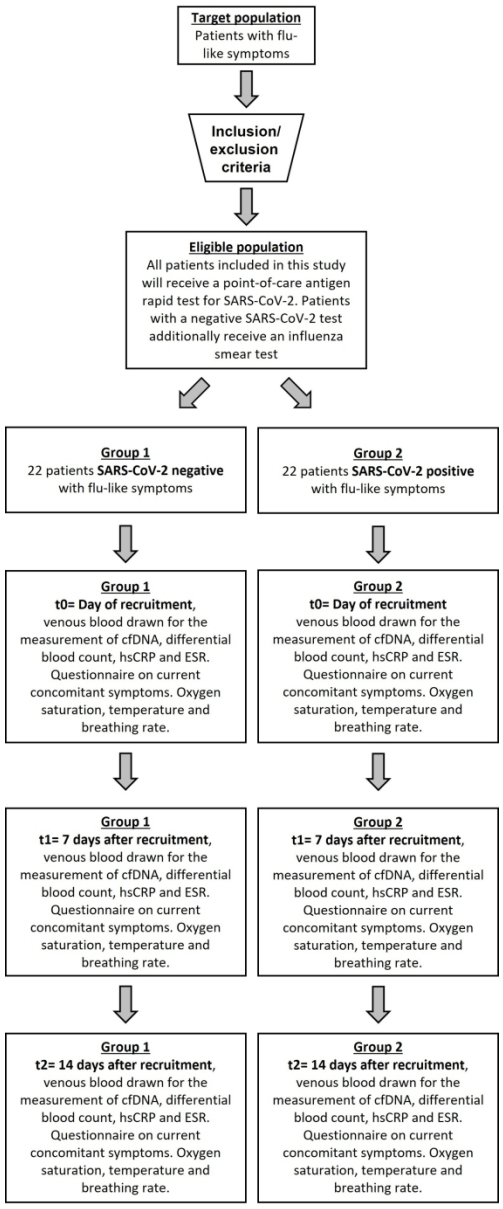

Figure 1: Flowchart of the study design.

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cfDNA as a surrogate marker for COVID-19 severity in patients with influenza-like symptoms with and without SARS-CoV-2 infections in general practice: A study protocol for a prospective cohort study

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cfDNA as a surrogate marker for COVID-19 severity in patients with influenza-like symptoms with and without SARS-CoV-2 infections in general practice: A study protocol for a prospective cohort study

Dorothea Dehnen[1], Elmo Neuberger[2], Jürgen in der Schmitten[1], Ekaterini Giagkou[1], Perikles Simon[2], Suzan Botzenhardt[1*]

[1]Institute of General Practice, Medical Faculty, University of Duisburg-Essen, Essen, Germany

[2]Department of Sports Medicine, Rehabilitation and Disease Prevention, Johannes Gutenberg University of Mainz, Mainz, Germany

***Corresponding author**

Suzan Botzenhardt, PhD

Institute for General Practice

University Hospital Essen

Hufelandstr. 55

45147 Essen / Germany

Tel: +49-201-723-8036, Fax: +49-201 723-8044

Email: suzan.botzenhardt@uk-essen.de

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Abstract

Introduction: The clinical course of patients with a SARS-CoV-2 (severe acute respiratory syndrome coronavirus type 2, COVID-19) infection varies widely, from symptom-free to severe courses that can lead to death. Laboratory values of SARS-CoV-2 patients such as lymphocyte counts or C-reactive protein (CRP) do not allow a prediction of the actual course of the disease. To identify a possible predictive marker for the differentiation and prognosis of illness with influenza-like symptoms with and without SARS-CoV-2 infections in general practice we will analyse the concentrations of cell-free DNA (cfDNA) levels, laboratory and clinical parameters, temperature, oxygen saturation, breathing rate and concomitant symptoms in patients with flu-like symptoms with and without a SARS-CoV-2 infection.

Methods and analysis: This is a single-centre, two-arm, parallel longitudinal cohort study with a total of 44 patients. 22 patients with flu-like symptoms without a SARS-CoV-2 infection and 22 patients with flu-like symptoms with a SARS-CoV-2 infection will be recruited by their general practitioners. The primary objective is to compare the concentrations of cfDNA levels in ambulatory patients in general practice with flu-like symptoms with SARS-CoV-2 infection with those with flu like symptoms without a SARS-CoV-2 infection during the disease (day 7 and day 14). The secondary objective is to determine whether there is a correlation between cfDNA concentrations on the one hand, and laboratory and clinical parameters on the other hand. cfDNA, differential blood count, high-sensitive CRP (hsCRP) and erythrocyte sedimentation rat (ESR) will be measured in blood samples, concomitant symptoms will be surveyed via a self-assessment questionnaire, and oxygen saturation, breathing rate and examination of the lungs will be reported by treating physicians.

Ethics and dissemination: Ethical approval was issued on 2021/03/01 by the Ethics Committee Essen under the number 21-9916-BO. Informed consent will be obtained from all participants in this study. The study was registered in the German Clinical Trials Registry (DRKS) in March 2021 under the number DRKS00024722.

Article Summary

Strengths and limitations of this study

- Measurement of cfDNA is cost-effective and requires only minimal amounts of blood, which in the future can be collected in the primary care physician's office.
- Clinical and serological parameters (hsCRP, ESR and a differential blood count) are collected in a setting (family practice) where patients with mild or moderate symptoms are predominantly treated,

but where no suitable prognostic markers are available at this time.

- The success of the study should be ensured by the close follow-up and home visits of the patients by the general practitioner and the short duration of the study.
- cfDNA is already a well-established biomarker that is associated with various diseases and has been used in different research areas, such as oncology, non-invasive prenatal diagnosis, organ transplantation, autoimmune diseases, trauma, coronary heart disease and diabetes.
- Limitations of the study include that patients with a SARS-CoV-2 infection could have a severe disease course requiring hospital treatment which could eventually lead to proceed with the study per protocol, which may in worst case lead to missing laboratory values at t1 and t2. However, analysis will be done according to intention to treat and hospitalisation as a main outcome will be assessed unless a patient withdraws his or her consent.

Keywords: SARS-CoV-2 infection, cell-free DNA (cfDNA), flu/influenza, predictive marker, general practice.

Introduction

The current SARS-CoV-2 (severe acute respiratory syndrome coronavirus type 2, COVID-19) pandemic is confronting humanity with a new dimension of medical, economic and social problems [1]. Among humans, the virus spreads rapidly and causes varying degrees of severity of symptoms and illness in patients [2, 3]. Thus, the clinical course of patients with a SARS-CoV-2 infection can vary widely, from symptom-free courses to severe courses that can lead to death. The incubation period of the virus ranges from 1 to 14 days, the duration of viral excretion can last from 8 to 37 days, and the time from disease onset to discharge or death ranges from 15-25 days [4, 5]. Furthermore, it has been shown that mortality rates correlate with increasing age and pre-existing concomitant diseases, such as cardiovascular disease, diabetes, overweight and hypertension [2, 3, 6]. In the course of analysing laboratory values of SARS-CoV-2 patients, such as measurements of lymphocyte counts, C-reactive protein (CRP), as well as secondary bacterial infections it was found that the analysis and evaluation of these do not allow an assessment of the actual course of the disease [7, 8]. In order to be able to better assess the course of this disease, it would be important to find a predictive marker that could be used to determine the severity of the disease at the earliest possible stage.

One marker that could play an important role in this determination is cell-free DNA (cfDNA), which is usually released from cells by apoptosis, necrosis, NETosis, as well as active secretion [9, 10]. It comprises a high variability of fragmented molecules that contain valuable information about gene expression and the nucleosome pattern in relation to their tissue of origin [11–13]. Numerous studies have already demonstrated that cfDNA levels are associated with various diseases and have been used in various research areas, such as oncology, non-invasive prenatal diagnosis, organ transplantation, autoimmune diseases, trauma, coronary heart disease and diabetes [11–16].

Recent studies have investigated the role of cfDNA as a potential marker for therapeutic targets of SARS-CoV-2 in order to develop new therapeutic strategies for the disease [17]. In their study, Chen et al. profiled and analysed for the first-time plasma cell-free DNA (cfDNA) of mild and severe COVID-19 patients. They found that in comparison between mild and severe COVID-19 patients, Interleukin-37 signalling was one of the most relevant pathways. Their data thus revealed potential tissue involvement, provided insights into mechanism on COVID-19 progression, and highlighted utility of cfDNA as a non-invasive biomarker for disease severity inspections [18]. In a further study, Andargie et al. showed that cfDNA levels correlated positively with COVID-19 disease severity, C-reactive protein and D-dimer, and that the cfDNA profile at admission identified patients who subsequently required intensive care or died during hospitalization. They conclude that cfDNA could be used as a potential diagnostic biomarker to map sources of injury and as a prognostic biomarker to predict COVID-19 trajectory and outcome by providing mechanistic information about COVID-19-induced tissue injury [19]. However, in this study, cfDNA levels were not measured in patients before they presented at the hospital. Our study will instead focus on patients who visit their GP's office with mild flu-like symptoms. In addition to the studies on cfDNA levels and SARS-CoV-2, further studies have measured the impact of influenza on cfDNA levels. Again, it was shown that patients had significantly increased cfDNA levels [19, 20]. The aim of our study is to determine if cfDNA levels differ in patients with mild or moderate flu symptoms when either SARS-CoV-2 infection or infection with another common respiratory pathogen is present or if these concentrations are similar to each other.

Methods

Design

This is a single-centre, two-arm, parallel cohort study with a 1:1 allocation ratio to be conducted between

August 2021 and April 2022. 22 patients with flu-like symptoms without a SARS-CoV-2 infection and 22 patients with flu-like symptoms with a SARS-CoV-2 infection will be included (n=44, age over 18 years) (see Figure 1). Due to our study design, no randomisation is needed. Thus, no blinding will be performed.

insert Figure 1 here

Patients

Setting of the study and characteristics of participants

This single-centre study will be conducted at the University Hospital in Essen. Patients who visit their general practice in Mülheim an der Ruhr with flu-like symptoms will be asked to complete a screening questionnaire to check if they met the inclusion criteria. If they meet the eligibility criteria, they will be included in the study. Written informed consent will be obtained by the principal investigator from all patients willing to participate in the study.

Inclusion and exclusion criteria

All persons enrolled in the study must provide full written informed consent and are required to complete a baseline screening questionnaire to assess their eligibility.

Inclusion criteria are:

1. Age 18-99 years,
2. Consent given by the patient or legal representative for blood draw, oropharyngeal or nasopharyngeal swab for a rapid SARS-CoV-2 antigen test and subsequent qPCR, if applicable,
3. Sufficient knowledge of the German language to understand the study content and instructions.

Exclusion criteria are:

1. Severe acute or chronic illness with known elevated cfDNA levels due to the underlying disease, e.g.:
    a. Tumour disease
    b. Severe renal insufficiency
    c. Severe/moderate inflammatory diseases
    d. Autoimmune diseases
    e. Rheumatological diseases

Intervention description

All patients included in this study will receive a point-of-care antigen rapid test for SARS-CoV-2 (Roche SARS-CoV-2 Rapid Antigen Test) and a subsequent RT-PCR on the day of initial presentation. Afterwards, patients will be assigned either to the group “SARS-CoV-2 positive with flu-like symptoms” or to the group “SARS-CoV-2 negative with flu-like symptoms” depending on the test results. For cost reasons, the patients in the SARS-CoV-2 negative with flu symptoms group cannot be tested for other respiratory pathogens. However, data from the Robert Koch Institute indicate the frequency of possible respiratory pathogens. Thus, in the 10th calendar week of 2022, a total of 72 (60%) of the 121 sent in sentinel samples (national reference centre influenza) identified respiratory viruses, including most frequently SARS-CoV-2 (n=32, 26 %), followed by rhinoviruses (n=20, 17 %), human metapneumoviruses (hMPV) (n=18, 15 %), human seasonal coronaviruses (hCoV) (n=6, 5%), influenza A(H3N2) viruses (n=4, 3%), parainfluenza viruses (PIV) (n=3, 2%), and respiratory syncytial viruses (RSV) (n=2, 2%) [21]. Taking into account the other weekly reports from 2022, human metapneumoviruses (7-16%), rhinoviruses (7-22%) and human seasonal coronaviruses (1-17%) dominated in addition to SARS-CoV-2. Influenza viruses in particular play a subordinate role as pathogens of respiratory tract infections (<5%) [22].

All patients will have venous blood (15.3 mL) collected on three different visits to determine cfDNA concentrations as well as the inflammation markers hsCRP, ESR and a differential blood count (t0= day of recruitment, t1= after 7 days, t2= after 14 days). In addition, temperature, oxygen saturation and respiratory rate will be measured at all three visits and patients must complete a questionnaire about current symptoms [23]. The blood samples t0 were collected at the day when the patients came to the General practice with having symptoms occurring in the last 24-48 hours. Blood samples will then be centrifuged directly at the general practitioner’s office at 1600 rpm to obtain the necessary blood plasma to avoid possible lysis of the cells. The samples will be stored at -20 °C until the required case numbers are reached and will thereafter be transported to Mainz. Once there, the samples will be stored in the freezer at -80 °C until the further analysis for the same purpose of the study objectives is performed. The cfDNA concentrations, including non-disease-specific qualitative aspects such as integrity of DNA, will be determined at the Department of Sports Medicine, Prevention and Rehabilitation at the Johannes Gutenberg University Mainz using a self-established qPCR [24]. The material will be processed in a S2 laboratory.

After six months, patients with a SARS-CoV-2 infection will be contacted again by telephone to inquire about possible long-term symptoms (post-COVID-19-syndrome according to the NICE guidelines [25]) and again to

determine cfDNA levels in patients who remain symptomatic. Special note will be made of whether there has been a new SARS-CoV-2 infection or a vaccination against SARS-CoV-2 in the meantime.

Analysis will be done intention to treat, as it may not be possible to retrieve blood samples from the patients at t1 or t2 due to hospitalisation or other circumstances. All participants can discontinue their participation in the trial at any time for any reason without prejudice to current or future medical care. The investigator may discontinue patients' participation in the trial for any reason for their safety or in their best interest. If participants express a desire to withdraw from the study, they will receive instructions to complete an "end-of-study" visit, which will also be voluntary. All participants can receive any concomitant treatment at any time during the trial. However, participants must indicate at each study visit whether they are receiving any concomitant drug therapy.

In the context of this research project, the participants will be insured for potential damages of the biomaterial collection as well as for commuting accidents in accordance with § 2 Para. 1 No. 13b Social Security Code (SGB) VII. After the trial, the patients will receive their primary care by the general practitioner as usual. There is thus no need to provide any additional post-trial care.

Measurement of cfDNA concentrations

CfDNA concentrations will be determined at the Department of Sports Medicine, Prevention and Rehabilitation at the Johannes Gutenberg University Mainz, in a S2 laboratory. We will use validated qPCR assays to determine the concentrations of a 90 bp and 222 bp fragment in diluted EDTA plasma samples without prior DNA isolation [20]. As described in Neuberger et al. the assays specifically target repetitive sequences, which facilitates highly sensitive cfDNA detection from small amounts of diluted plasma. No DNA isolation is required which saves time, costs, and avoids the loss of DNA due to the isolation procedure. The assays show repeatability ≤ 11.6% (95% CI 8.1–20.3), and intermediate precision ≤ 12.1% (95% CI 9.2–17.7). Moreover, the robustness of the assays was demonstrated by incurred sample reanalysis, indicating sufficient validity and sensitivity to quantify cfDNA in the study samples. The blood samples t0 were collected at the day when the patients came to the General practice with having symptoms occurring in the last 24-48 hours. These samples than were directly centrifuged in the General practice.

Participant timeline

The symptomatic participant timeline is presented in Table 1.

Table 1: Research timeline for each participant

Timepoint	T0	T1	T2	T3 (when symptomatic 6 months after positive PCR result)
Consent collection	X			
Demographics, medical history, disease characteristics	X			
Point-of-care antigen rapid test for SARS-CoV-2	X			
Additional RT-qPCR	X			
cfDNA determinations	X	X	X	X
hsCRP, ESR, temperature, oxygen saturation and breathing rate	X	X	X	
Differential blood count	X	X	X	
Concomitant symptoms	X	X	X	
Questionnaire	X	X	X	X

Outcomes

Main outcome measures

The primary outcome is the determination of cfDNA concentrations in patients with flu-like symptoms with SARS-CoV-2 infection with those with flu like symptoms without a SARS-CoV-2 infection in general practice using a self-established qPCR from EDTA plasma. These cfDNA concentrations will be determined at t0 (day 0), t1 (day 7) and t2 (day 14). We investigate the difference in cfDNA at t0 between the two cohorts as primary outcome.

Secondary outcome measures

The secondary objective is to analyse whether there is a correlation between cfDNA concentrations with symptoms/wellness and the severity of the disease. To this end, we will determine whether there is an

association between cfDNA concentrations and the variables mentioned below:

1. High-sensitivity CRP (hsCRP, collected at t0, t1 and t2). hsCRP is a routine inflammatory biomarker and will be measured in the patients' blood samples.
2. Erythrocyte sedimentation rate (ESR, collected at t0, t1 and t2) at day 0, day 7 and day 14. ESR is a routine inflammatory biomarker and will be measured in the patients' blood samples.
3. Differential blood count, especially regarding lymphocyte and neutrophil granulocytes. Lymphopenia and neutropenia appear to be associated with a severe COVID-19 course [26].
4. Temperature taken at day 0, day 7 and day 14. Several studies suggest that high fever increases the risk of ARDS and should be controlled accordingly at an early stage [27, 28].
5. Oxygen saturation measured at day 0, day 7 and day 14, because impairment of oxygen is associated with critical illness [29]. Thus, a target spO2 of 92-96 % is recommended [30].
6. Breathing rate measured at day 0, day 7 and day 14. It has long been known that determining the respiratory rate is a simple way to assess the prognosis in pneumonia or other lung diseases [31].
7. Concomitant symptoms (collected at t_0, t_1 and t_2). Concomitant symptoms will be queried via a self-assessment questionnaire [23] where the patients can report any current concomitant symptoms which then have to be rated on a Likert scale.

To assess the severity of dyspnoea, the American Thoracic Society's Dyspnea Scale will be used. The examiner will apply the WHO Clinical Progression Scale to assess the patient's general condition [32]. After six months, the recruited patients who were SARS-CoV-2 positive will be contacted again by telephone and asked about their condition. In symptoms that could be attributed to a post-Covid-19 syndrome are present, the cfDNA concentration will be determined again in these patients.

Safety

Adverse event reporting and harms

The risk of the venous blood sampling required for the cfDNA determination can be considered minimal. As with any other venous blood draw, pain may occur during the blood draw. Bruising may also occur, especially if there is insufficient compression on the puncture site after blood collection. In very rare cases, the blood draw may result in infection of the puncture site (thrombophlebitis) or nerve injury. However, there are no serious complications associated with blood sampling. The swab for the SARS-CoV-2 Ag rapid test and the subsequent RT-PCR will be performed by a general practitioner who is experienced in this procedure. Rarely,

minor bleeding can occur during nasopharyngeal swabbing; serious injuries do not occur if the procedure is performed correctly. The investigator will assess the severity of each adverse event and will report all serious and non-serious adverse events in the electronic case report form. The investigator will also assess the causal relationship of the serious adverse events to the trial intervention. Termination criteria have not been defined, as it does not seem reasonable for the planned study with a short survey period.

Sample size calculation

With respect to the group with flu-like symptoms without a SARS-CoV-2 infection, we expect –with a relatively high variance– a twofold increase in cfDNA concentrations compared to healthy subjects. A group comparable to this can be found, for example, in the group of chronic inflammatory and currently non-acute diseases, such as systemic lupus erythematosus. In one study, we showed that lupus patients had a mean cfDNA level of 44.7 ng/mL with a standard deviation of 53.5 ng/mL [16]. We hypothesize that a SARS-CoV-2 infection will increase the levels by 100% once more compared with the cohort with influenza-like symptoms without a SARS-CoV-2 infection. Furthermore, we want to be sure enough to obtain group sizes of at least 22 participants in each intervention group (total n=44) and to adjust for any dropouts during the study. Sample size is planned by a two-sample t-test on a two-sided significance level of α=5 % to achieve a power of more than 80 %. We therefore plan to include a total of around 35 patients of comparable age and sex in both the group with a positive and the group with a negative test.

Recruitment will take place in the general practice in Mülheim an der Ruhr via the respective principal physician. We plan to include a total of 44 patients until April 2022.

Plans to promote participant retention and complete follow-up

Participants will benefit from the study as they will know immediately whether a SARS-CoV-2 infection is present via point-of-care diagnostics (Roche SARS-CoV-2 Rapid Antigen Test: sensitivity: 96.52 %, specificity: 99.68 %) which will be subsequently validated by RT-qPCR. During the study, the patients will be cared for in their home environment by their general practitioner. If participants express a desire to withdraw from the study, they will be asked to complete an “end-of-study” visit. Data collected up to the time of withdrawal will remain in the trial database and be included in data analysis, unless otherwise indicated by the participant.

Data management

Trial data will be collected in the electronic case report form by the principal investigator at the Institute of

General Practice at the University Hospital in Essen. Source documents, defined as any original document or object making it possible to prove the existence or accuracy of data or facts recorded during the research, will be kept by the principal physician according to the regulations in force. All questionnaire data will be entered twice by two different persons to ensure the dual control principle. Using a software tool, a third person will check the agreement between the two datasets resulting from the double entry. In cases where entries deviate from one another, the third person will determine the correct entry by looking at the questionnaire. In cases where the questionnaire answers are ambiguous, two persons will decide what should be entered by discussion until a consensus is reached. All data concerning participant information will be stored in locked file cabinets accessible only by the principal investigator. All collected data will be pseudonymised and will therefore be traceable only by means of a code. All files containing names or other personal identifiers, such as the informed consent forms, will be stored separately from the data containing this code number.

Statistical methods

Statistical methods for primary and secondary outcomes

The statistical analysis of the data will be performed with the statistical program SPSS, R or SAS using a pseudonymised dataset. A correlation of the cfDNA concentration with the presence or absence of a SARS-CoV-2 infection, as well as a correlation of these results with the data obtained from the questionnaires will be determined. Descriptive statistics will summarise all study variables.

All data will be tested for normal distribution before and after log transformation using the Kolmogorov-Smirnov test with the Lilliefors correction. If the assumption of normally distributed data cannot be rejected, arithmetic group means ± standard deviations will be calculated. If the normality test fails in at least one of the study groups compared, all data will be expressed as group medians with inter-quartile ranges given in parentheses. Between-group comparisons of primary and secondary outcomes will be performed at t_0 and at two consecutive time points independently (t_1, t_2) using unpaired Student's t-tests (comparing two groups, normally distributed) or the Mann-Whitney U test (comparing two groups, not normally distributed) or analysis of variance (three groups) and chi-square tests for numerical and categorical data, respectively. P values will be considered statistically significant if $p \leq 0.05$. We plan to perform a subgroup analysis for the concomitant symptoms and the cfDNA levels at t_0, t_1 and t_2 in the event of differences within each arm.

Statistical analyses will be carried out according to the intention-to-treat approach and therefore will include all participants. The extent of missing data will be analysed. We will explore missing data patterns and determine

the type of missing data. We will use multiple imputation to substitute missing values.

Patient and Public Involvement

No patients were involved in the development of the research questions. The results of the temperature measurement, the oxygen saturation and breathing rate of each individual patient will be communicated to the patient directly after the examination by the attending physician. The laboratory and clinical parameters and the levels of the cfDNA will be disseminated after they have been measured.

Declarations

Ethics and dissemination

Ethical approval was issued on 2021/03/01 by the Ethics Committee Essen under the number 21-9916-BO. Findings will be disseminated initially to the impacted community, then by publication in scientific journals and on international congresses.

Registration: The study is registered with DRKS under the number DRKS00024722. Informed consent will be obtained from all participants in this study.

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Author statement

DD is the principal investigator; she conceived the study, obtained funding, led the proposal and protocol development, and assisted in manuscript preparation and revision. EN contributed to the study concept and design and assisted in manuscript preparation and revision. JidS assisted in manuscript revision. EG contributed to the study concept and design and assisted in manuscript preparation. PS contributed to the acquisition and analysis of qualitative data and the development of the intervention and assisted in manuscript preparation and revision. SB contributed to the study concept and design and drafted the manuscript. All authors read and approved the final manuscript.

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Conflicts of interests

The authors declare that they have no conflicts of interests.

Consent for publication

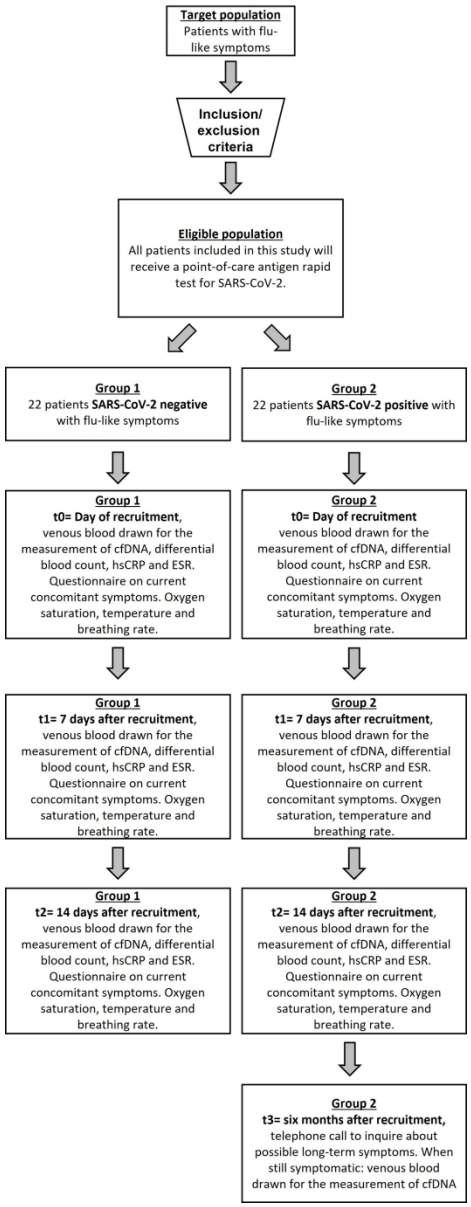
Not required.

Data Statement

The study principal investigator and the co-investigators will have access to the full study data and materials. The authors will be willing to share the individual-level study data after completion and publication of primary and secondary analyses.

Figure Legends

Figure 1: Flowchart of the study design.



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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page # (track changes)
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2 (study protocol, what will be done)
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	2-5
Objectives	3	State specific objectives, including any prespecified hypotheses	5-6
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5-9
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6 (eligibility criteria, selection of participants), 7/10-11 (follow-up)
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	n/a
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	9-10
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	11-12
Bias	9	Describe any efforts to address potential sources of bias	7-8
Study size	10	Explain how the study size was arrived at	10-11
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7/10-11
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	12-13
		(*b*) Describe any methods used to examine subgroups and interactions	12-13
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, explain how loss to follow-up was addressed	7/10

		(*e*) Describe any sensitivity analyses	7/10-11
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	n/a (study protocol)
		(b) Give reasons for non-participation at each stage	n/a (study protocol)
		(c) Consider use of a flow diagram	Figure 1 (page 4)
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	n/a (study protocol)
		(b) Indicate number of participants with missing data for each variable of interest	n/a (study protocol)
		(c) Summarise follow-up time (eg, average and total amount)	n/a (study protocol)
Outcome data	15*	Report numbers of outcome events or summary measures over time	n/a (study protocol)
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	n/a (study protocol)
		(*b*) Report category boundaries when continuous variables were categorized	n/a (study protocol)
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a (study protocol)
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	n/a (study protocol)
Discussion			
Key results	18	Summarise key results with reference to study objectives	n/a (study protocol)
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	n/a (study protocol)
Generalisability	21	Discuss the generalisability (external validity) of the study results	n/a (study protocol)
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

cfDNA as a surrogate marker for COVID-19 severity in patients with influenza-like symptoms with and without SARS-CoV-2 infections in general practice: A study protocol for a prospective cohort study

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Complete List of Authors:	Dehnen, Dorothea; University of Duisburg-Essen Faculty of Medicine Neuberger, Elmo; Johannes Gutenberg Universitat Mainz, Department of Sports Medicine, Rehabilitation and Disease Prevention in der Schmitten, Jürgen; University of Duisburg-Essen Faculty of Medicine Giagkou, Ekaterini; University of Duisburg-Essen Faculty of Medicine Simon, Perikles; Johannes Gutenberg Universitat Mainz, Department of Sports Medicine, Rehabilitation and Disease Prevention Botzenhardt, Suzan; University of Duisburg-Essen Faculty of Medicine,
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cfDNA as a surrogate marker for COVID-19 severity in patients with influenza-like symptoms with and without SARS-CoV-2 infections in general practice: A study protocol for a prospective cohort study

Dorothea Dehnen[1], Elmo Neuberger[2], Jürgen in der Schmitten[1], Ekaterini Giagkou[1], Perikles Simon[2], Suzan Botzenhardt[1*]

[1]Institute of General Practice, Medical Faculty, University of Duisburg-Essen, Essen, Germany

[2]Department of Sports Medicine, Rehabilitation and Disease Prevention, Johannes Gutenberg University of Mainz, Mainz, Germany

***Corresponding author**

Suzan Botzenhardt, PhD

Institute for General Practice

University Hospital Essen

Hufelandstr. 55

45147 Essen / Germany

Tel: +49-201-723-8036, Fax: +49-201 723-8044

Email: suzan.botzenhardt@uk-essen.de

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Abstract

Introduction: The clinical course of patients with a SARS-CoV-2 (severe acute respiratory syndrome coronavirus type 2, COVID-19) infection varies widely, from symptom-free to severe courses that can lead to death. Laboratory values of SARS-CoV-2 patients such as lymphocyte counts or C-reactive protein (CRP) do not allow a prediction of the actual course of the disease. To identify a possible predictive marker for the differentiation and prognosis of illness with influenza-like symptoms with and without SARS-CoV-2 infections in general practice we will analyse the concentrations of cell-free DNA (cfDNA) levels, laboratory and clinical parameters, temperature, oxygen saturation, breathing rate and concomitant symptoms in patients with flu-like symptoms with and without a SARS-CoV-2 infection.

Methods and analysis: This is a single-centre, two-arm, parallel longitudinal cohort study with a total of 44 patients. 22 patients with flu-like symptoms without a SARS-CoV-2 infection and 22 patients with flu-like symptoms with a SARS-CoV-2 infection will be recruited. The primary objective is to compare cfDNA levels in ambulatory patients in general practice with flu-like symptoms with SARS-CoV-2 infection with those with flu like symptoms without a SARS-CoV-2 infection during the disease (day 7 and day 14). The secondary objective is to determine whether there is a correlation between cfDNA concentrations on the one hand, and laboratory and clinical parameters on the other hand. cfDNA, differential blood count, high-sensitive CRP (hsCRP) and erythrocyte sedimentation rat (ESR) will be measured in blood samples, concomitant symptoms will be surveyed via a self-assessment questionnaire, and oxygen saturation, breathing rate and examination of the lungs will be reported by treating physicians.

Ethics and dissemination: Ethical approval was issued on 2021/03/01 by the Ethics Committee Essen under the number 21-9916-BO. Findings will be published in peer-reviewed open-access journals and presented at national and international conferences.

Trial registration number: The study was registered in the German Clinical Trials Registry (DRKS) in March 2021 under the number DRKS00024722.

Article Summary

Strengths and limitations of this study

- Measurement of cfDNA is cost-effective and requires only minimal amounts of blood, which in the future can be collected in the primary care physician's office.

- Clinical and serological parameters (hsCRP, ESR and a differential blood count) are collected in a setting (family practice) where patients with mild or moderate symptoms are predominantly treated, but where no suitable prognostic markers are available at this time.
- The success of the study should be ensured by the close follow-up and home visits of the patients by the general practitioner and the short duration of the study.
- cfDNA is already a well-established biomarker that is associated with various diseases and has been used in different research areas, such as oncology, non-invasive prenatal diagnosis, organ transplantation, autoimmune diseases, trauma, coronary heart disease and diabetes.
- The limitation of the study due to missing laboratory values at t1 and t2 in the case of a severe disease course requiring hospitalisation is addressed by conducting the analysis according to the intention-to-treat principle and assessing hospitalisation as the main outcome."

Keywords: SARS-CoV-2 infection, cell-free DNA (cfDNA), flu/influenza, predictive marker, general practice.

Introduction

The current SARS-CoV-2 (severe acute respiratory syndrome coronavirus type 2, COVID-19) pandemic is confronting humanity with a new dimension of medical, economic and social problems [1]. Among humans, the virus spreads rapidly and causes varying degrees of severity of symptoms and illness in patients [2, 3]. Thus, the clinical course of patients with a SARS-CoV-2 infection can vary widely, from symptom-free courses to severe courses that can lead to death. The incubation period of the virus ranges from 1 to 14 days, the duration of viral excretion can last from 8 to 37 days, and the time from disease onset to discharge or death ranges from 15-25 days [4, 5]. Furthermore, it has been shown that mortality rates correlate with increasing age and pre-existing concomitant diseases, such as cardiovascular disease, diabetes, overweight and hypertension [2, 3, 6]. In the course of analysing laboratory values of SARS-CoV-2 patients, such as measurements of lymphocyte counts, C-reactive protein (CRP), as well as secondary bacterial infections it was found that the analysis and evaluation of these do not allow an assessment of the actual course of the disease [7, 8]. In order to be able to better assess the course of this disease, it would be important to find a predictive marker that could be used to determine the severity of the disease at the earliest possible stage.

One marker that could play an important role in this determination is cell-free DNA (cfDNA), which is usually released from cells by apoptosis, necrosis, NETosis, as well as active secretion [9, 10]. It comprises a high variability of fragmented molecules that contain valuable information about gene expression and the nucleosome pattern in relation to their tissue of origin [11–13]. Numerous studies have already demonstrated that cfDNA levels are associated with various diseases and have been used in various research areas, such as oncology, non-invasive prenatal diagnosis, organ transplantation, autoimmune diseases, trauma, coronary heart disease and diabetes [11–16].

Recent studies have investigated the role of cfDNA as a potential marker for therapeutic targets of SARS-CoV-2 in order to develop new therapeutic strategies for the disease [17]. In their study, Chen et al. profiled and analysed for the first-time plasma cell-free DNA (cfDNA) of mild and severe COVID-19 patients. They found that in comparison between mild and severe COVID-19 patients, Interleukin-37 signalling was one of the most relevant pathways. Their data thus revealed potential tissue involvement, provided insights into mechanism on COVID-19 progression, and highlighted utility of cfDNA as a non-invasive biomarker for disease severity inspections [18]. In a further study, Andargie et al. showed that cfDNA levels correlated positively with COVID-19 disease severity, C-reactive protein and D-dimer, and that the cfDNA profile at admission identified patients who subsequently required intensive care or died during hospitalization. They conclude that cfDNA could be used as a potential diagnostic biomarker to map sources of injury and as a prognostic biomarker to predict COVID-19 trajectory and outcome by providing mechanistic information about COVID-19-induced tissue injury [19]. However, in this study, cfDNA levels were not measured in patients before they presented at the hospital. Our study will instead focus on patients who visit their GP's office with mild flu-like symptoms. In addition to the studies on cfDNA levels and SARS-CoV-2, further studies have measured the impact of influenza on cfDNA levels. Again, it was shown that patients had significantly increased cfDNA levels [19, 20]. The aim of our study is to determine if cfDNA levels differ in patients with mild or moderate flu symptoms when either SARS-CoV-2 infection or infection with another common respiratory pathogen is present or if these concentrations are similar to each other.

Methods

Design

This is a single-centre, two-arm, parallel cohort study with a 1:1 allocation ratio to be conducted between

August 2021 and April 2022. 22 patients with flu-like symptoms without a SARS-CoV-2 infection and 22 patients with flu-like symptoms with a SARS-CoV-2 infection will be included (n=44, age over 18 years) (see Figure 1). Due to our study design, no randomisation is needed. Thus, no blinding will be performed.

insert Figure 1 here

Patients

Setting of the study and characteristics of participants

This single-centre study will be conducted at the University Hospital in Essen. Patients who visit their general practice in Mülheim an der Ruhr with flu-like symptoms will be asked to complete a screening questionnaire to check if they met the inclusion criteria. If they meet the eligibility criteria, they will be included in the study. Written informed consent will be obtained by the principal investigator from all patients willing to participate in the study.

Inclusion and exclusion criteria

All persons enrolled in the study must provide full written informed consent and are required to complete a baseline screening questionnaire to assess their eligibility.

Inclusion criteria are:

1. Age 18-99 years,
2. Consent given by the patient or legal representative for blood draw, oropharyngeal or nasopharyngeal swab for a rapid SARS-CoV-2 antigen test and subsequent qPCR, if applicable,
3. Sufficient knowledge of the German language to understand the study content and instructions.

Exclusion criteria are:

1. Severe acute or chronic illness with known elevated cfDNA levels due to the underlying disease, e.g.:
    a. Tumour disease
    b. Severe renal insufficiency
    c. Severe/moderate inflammatory diseases
    d. Autoimmune diseases
    e. Rheumatological diseases

Intervention description

All patients included in this study will receive a point-of-care antigen rapid test for SARS-CoV-2 (Roche SARS-CoV-2 Rapid Antigen Test) and a subsequent RT-PCR on the day of initial presentation. Afterwards, patients will be assigned either to the group “SARS-CoV-2 positive with flu-like symptoms” or to the group “SARS-CoV-2 negative with flu-like symptoms” depending on the test results. For cost reasons, the patients in the SARS-CoV-2 negative with flu symptoms group cannot be tested for other respiratory pathogens. However, data from the Robert Koch Institute indicate the frequency of possible respiratory pathogens. Thus, in the 10th calendar week of 2022, a total of 72 (60%) of the 121 sent in sentinel samples (national reference centre influenza) identified respiratory viruses, including most frequently SARS-CoV-2 (n=32, 26 %), followed by rhinoviruses (n=20, 17 %), human metapneumoviruses (hMPV) (n=18, 15 %), human seasonal coronaviruses (hCoV) (n=6, 5%), influenza A(H3N2) viruses (n=4, 3%), parainfluenza viruses (PIV) (n=3, 2%), and respiratory syncytial viruses (RSV) (n=2, 2%) [21]. Taking into account the other weekly reports from 2022, human metapneumoviruses (7-16%), rhinoviruses (7-22%) and human seasonal coronaviruses (1-17%) dominated in addition to SARS-CoV-2. Influenza viruses in particular play a subordinate role as pathogens of respiratory tract infections (<5%) [22].

All patients will have venous blood (15.3 mL) collected on three different visits to determine cfDNA concentrations as well as the inflammation markers hsCRP, ESR and a differential blood count (t0= day of recruitment, t1= after 7 days, t2= after 14 days). In addition, temperature, oxygen saturation and respiratory rate will be measured at all three visits and patients must complete a questionnaire about current symptoms [23]. The blood samples t0 were collected at the day when the patients came to the General practice with having symptoms occurring in the last 24-48 hours. Blood samples will then be centrifuged directly at the general practitioner’s office at 1600 rpm to obtain the necessary blood plasma to avoid possible lysis of the cells. The samples will be stored at -20 °C until the required case numbers are reached and will thereafter be transported to Mainz. Once there, the samples will be stored in the freezer at -80 °C until the further analysis for the same purpose of the study objectives is performed. The cfDNA concentrations, including non-disease-specific qualitative aspects such as integrity of DNA, will be determined at the Department of Sports Medicine, Prevention and Rehabilitation at the Johannes Gutenberg University Mainz using a self-established qPCR [24]. The material will be processed in a S2 laboratory.

After six months, patients with a SARS-CoV-2 infection will be contacted again by telephone to inquire about possible long-term symptoms (post-COVID-19-syndrome according to the NICE guidelines [25]) and again to

determine cfDNA levels in patients who remain symptomatic. Special note will be made of whether there has been a new SARS-CoV-2 infection or a vaccination against SARS-CoV-2 in the meantime.

Analysis will be done intention to treat, as it may not be possible to retrieve blood samples from the patients at t1 or t2 due to hospitalisation or other circumstances. All participants can discontinue their participation in the trial at any time for any reason without prejudice to current or future medical care. The investigator may discontinue patients' participation in the trial for any reason for their safety or in their best interest. If participants express a desire to withdraw from the study, they will receive instructions to complete an "end-of-study" visit, which will also be voluntary. All participants can receive any concomitant treatment at any time during the trial. However, participants must indicate at each study visit whether they are receiving any concomitant drug therapy.

In the context of this research project, the participants will be insured for potential damages of the biomaterial collection as well as for commuting accidents in accordance with § 2 Para. 1 No. 13b Social Security Code (SGB) VII. After the trial, the patients will receive their primary care by the general practitioner as usual. There is thus no need to provide any additional post-trial care.

Measurement of cfDNA concentrations

CfDNA concentrations will be determined at the Department of Sports Medicine, Prevention and Rehabilitation at the Johannes Gutenberg University Mainz, in a S2 laboratory. We will use validated qPCR assays to determine the concentrations of a 90 bp and 222 bp fragment in diluted EDTA plasma samples without prior DNA isolation [20]. As described in Neuberger et al. the assays specifically target repetitive sequences, which facilitates highly sensitive cfDNA detection from small amounts of diluted plasma. No DNA isolation is required which saves time, costs, and avoids the loss of DNA due to the isolation procedure. The assays show repeatability ≤ 11.6% (95% CI 8.1–20.3), and intermediate precision ≤ 12.1% (95% CI 9.2–17.7). Moreover, the robustness of the assays was demonstrated by incurred sample reanalysis, indicating sufficient validity and sensitivity to quantify cfDNA in the study samples. The blood samples t0 were collected at the day when the patients came to the General practice with having symptoms occurring in the last 24-48 hours. These samples than were directly centrifuged in the General practice.

Participant timeline

The symptomatic participant timeline is presented in Table 1.

Table 1: Research timeline for each participant

Timepoint	T0	T1	T2	T3 (when symptomatic 6 months after positive PCR result)
Consent collection	X			
Demographics, medical history, disease characteristics	X			
Point-of-care antigen rapid test for SARS-CoV-2	X			
Additional RT-qPCR	X			
cfDNA determinations	X	X	X	X
hsCRP, ESR, temperature, oxygen saturation and breathing rate	X	X	X	
Differential blood count	X	X	X	
Concomitant symptoms	X	X	X	
Questionnaire	X	X	X	X

Outcomes

Main outcome measures

The primary outcome is the determination of cfDNA concentrations in patients with flu-like symptoms with SARS-CoV-2 infection with those with flu like symptoms without a SARS-CoV-2 infection in general practice using a self-established qPCR from EDTA plasma. These cfDNA concentrations will be determined at t0 (day 0), t1 (day 7) and t2 (day 14). We investigate the difference in cfDNA at t0 between the two cohorts as primary outcome.

Secondary outcome measures

The secondary objective is to analyse whether there is a correlation between cfDNA concentrations with symptoms/wellness and the severity of the disease. To this end, we will determine whether there is an

association between cfDNA concentrations and the variables mentioned below:

1. High-sensitivity CRP (hsCRP, collected at t0, t1 and t2). hsCRP is a routine inflammatory biomarker and will be measured in the patients' blood samples.
2. Erythrocyte sedimentation rate (ESR, collected at t0, t1 and t2) at day 0, day 7 and day 14. ESR is a routine inflammatory biomarker and will be measured in the patients' blood samples.
3. Differential blood count, especially regarding lymphocyte and neutrophil granulocytes. Lymphopenia and neutropenia appear to be associated with a severe COVID-19 course [26].
4. Temperature taken at day 0, day 7 and day 14. Several studies suggest that high fever increases the risk of ARDS and should be controlled accordingly at an early stage [27, 28].
5. Oxygen saturation measured at day 0, day 7 and day 14, because impairment of oxygen is associated with critical illness [29]. Thus, a target spO2 of 92-96 % is recommended [30].
6. Breathing rate measured at day 0, day 7 and day 14. It has long been known that determining the respiratory rate is a simple way to assess the prognosis in pneumonia or other lung diseases [31].
7. Concomitant symptoms (collected at t_0, t_1 and t_2). Concomitant symptoms will be queried via a self-assessment questionnaire [23] where the patients can report any current concomitant symptoms which then have to be rated on a Likert scale.

To assess the severity of dyspnoea, the American Thoracic Society's Dyspnea Scale will be used. The examiner will apply the WHO Clinical Progression Scale to assess the patient's general condition [32]. After six months, the recruited patients who were SARS-CoV-2 positive will be contacted again by telephone and asked about their condition. In symptoms that could be attributed to a post-Covid-19 syndrome are present, the cfDNA concentration will be determined again in these patients.

Safety

Adverse event reporting and harms

The risk of the venous blood sampling required for the cfDNA determination can be considered minimal. As with any other venous blood draw, pain may occur during the blood draw. Bruising may also occur, especially if there is insufficient compression on the puncture site after blood collection. In very rare cases, the blood draw may result in infection of the puncture site (thrombophlebitis) or nerve injury. However, there are no serious complications associated with blood sampling. The swab for the SARS-CoV-2 Ag rapid test and the subsequent RT-PCR will be performed by a general practitioner who is experienced in this procedure. Rarely,

minor bleeding can occur during nasopharyngeal swabbing; serious injuries do not occur if the procedure is performed correctly. The investigator will assess the severity of each adverse event and will report all serious and non-serious adverse events in the electronic case report form. The investigator will also assess the causal relationship of the serious adverse events to the trial intervention. Termination criteria have not been defined, as it does not seem reasonable for the planned study with a short survey period.

Sample size calculation

With respect to the group with flu-like symptoms without a SARS-CoV-2 infection, we expect –with a relatively high variance– a twofold increase in cfDNA concentrations compared to healthy subjects. A group comparable to this can be found, for example, in the group of chronic inflammatory and currently non-acute diseases, such as systemic lupus erythematosus. In one study, we showed that lupus patients had a mean cfDNA level of 44.7 ng/mL with a standard deviation of 53.5 ng/mL [16]. We hypothesize that a SARS-CoV-2 infection will increase the levels by 100% once more compared with the cohort with influenza-like symptoms without a SARS-CoV-2 infection. Furthermore, we want to be sure enough to obtain group sizes of at least 22 participants in each intervention group (total n=44) and to adjust for any dropouts during the study. Sample size is planned by a two-sample t-test on a two-sided significance level of α=5 % to achieve a power of more than 80 %. We therefore plan to include a total of around 35 patients of comparable age and sex in both the group with a positive and the group with a negative test.

Recruitment will take place in the general practice in Mülheim an der Ruhr via the respective principal physician. We plan to include a total of 44 patients until April 2022.

Plans to promote participant retention and complete follow-up

Participants will benefit from the study as they will know immediately whether a SARS-CoV-2 infection is present via point-of-care diagnostics (Roche SARS-CoV-2 Rapid Antigen Test: sensitivity: 96.52 %, specificity: 99.68 %) which will be subsequently validated by RT-qPCR. During the study, the patients will be cared for in their home environment by their general practitioner. If participants express a desire to withdraw from the study, they will be asked to complete an “end-of-study” visit. Data collected up to the time of withdrawal will remain in the trial database and be included in data analysis, unless otherwise indicated by the participant.

Data management

Trial data will be collected in the electronic case report form by the principal investigator at the Institute of

General Practice at the University Hospital in Essen. Source documents, defined as any original document or object making it possible to prove the existence or accuracy of data or facts recorded during the research, will be kept by the principal physician according to the regulations in force. All questionnaire data will be entered twice by two different persons to ensure the dual control principle. Using a software tool, a third person will check the agreement between the two datasets resulting from the double entry. In cases where entries deviate from one another, the third person will determine the correct entry by looking at the questionnaire. In cases where the questionnaire answers are ambiguous, two persons will decide what should be entered by discussion until a consensus is reached. All data concerning participant information will be stored in locked file cabinets accessible only by the principal investigator. All collected data will be pseudonymised and will therefore be traceable only by means of a code. All files containing names or other personal identifiers, such as the informed consent forms, will be stored separately from the data containing this code number.

Statistical methods

Statistical methods for primary and secondary outcomes

The statistical analysis of the data will be performed with the statistical program SPSS, R or SAS using a pseudonymised dataset. A correlation of the cfDNA concentration with the presence or absence of a SARS-CoV-2 infection, as well as a correlation of these results with the data obtained from the questionnaires will be determined. Descriptive statistics will summarise all study variables.

All data will be tested for normal distribution before and after log transformation using the Kolmogorov-Smirnov test with the Lilliefors correction. If the assumption of normally distributed data cannot be rejected, arithmetic group means ± standard deviations will be calculated. If the normality test fails in at least one of the study groups compared, all data will be expressed as group medians with inter-quartile ranges given in parentheses. Between-group comparisons of primary and secondary outcomes will be performed at t_0 and at two consecutive time points independently (t_1, t_2) using unpaired Student's t-tests (comparing two groups, normally distributed) or the Mann-Whitney U test (comparing two groups, not normally distributed) or analysis of variance (three groups) and chi-square tests for numerical and categorical data, respectively. P values will be considered statistically significant if $p \leq 0.05$. We plan to perform a subgroup analysis for the concomitant symptoms and the cfDNA levels at t_0, t_1 and t_2 in the event of differences within each arm.

Statistical analyses will be carried out according to the intention-to-treat approach and therefore will include all participants. The extent of missing data will be analysed. We will explore missing data patterns and determine

the type of missing data. We will use multiple imputation to substitute missing values.

Patient and Public Involvement

No patients were involved in the development of the research questions. The results of the temperature measurement, the oxygen saturation and breathing rate of each individual patient will be communicated to the patient directly after the examination by the attending physician. The laboratory and clinical parameters and the levels of the cfDNA will be disseminated after they have been measured.

Declarations

Ethics and dissemination

Ethical approval was issued on 2021/03/01 by the Ethics Committee Essen under the number 21-9916-BO. Findings will be disseminated initially to the impacted community, then by publication in scientific journals and on international congresses.

Registration: The study is registered with DRKS under the number DRKS00024722. Informed consent will be obtained from all participants in this study.

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Author statement

DD is the principal investigator; she conceived the study, obtained funding, led the proposal and protocol development, and assisted in manuscript preparation and revision. EN contributed to the study concept and design and assisted in manuscript preparation and revision. JidS assisted in manuscript revision. EG contributed to the study concept and design and assisted in manuscript preparation. PS contributed to the acquisition and analysis of qualitative data and the development of the intervention and assisted in manuscript preparation and revision. SB contributed to the study concept and design and drafted the manuscript. All authors read and approved the final manuscript.

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Conflicts of interests

The authors declare that they have no conflicts of interests.

Consent for publication

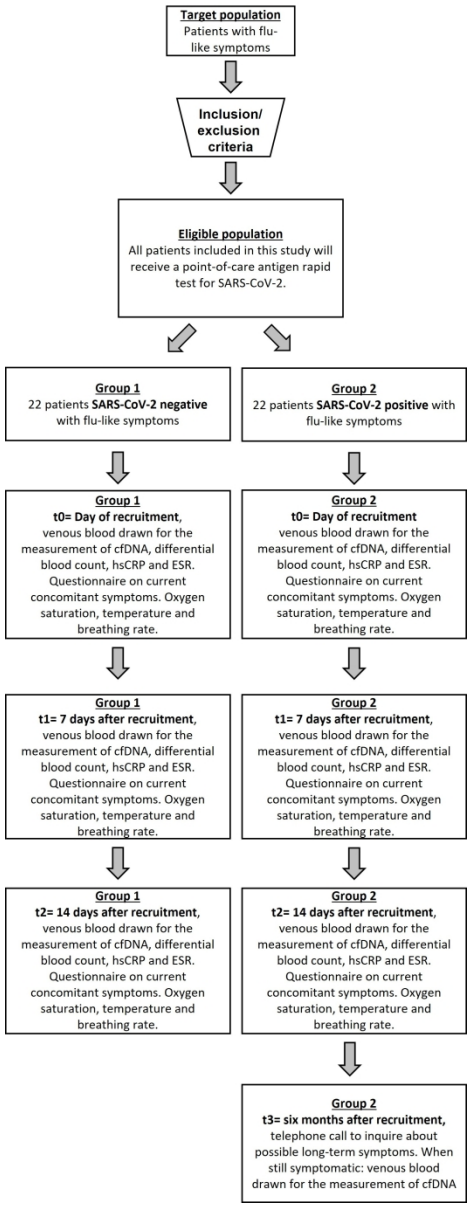
Not required.

Data Statement

The study principal investigator and the co-investigators will have access to the full study data and materials. The authors will be willing to share the individual-level study data after completion and publication of primary and secondary analyses.

Figure Legends

Figure 1: Flowchart of the study design.



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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page # (track changes)
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2 (study protocol, what will be done)
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	2-5
Objectives	3	State specific objectives, including any prespecified hypotheses	5-6
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5-9
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6 (eligibility criteria, selection of participants), 7/10-11 (follow-up)
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	n/a
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	9-10
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	11-12
Bias	9	Describe any efforts to address potential sources of bias	7-8
Study size	10	Explain how the study size was arrived at	10-11
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7/10-11
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	12-13
		(*b*) Describe any methods used to examine subgroups and interactions	12-13
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, explain how loss to follow-up was addressed	7/10

		(*e*) Describe any sensitivity analyses	7/10-11
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	n/a (study protocol)
		(b) Give reasons for non-participation at each stage	n/a (study protocol)
		(c) Consider use of a flow diagram	Figure 1 (page 4)
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	n/a (study protocol)
		(b) Indicate number of participants with missing data for each variable of interest	n/a (study protocol)
		(c) Summarise follow-up time (eg, average and total amount)	n/a (study protocol)
Outcome data	15*	Report numbers of outcome events or summary measures over time	n/a (study protocol)
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	n/a (study protocol)
		(*b*) Report category boundaries when continuous variables were categorized	n/a (study protocol)
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a (study protocol)
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	n/a (study protocol)
Discussion			
Key results	18	Summarise key results with reference to study objectives	n/a (study protocol)
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	n/a (study protocol)
Generalisability	21	Discuss the generalisability (external validity) of the study results	n/a (study protocol)
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.