

BMJ Open

BMJ Open is committed to open peer review. As part of this commitment we make the peer review history of every article we publish publicly available.

When an article is published we post the peer reviewers' comments and the authors' responses online. We also post the versions of the paper that were used during peer review. These are the versions that the peer review comments apply to.

The versions of the paper that follow are the versions that were submitted during the peer review process. They are not the versions of record or the final published versions. They should not be cited or distributed as the published version of this manuscript.

BMJ Open is an open access journal and the full, final, typeset and author-corrected version of record of the manuscript is available on our site with no access controls, subscription charges or pay-per-view fees (<http://bmjopen.bmj.com>).

If you have any questions on BMJ Open's open peer review process please email info.bmjopen@bmj.com

BMJ Open

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2021-058647
Article Type:	Protocol
Date Submitted by the Author:	25-Oct-2021
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,
Keywords:	MOLECULAR BIOLOGY, Cell biology < NATURAL SCIENCE DISCIPLINES, GENERAL MEDICINE (see Internal Medicine), COVID-19

SCHOLARONE™
Manuscripts

1
2
3 **cfDNA as a predictive marker for the prognosis of severe courses in**
4 **patients with influenza-like symptoms with and without SARS-CoV-2**
5
6 **infections in general practice: A prospective cohort study**
7
8
9

10
11
12
13 Dorothea Dehnen¹, Elmo Neuberger², Jürgen in der Schmitt¹, Ekaterini Giagkou¹, Perikles Simon², Suzan
14 Botzenhardt^{1*}
15

16
17
18
19 ¹Institute of General Practice, Medical Faculty, University of Duisburg-Essen, Essen, Germany

20
21 ²Department of Sports Medicine, Rehabilitation and Disease Prevention, Johannes Gutenberg University of
22 Mainz, Mainz, Germany
23

24
25
26 ***Corresponding author**

27 Suzan Botzenhardt, PhD

28 Institute for General Practice

29 University Hospital Essen

30 Hufelandstr. 55

31 45147 Essen / Germany

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

33 Email: suzan.botzenhardt@uk-essen.de

34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
Word count: 3236 words

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-

1
2
3 invasive prenatal diagnosis, organ transplantation, autoimmune diseases, trauma, coronary heart disease and
4 diabetes [11–16].

5
6
7 Recent studies have investigated the role of cfDNA as a potential marker for therapeutic targets of SARS-
8
9 CoV-2 in order to develop new therapeutic strategies for the disease [17]. In their study, Chen et al.
10 systematically analysed whole-genome sequencing (WGS) of cfDNA in patients with mild and severe SARS-
11
12 CoV-2 courses to explore the clinical value of cfDNA in SARS-CoV-2. They showed that significantly different
13
14 levels were measured between mild and severe SARS-CoV-2 courses in patients, which could indicate the
15
16 involvement of potential genes, tissues and signalling pathways in disease progression and severity [18]. In a
17
18 further study, Andargie et al. showed that cfDNA levels correlated positively with COVID-19 disease severity,
19
20 C-reactive protein and D-dimer, and that the cfDNA profile at admission identified patients who subsequently
21
22 required intensive care or died during hospitalization. They conclude that cfDNA could be used as a potential
23
24 diagnostic biomarker to map sources of injury and as a prognostic biomarker to predict COVID-19 trajectory
25
26 and outcome by providing mechanistic information about COVID-19-induced tissue injury [19]. However, in
27
28 this study, cfDNA levels were not measured in patients before they presented at the hospital. Our study will
29
30 instead focus on patients who visit their GP's office with mild flu-like symptoms. In addition to the studies on
31
32 cfDNA levels and SARS-CoV-2, further studies have measured the impact of influenza on cfDNA levels. Again,
33
34 it was shown that patients had significantly increased cfDNA levels [19, 20].
35
36
37
38

39 Methods

40 Design

41
42 This is a single-centre, two-arm, parallel cohort study with a 1:1 allocation ratio. 22 patients with flu-like
43
44 symptoms without a SARS-CoV-2 infection and 22 patients with flu-like symptoms with a SARS-CoV-2
45
46 infection will be included (n=44, age over 18 years) (see Figure 1). Due to our study design, no randomisation
47
48 is needed. Thus, no blinding will be performed.
49
50
51
52

53 ***insert Figure 1 here***
54
55
56
57
58
59
60

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-

1
2
3 CoV-2 Rapid Antigen Test) and a subsequent RT-PCR on the day of initial presentation. If the rapid antigen
4 test result is negative, the swab will be tested by RT-PCR not only for SARS-CoV-2 but also for influenza in
5 order to exclude both a SARS-CoV-2 infection and an influenza infection in the control group. Afterwards,
6
7 patients will be assigned either to the group “SARS-CoV-2 positive with flu-like symptoms” or to the group
8
9 “SARS-CoV-2 negative with flu-like symptoms” depending on the test results. All patients will have venous
10
11 blood (22.4 mL) collected on three different visits to determine cfDNA concentrations as well as the
12
13 inflammation markers hsCRP, ESR and a differential blood count (t0= day of recruitment, t1= after 7 days, t2=
14
15 after 14 days). In addition, temperature, oxygen saturation and respiratory rate will be measured at all three
16
17 visits and patients must complete a questionnaire about current symptoms [21].
18
19

20
21 Blood samples will be centrifuged directly at the general practitioner’s office at 1600 rpm to obtain the
22
23 necessary blood plasma to avoid possible lysis of the cells. The samples will be stored at -20 °C until the
24
25 required case numbers are reached and will thereafter be transported to Mainz. Once there, the samples will
26
27 be stored in the freezer at -80 °C until the further analysis for the same purpose of the study objectives is
28
29 performed. The cfDNA concentrations, including non-disease-specific qualitative aspects such as integrity of
30
31 DNA, will be determined at the Department of Sports Medicine, Prevention and Rehabilitation at the Johannes
32
33 Gutenberg University Mainz using a self-established qPCR [22]. The material will be processed in an S2
34
35 laboratory.

36
37 After three and six months, patients with a SARS-CoV-2 infection will be contacted again by telephone to
38
39 inquire about possible long-term symptoms and to again determine cfDNA levels in patients who remain
40
41 symptomatic.

42
43 All participants can discontinue their participation in the trial at any time for any reason without prejudice to
44
45 current or future medical care. The investigator may discontinue patients’ participation in the trial for any reason
46
47 for their safety or in their best interest. If participants express a desire to withdraw from the study, they will
48
49 receive instructions to complete an “end-of-study” visit, which will also be voluntary.

50
51 All participants can receive any concomitant treatment at any time during the trial. However, participants must
52
53 indicate at each study visit whether they are receiving any concomitant drug therapy.

54
55 In the context of this research project, the participants will be insured for potential damages of the biomaterial
56
57 collection as well as for commuting accidents in accordance with § 2 Para. 1 No. 13b Social Security Code
58
59 (SGB) VII. After the trial, the patients will receive their primary care by the general practitioner as usual. There
60
61 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 spO_2 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

1
2
3 serious complications associated with blood sampling. The swab for the SARS-CoV-2 Ag rapid test and the
4 subsequent RT-PCR will be performed by a general practitioner who is experienced in this procedure. Rarely,
5 minor bleeding can occur during nasopharyngeal swabbing; serious injuries do not occur if the procedure is
6 performed correctly. The investigator will assess the severity of each adverse event and will report all serious
7 and non-serious adverse events in the electronic case report form. The investigator will also assess the causal
8 relationship of the serious adverse events to the trial intervention. Termination criteria have not been defined,
9 as it does not seem reasonable for the planned study with a short survey period.
10
11
12
13
14
15
16
17
18

19 Sample size calculation

20
21 With respect to the group with flu-like symptoms without a SARS-CoV-2 infection, we expect –with a relatively
22 high variance– a twofold increase in cfDNA concentrations compared to healthy subjects. A group comparable
23 to this can be found, for example, in the group of chronic inflammatory and currently non-acute diseases, such
24 as systemic lupus erythematosus. In one study, we showed that lupus patients had a mean cfDNA level of
25 44.7 ng/mL with a standard deviation of 53.5 ng/mL [16]. We hypothesize that a SARS-CoV-2 infection will
26 increase the levels by 100% once more compared with the cohort with influenza-like symptoms without a
27 SARS-CoV-2 infection. Furthermore, we want to be sure enough to obtain group sizes of at least
28 22 participants in each intervention group (total n=44) and to adjust for any dropouts during the study. Sample
29 size is planned by a two-sample t-test on a two-sided significance level of $\alpha=5\%$ to achieve a power of more
30 than 80%. We therefore plan to include a total of around 35 patients of comparable age and sex in both the
31 group with a positive and the group with a negative test.
32
33
34
35
36
37
38
39
40
41

42 Recruitment will take place in the general practice in Mülheim an der Ruhr via the respective principal
43 physician. We plan to include a total of 44 patients until February 2022.
44
45
46
47

48 Plans to promote participant retention and complete follow-up

49
50 Participants will benefit from the study as they will know immediately whether a SARS-CoV-2 infection is
51 present via point-of-care diagnostics (Roche SARS-CoV-2 Rapid Antigen Test: sensitivity: 96.52%, specificity:
52 99.68%) which will be subsequently validated by RT-qPCR. Those with a negative result in the point-of-care
53 diagnostic will be additionally tested for influenza. During the study, the patients will be cared for in their home
54 environment by their general practitioner. If participants express a desire to withdraw from the study, they will
55 be asked to complete an “end-of-study” visit. Data collected up to the time of withdrawal will remain in the trial
56
57
58
59
60

1
2
3 database and be included in data analysis, unless otherwise indicated by the participant.
4
5

6 Data management 7

8 Trial data will be collected in the electronic case report form by the principal investigator at the Institute of
9 General Practice at the University Hospital in Essen. Source documents, defined as any original document or
10 object making it possible to prove the existence or accuracy of data or facts recorded during the research, will
11 be kept by the principal physician according to the regulations in force. All questionnaire data will be entered
12 twice by two different persons to ensure the dual control principle. Using a software tool, a third person will
13 check the agreement between the two datasets resulting from the double entry. In cases where entries deviate
14 from one another, the third person will determine the correct entry by looking at the questionnaire. In cases
15 where the questionnaire answers are ambiguous, two persons will decide what should be entered by
16 discussion until a consensus is reached. All data concerning participant information will be stored in locked file
17 cabinets accessible only by the principal investigator. All collected data will be pseudonymised and will
18 therefore be traceable only by means of a code. All files containing names or other personal identifiers, such
19 as the informed consent forms, will be stored separately from the data containing this code number.
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34

35 Statistical methods 36

37 **Statistical methods for primary and secondary outcomes** 38

39 The statistical analysis of the data will be performed with the statistical program SPSS, R or SAS using a
40 pseudonymised dataset. A correlation of the cfDNA concentration with the presence or absence of a SARS-
41 CoV-2 infection, as well as a correlation of these results with the data obtained from the questionnaires will be
42 determined. Descriptive statistics will summarise all study variables.
43
44
45
46

47 All data will be tested for normal distribution before and after log transformation using the Kolmogorov-Smirnov
48 test with the Lilliefors correction. If the assumption of normally distributed data cannot be rejected, arithmetic
49 group means \pm standard deviations will be calculated. If the normality test fails in at least one of the study
50 groups compared, all data will be expressed as group medians with data ranges given in parentheses.
51 Between-group comparisons of primary and secondary outcomes will be performed at t_0 and at two
52 consecutive time points independently (t_1 , t_2) using unpaired Student's t-tests (comparing two groups, normally
53 distributed) or the Mann-Whitney U test (comparing two groups, not normally distributed) or analysis of
54
55
56
57
58
59
60

1
2
3 variance (three groups) and chi-square tests for numerical and categorical data, respectively. P values will be
4 considered statistically significant if $p \leq 0.05$. We plan to perform a subgroup analysis for the concomitant
5 symptoms and the cfDNA levels at t_0 , t_1 and t_2 in the event of differences within each arm.
6
7

8
9 Statistical analyses will be carried out according to the intention-to-treat approach and therefore will include all
10 participants. The extent of missing data will be analysed. We will explore missing data patterns and determine
11 the type of missing data. We will use multiple imputation to substitute missing values.
12
13
14
15

16 **Patient and Public Involvement**

17
18 No patients were involved in the development of the research questions. The results of the temperature
19 measurement, the oxygen saturation and breathing rate of each individual patient will be communicated to the
20 patient directly after the examination by the attending physician. The laboratory and clinical parameters and
21 the levels of the cfDNA will be disseminated after they have been measured.
22
23
24
25
26

27 **Declarations**

28 **Ethics and dissemination**

29
30 Ethical approval was issued on 2021/03/01 by the Ethics Committee Essen under the number 21-9916-BO.
31 Findings will be disseminated initially to the impacted community, then by publication in scientific journals and
32 on international congresses.
33
34
35
36
37

38 **Registration:** The study is registered with DRKS under the number DRKS00024722. Informed consent will be
39 obtained from all participants in this study.
40
41
42

43 **References**

- 44
45
46 1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia
47 in China, 2019. *N Engl J Med*. 2020;382:727–33. doi:10.1056/NEJMoa2001017.
48
49
- 50
51 2. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting
52 Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New
53 York City Area. *JAMA*. 2020;323:2052. doi:10.1001/jama.2020.6775.
54
55
- 56
57 3. Tian H, Liu Y, Li Y, Wu C-H, Chen B, Kraemer MUG, et al. An investigation of transmission control
58 measures during the first 50 days of the COVID-19 epidemic in China. *Science* (80-). 2020;368:638–42.
59
60

1
2
3 doi:10.1126/science.abb6105.
4

5 4. Young BE, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, et al. Epidemiologic Features and Clinical
6 Course of Patients Infected With SARS-CoV-2 in Singapore. *JAMA*. 2020;323:1488.
7

8
9 doi:10.1001/jama.2020.3204.
10

11 5. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult
12 inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. 2020;395:1054–62.
13

14
15 doi:10.1016/S0140-6736(20)30566-3.
16

17 6. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, et al. Baseline Characteristics and
18 Outcomes of 1591 Patients Infected With SARS-CoV-2 Admitted to ICUs of the Lombardy Region, Italy.
19

20
21 *JAMA*. 2020;323:1574. doi:10.1001/jama.2020.5394.
22

23 7. Vincent J-L, Taccone FS. Understanding pathways to death in patients with COVID-19. *Lancet Respir*
24
25 *Med*. 2020;8:430–2. doi:10.1016/S2213-2600(20)30165-X.
26

27 8. Phua J, Weng L, Ling L, Egi M, Lim C-M, Divatia JV, et al. Intensive care management of coronavirus
28
29 disease 2019 (COVID-19): challenges and recommendations. *Lancet Respir Med*. 2020;8:506–17.
30

31
32 doi:10.1016/S2213-2600(20)30161-2.
33

34 9. Breitbach S, Sterzing B, Magallanes C, Tug S, Simon P. Direct measurement of cell-free DNA from
35
36 serially collected capillary plasma during incremental exercise. *J Appl Physiol*. 2014;117:119–30.
37

38
39 doi:10.1152/jappphysiol.00002.2014.
40

41 10. Breitbach S, Tug S, Simon P. Circulating cell-free DNA: an up-coming molecular marker in exercise
42
43 physiology. *Sports Med*. 2012;42:565–86. doi:10.2165/11631380-000000000-00000.
44

45 11. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an In Vivo Nucleosome
46
47 Footprint that Informs Its Tissues-Of-Origin. *Cell*. 2016;164:57–68.
48

49 12. Sun K, Jiang P, Cheng SH, Cheng THT, Wong J, Wong VWS, et al. Orientation-aware plasma cell-free
50
51 DNA fragmentation analysis in open chromatin regions informs tissue of origin. *Genome Res*. 2019;29:418–
52
53 27. doi:10.1101/gr.242719.118.
54

55 13. Sun K, Jiang P, Chan KCA, Wong J, Cheng YKY, Liang RHS, et al. Plasma DNA tissue mapping by
56
57 genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments.
58
59 *Proc Natl Acad Sci*. 2015;112:E5503–12. doi:10.1073/pnas.1508736112.
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

14. Michal M, Simon P, Gori T, König J, Wild PS, Wiltink J, et al. Psychodynamic Motivation and Training program (PMT) for the secondary prevention in patients with stable coronary heart disease: study protocol for a randomized controlled trial of feasibility and effects. *Trials*. 2013;14:314. doi:10.1186/1745-6215-14-314.
15. Helmig S, Frühbeis C, Krämer-Albers E-M, Simon P, Tug S. Release of bulk cell free DNA during physical exercise occurs independent of extracellular vesicles. *Eur J Appl Physiol*. 2015;115.
16. Tug S, Helmig S, Menke J, Zahn D, Kubiak T, Schwarting A, et al. Correlation between cell free DNA levels and medical evaluation of disease progression in systemic lupus erythematosus patients. *Cell Immunol*. 2014;292.
17. Zuo Y, Yalavarthi S, Shi H, Gockman K, Zuo M, Madison JA, et al. Neutrophil extracellular traps in COVID-19. *JCI Insight*. 2020. doi:10.1172/jci.insight.138999.
18. Chen X. Time-series plasma cell-free DNA analysis reveals disease severity of COVID19 patients. 2020.
19. Andargie TE, Tsuji N, Seifuddin F, Jang MK, Yuen PST, Kong H, et al. Cell-free DNA maps COVID-19 tissue injury and risk of death, and can cause tissue injury. *JCI Insight*. 2021. doi:10.1172/jci.insight.147610.
20. Zhang N. Circulating rather than alveolar extracellular DNA levels predict outcomes in influenza: a cohort study. 2020.
21. Jeong H, Lee J, Kim J, Choen S, Sohn KM, Kim Y-S, et al. Self-Assessment Questionnaire for Efficient and Safe Evaluation of Patients with Mild COVID-19. *Infect Chemother*. 2020;52:212. doi:10.3947/ic.2020.52.2.212.
22. Breitbach S, Sterzing B, Magallanes C, Tug S, Simon P. Direct measurement of cell-free DNA from serially collected capillary plasma during incremental exercise. *J Appl Physiol*. 2014;117.
23. Singh K, Mittal S, Gollapudi S, Butzmann A, Kumar J, Ohgami RS. A meta-analysis of SARS-CoV-2 patients identifies the combinatorial significance of D-dimer, C-reactive protein, lymphocyte, and neutrophil values as a predictor of disease severity. *Int J Lab Hematol*. 2021;43:324–8. doi:10.1111/ijlh.13354.
24. Suardi LR, Pallotto C, Esperti S, Tazzioli E, Baragli F, Salomoni E, et al. Risk factors for non-invasive/invasive ventilatory support in patients with COVID-19 pneumonia: A retrospective study within a multidisciplinary approach. *Int J Infect Dis*. 2020;100:258–63. doi:10.1016/j.ijid.2020.09.012.

- 1
2
3 25. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk Factors Associated With Acute Respiratory
4 Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China.
5 JAMA Intern Med. 2020;180:934. doi:10.1001/jamainternmed.2020.0994.
6
7
8
9 26. Petrilli CM, Jones SA, Yang J, Rajagopalan H, O'Donnell L, Chernyak Y, et al. Factors associated with
10 hospital admission and critical illness among 5279 people with coronavirus disease 2019 in New York City:
11 prospective cohort study. BMJ. 2020;:m1966. doi:10.1136/bmj.m1966.
12
13
14
15 27. Shenoy N, Luchtel R, Gulani P. Considerations for target oxygen saturation in COVID-19 patients: are
16 we under-shooting? BMC Med. 2020;18:260. doi:10.1186/s12916-020-01735-2.
17
18
19
20 28. Strauß R, Ewig S, Richter K, König T, Heller G, Bauer TT. The Prognostic Significance of Respiratory
21 Rate in Patients With Pneumonia. Dtsch Arzteblatt Online. 2014. doi:10.3238/arztebl.2014.0503.
22
23
24 29. Marshall JC, Murthy S, Diaz J, Adhikari NK, Angus DC, Arabi YM, et al. A minimal common outcome
25 measure set for COVID-19 clinical research. Lancet Infect Dis. 2020;20:e192–7. doi:10.1016/S1473-
26 3099(20)30483-7.
27
28
29
30

31 **Acknowledgements**

32 We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen.
33

34 **Author statement**

35 DD is the principal investigator; she conceived the study, obtained funding, led the proposal and protocol
36 development, and assisted in manuscript preparation and revision. EN contributed to the study concept and
37 design and assisted in manuscript preparation and revision. JidS assisted in manuscript revision. EG
38 contributed to the study concept and design and assisted in manuscript preparation. PS contributed to the
39 acquisition and analysis of qualitative data and the development of the intervention and assisted in manuscript
40 preparation and revision. SB contributed to the study concept and design and drafted the manuscript. All
41 authors read and approved the final manuscript.
42
43
44
45
46
47
48
49
50
51

52 **Funding**

53 This research received no specific grant from any funding agency in the public, commercial or not-for-profit
54 sectors. This study is funded entirely by the University of Essen and the University of Mainz.
55
56
57
58
59

60 **Conflicts of interests**

1
2
3 The authors declare that they have no conflicts of interests.
4

5
6 **Consent for publication**
7

8 Not required.
9

10
11
12 **Data Statement**
13

14 The study principal investigator and the co-investigators will have access to the full study data and materials.

15
16 The authors will be willing to share the individual-level study data after completion and publication of primary
17
18 and secondary analyses.
19

20
21
22
23 **Figure Legends**
24

25 Figure 1: Flowchart of the study design.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

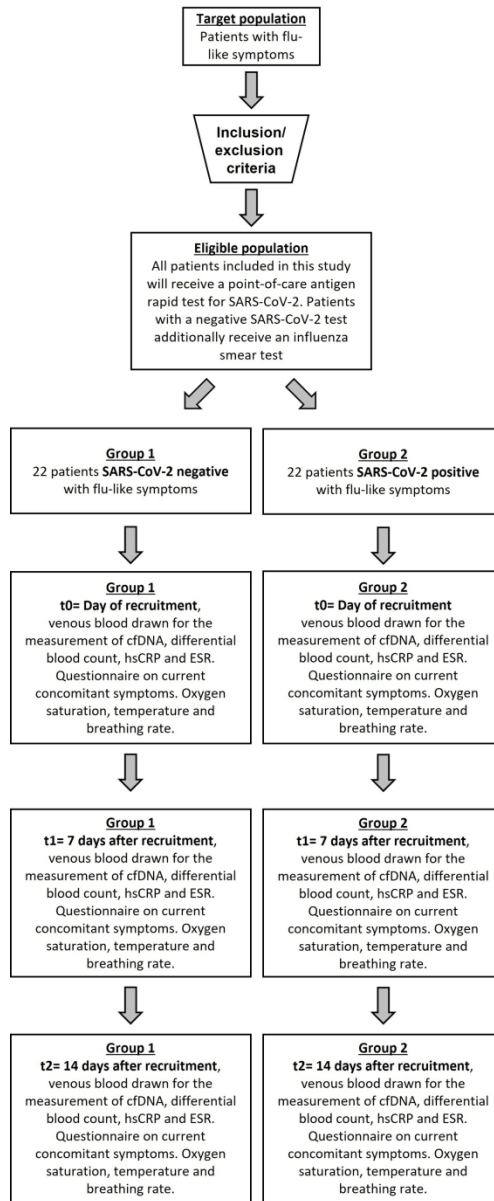


Figure 1: Flowchart of the study design.

119x289mm (300 x 300 DPI)

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2021-058647.R1
Article Type:	Protocol
Date Submitted by the Author:	17-Mar-2022
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,
Primary Subject Heading:	General practice / Family practice
Secondary Subject Heading:	Infectious diseases
Keywords:	MOLECULAR BIOLOGY, Cell biology < NATURAL SCIENCE DISCIPLINES, GENERAL MEDICINE (see Internal Medicine), COVID-19

SCHOLARONE™
Manuscripts

1
2
3 **cfDNA as a surrogate marker for COVID-19 severity in patients with**
4 **influenza-like symptoms with and without SARS-CoV-2 infections in**
5 **general practice: A study protocol for a prospective cohort study**
6
7
8
9

10
11
12
13 Dorothea Dehnen¹, Elmo Neuberger², Jürgen in der Schmitt¹, Ekaterini Giagkou¹, Perikles Simon², Suzan
14 Botzenhardt^{1*}
15

16
17
18
19 ¹Institute of General Practice, Medical Faculty, University of Duisburg-Essen, Essen, Germany

20
21 ²Department of Sports Medicine, Rehabilitation and Disease Prevention, Johannes Gutenberg University of
22 Mainz, Mainz, Germany
23

24
25
26 ***Corresponding author**

27 Suzan Botzenhardt, PhD

28 Institute for General Practice

29 University Hospital Essen

30 Hufelandstr. 55

31 45147 Essen / Germany

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

33 Email: suzan.botzenhardt@uk-essen.de

34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
Word count: 3916 words

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 rate (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,

1
2
3 but where no suitable prognostic markers are available at this time.

- 4
5 • The success of the study should be ensured by the close follow-up and home visits of the patients by
6 the general practitioner and the short duration of the study.
7
8
9 • cfDNA is already a well-established biomarker that is associated with various diseases and has been
10 used in different research areas, such as oncology, non-invasive prenatal diagnosis, organ
11 transplantation, autoimmune diseases, trauma, coronary heart disease and diabetes.
12
13
14 • Limitations of the study include that patients with a SARS-CoV-2 infection could have a severe disease
15 course requiring hospital treatment which could eventually lead to proceed with the study per protocol,
16 which may in worst case lead to missing laboratory values at t1 and t2. However, analysis will be done
17 according to intention to treat and hospitalisation as a main outcome will be assessed unless a patient
18 withdraws his or her consent.
19
20
21
22
23
24
25

26 **Keywords:** SARS-CoV-2 infection, cell-free DNA (cfDNA), flu/influenza, predictive marker, general practice.
27
28
29
30

31 Introduction

32
33
34 The current SARS-CoV-2 (severe acute respiratory syndrome coronavirus type 2, COVID-19) pandemic is
35 confronting humanity with a new dimension of medical, economic and social problems [1]. Among humans,
36 the virus spreads rapidly and causes varying degrees of severity of symptoms and illness in patients [2, 3].
37
38 Thus, the clinical course of patients with a SARS-CoV-2 infection can vary widely, from symptom-free courses
39 to severe courses that can lead to death. The incubation period of the virus ranges from 1 to 14 days, the
40 duration of viral excretion can last from 8 to 37 days, and the time from disease onset to discharge or death
41 ranges from 15-25 days [4, 5]. Furthermore, it has been shown that mortality rates correlate with increasing
42 age and pre-existing concomitant diseases, such as cardiovascular disease, diabetes, overweight and
43 hypertension [2, 3, 6]. In the course of analysing laboratory values of SARS-CoV-2 patients, such as
44 measurements of lymphocyte counts, C-reactive protein (CRP), as well as secondary bacterial infections it
45 was found that the analysis and evaluation of these do not allow an assessment of the actual course of the
46 disease [7, 8]. In order to be able to better assess the course of this disease, it would be important to find a
47 predictive marker that could be used to determine the severity of the disease at the earliest possible stage.
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 One marker that could play an important role in this determination is cell-free DNA (cfDNA), which is usually
4 released from cells by apoptosis, necrosis, NETosis, as well as active secretion [9, 10]. It comprises a high
5 variability of fragmented molecules that contain valuable information about gene expression and the
6 nucleosome pattern in relation to their tissue of origin [11–13]. Numerous studies have already demonstrated
7 that cfDNA levels are associated with various diseases and have been used in various research areas, such
8 as oncology, non-invasive prenatal diagnosis, organ transplantation, autoimmune diseases, trauma, coronary
9 heart disease and diabetes [11–16].

10
11 Recent studies have investigated the role of cfDNA as a potential marker for therapeutic targets of SARS-
12 CoV-2 in order to develop new therapeutic strategies for the disease [17]. In their study, Chen et al. profiled
13 and analysed for the first-time plasma cell-free DNA (cfDNA) of mild and severe COVID-19 patients. They
14 found that in comparison between mild and severe COVID-19 patients, Interleukin-37 signalling was one of
15 the most relevant pathways. Their data thus revealed potential tissue involvement, provided insights into
16 mechanism on COVID-19 progression, and highlighted utility of cfDNA as a non-invasive biomarker for disease
17 severity inspections [18]. In a further study, Andargie et al. showed that cfDNA levels correlated positively with
18 COVID-19 disease severity, C-reactive protein and D-dimer, and that the cfDNA profile at admission identified
19 patients who subsequently required intensive care or died during hospitalization. They conclude that cfDNA
20 could be used as a potential diagnostic biomarker to map sources of injury and as a prognostic biomarker to
21 predict COVID-19 trajectory and outcome by providing mechanistic information about COVID-19-induced
22 tissue injury [19]. However, in this study, cfDNA levels were not measured in patients before they presented
23 at the hospital. Our study will instead focus on patients who visit their GP's office with mild flu-like symptoms.
24 In addition to the studies on cfDNA levels and SARS-CoV-2, further studies have measured the impact of
25 influenza on cfDNA levels. Again, it was shown that patients had significantly increased cfDNA levels [19, 20].
26 The aim of our study is to determine if cfDNA levels differ in patients with mild or moderate flu symptoms when
27 either SARS-CoV-2 infection or infection with another common respiratory pathogen is present or if these
28 concentrations are similar to each other.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53

54 **Methods**

56 **Design**

57 This is a single-centre, two-arm, parallel cohort study with a 1:1 allocation ratio to be conducted between
58
59
60

1
2
3 August 2021 and April 2022. 22 patients with flu-like symptoms without a SARS-CoV-2 infection and
4
5 22 patients with flu-like symptoms with a SARS-CoV-2 infection will be included (n=44, age over 18 years)
6
7 (see Figure 1). Due to our study design, no randomisation is needed. Thus, no blinding will be performed.
8
9
10

11 ***insert Figure 1 here***
12
13
14
15

16 **Patients**

17 **Setting of the study and characteristics of participants**

18
19 This single-centre study will be conducted at the University Hospital in Essen. Patients who visit their general
20
21 practice in Mülheim an der Ruhr with flu-like symptoms will be asked to complete a screening questionnaire to
22
23 check if they met the inclusion criteria. If they meet the eligibility criteria, they will be included in the study.
24
25 Written informed consent will be obtained by the principal investigator from all patients willing to participate in
26
27 the study.
28
29
30
31

32 **Inclusion and exclusion criteria**

33
34 All persons enrolled in the study must provide full written informed consent and are required to complete a
35
36 baseline screening questionnaire to assess their eligibility.
37
38

39 Inclusion criteria are:

- 40 1. Age 18-99 years,
- 41 42 2. Consent given by the patient or legal representative for blood draw, oropharyngeal or nasopharyngeal
43 swab for a rapid SARS-CoV-2 antigen test and subsequent qPCR, if applicable,
- 44 45 3. Sufficient knowledge of the German language to understand the study content and instructions.
46
47

48 Exclusion criteria are:

- 49 50 1. Severe acute or chronic illness with known elevated cfDNA levels due to the underlying disease, e.g.:
51
52 a. Tumour disease
53
54 b. Severe renal insufficiency
55
56 c. Severe/moderate inflammatory diseases
57
58 d. Autoimmune diseases
59
60 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

1
2
3 determine cfDNA levels in patients who remain symptomatic. Special note will be made of whether there has
4 been a new SARS-CoV-2 infection or a vaccination against SARS-CoV-2 in the meantime.

5
6
7 Analysis will be done intention to treat, as it may not be possible to retrieve blood samples from the patients
8 at t1 or t2 due to hospitalisation or other circumstances. All participants can discontinue their participation in
9 the trial at any time for any reason without prejudice to current or future medical care. The investigator may
10 discontinue patients' participation in the trial for any reason for their safety or in their best interest. If participants
11 express a desire to withdraw from the study, they will receive instructions to complete an "end-of-study" visit,
12 which will also be voluntary. All participants can receive any concomitant treatment at any time during the trial.
13 However, participants must indicate at each study visit whether they are receiving any concomitant drug
14 therapy.
15
16
17
18
19
20
21
22

23 In the context of this research project, the participants will be insured for potential damages of the biomaterial
24 collection as well as for commuting accidents in accordance with § 2 Para. 1 No. 13b Social Security Code
25 (SGB) VII. After the trial, the patients will receive their primary care by the general practitioner as usual. There
26 is thus no need to provide any additional post-trial care.
27
28
29
30

31 **Measurement of cfDNA concentrations**

32
33
34 CfDNA concentrations will be determined at the Department of Sports Medicine, Prevention and Rehabilitation
35 at the Johannes Gutenberg University Mainz, in a S2 laboratory. We will use validated qPCR assays to
36 determine the concentrations of a 90 bp and 222 bp fragment in diluted EDTA plasma samples without prior
37 DNA isolation [20]. As described in Neuberger et al. the assays specifically target repetitive sequences, which
38 facilitates highly sensitive cfDNA detection from small amounts of diluted plasma. No DNA isolation is required
39 which saves time, costs, and avoids the loss of DNA due to the isolation procedure. The assays show
40 repeatability $\leq 11.6\%$ (95% CI 8.1–20.3), and intermediate precision $\leq 12.1\%$ (95% CI 9.2–17.7). Moreover,
41 the robustness of the assays was demonstrated by incurred sample reanalysis, indicating sufficient validity
42 and sensitivity to quantify cfDNA in the study samples. The blood samples t0 were collected at the day when
43 the patients came to the General practice with having symptoms occurring in the last 24-48 hours. These
44 samples than were directly centrifuged in the General practice.
45
46
47
48
49
50
51
52
53
54
55

56 **Participant timeline**

57
58 The symptomatic participant timeline is presented in Table 1.
59
60

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 t₀, t₁ and t₂). 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,

1
2
3 minor bleeding can occur during nasopharyngeal swabbing; serious injuries do not occur if the procedure is
4 performed correctly. The investigator will assess the severity of each adverse event and will report all serious
5 and non-serious adverse events in the electronic case report form. The investigator will also assess the causal
6 relationship of the serious adverse events to the trial intervention. Termination criteria have not been defined,
7 as it does not seem reasonable for the planned study with a short survey period.
8
9
10
11
12

13 **Sample size calculation**

14
15
16 With respect to the group with flu-like symptoms without a SARS-CoV-2 infection, we expect –with a relatively
17 high variance– a twofold increase in cfDNA concentrations compared to healthy subjects. A group comparable
18 to this can be found, for example, in the group of chronic inflammatory and currently non-acute diseases, such
19 as systemic lupus erythematosus. In one study, we showed that lupus patients had a mean cfDNA level of
20 44.7 ng/mL with a standard deviation of 53.5 ng/mL [16]. We hypothesize that a SARS-CoV-2 infection will
21 increase the levels by 100% once more compared with the cohort with influenza-like symptoms without a
22 SARS-CoV-2 infection. Furthermore, we want to be sure enough to obtain group sizes of at least
23 22 participants in each intervention group (total n=44) and to adjust for any dropouts during the study. Sample
24 size is planned by a two-sample t-test on a two-sided significance level of $\alpha=5\%$ to achieve a power of more
25 than 80%. We therefore plan to include a total of around 35 patients of comparable age and sex in both the
26 group with a positive and the group with a negative test.
27
28
29
30
31
32
33
34
35
36

37 Recruitment will take place in the general practice in Mülheim an der Ruhr via the respective principal
38 physician. We plan to include a total of 44 patients until April 2022.
39
40
41

42 **Plans to promote participant retention and complete follow-up**

43
44 Participants will benefit from the study as they will know immediately whether a SARS-CoV-2 infection is
45 present via point-of-care diagnostics (Roche SARS-CoV-2 Rapid Antigen Test: sensitivity: 96.52%, specificity:
46 99.68%) which will be subsequently validated by RT-qPCR. During the study, the patients will be cared for in
47 their home environment by their general practitioner. If participants express a desire to withdraw from the
48 study, they will be asked to complete an “end-of-study” visit. Data collected up to the time of withdrawal will
49 remain in the trial database and be included in data analysis, unless otherwise indicated by the participant.
50
51
52
53
54
55
56

57 **Data management**

58
59 Trial data will be collected in the electronic case report form by the principal investigator at the Institute of
60

1
2
3 General Practice at the University Hospital in Essen. Source documents, defined as any original document or
4 object making it possible to prove the existence or accuracy of data or facts recorded during the research, will
5 be kept by the principal physician according to the regulations in force. All questionnaire data will be entered
6 twice by two different persons to ensure the dual control principle. Using a software tool, a third person will
7 check the agreement between the two datasets resulting from the double entry. In cases where entries deviate
8 from one another, the third person will determine the correct entry by looking at the questionnaire. In cases
9 where the questionnaire answers are ambiguous, two persons will decide what should be entered by
10 discussion until a consensus is reached. All data concerning participant information will be stored in locked file
11 cabinets accessible only by the principal investigator. All collected data will be pseudonymised and will
12 therefore be traceable only by means of a code. All files containing names or other personal identifiers, such
13 as the informed consent forms, will be stored separately from the data containing this code number.

24 **Statistical methods**

27 **Statistical methods for primary and secondary outcomes**

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

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

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

1
2
3 the type of missing data. We will use multiple imputation to substitute missing values.
4
5
6

7 **Patient and Public Involvement**

8
9 No patients were involved in the development of the research questions. The results of the temperature
10 measurement, the oxygen saturation and breathing rate of each individual patient will be communicated to the
11 patient directly after the examination by the attending physician. The laboratory and clinical parameters and
12 the levels of the cfDNA will be disseminated after they have been measured.
13
14
15
16

17 **Declarations**

18 **Ethics and dissemination**

19
20 Ethical approval was issued on 2021/03/01 by the Ethics Committee Essen under the number 21-9916-BO.
21 Findings will be disseminated initially to the impacted community, then by publication in scientific journals and
22 on international congresses.
23
24

25 **Registration:** The study is registered with DRKS under the number DRKS00024722. Informed consent will be
26 obtained from all participants in this study.
27
28
29

30 **References**

- 31
32
33
34
35
36
37 1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia
38 in China, 2019. *N Engl J Med.* 2020;382:727–33. doi:10.1056/NEJMoa2001017.
39
40
41 2. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting
42 Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New
43 York City Area. *JAMA.* 2020;323:2052. doi:10.1001/jama.2020.6775.
44
45
46 3. Tian H, Liu Y, Li Y, Wu C-H, Chen B, Kraemer MUG, et al. An investigation of transmission control
47 measures during the first 50 days of the COVID-19 epidemic in China. *Science (80-).* 2020;368:638–42.
48
49
50
51
52
53
54
55 4. Young BE, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, et al. Epidemiologic Features and Clinical
56 Course of Patients Infected With SARS-CoV-2 in Singapore. *JAMA.* 2020;323:1488.
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

5. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. 2020;395:1054–62. doi:10.1016/S0140-6736(20)30566-3.
6. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, et al. Baseline Characteristics and Outcomes of 1591 Patients Infected With SARS-CoV-2 Admitted to ICUs of the Lombardy Region, Italy. *JAMA*. 2020;323:1574. doi:10.1001/jama.2020.5394.
7. Vincent J-L, Taccone FS. Understanding pathways to death in patients with COVID-19. *Lancet Respir Med*. 2020;8:430–2. doi:10.1016/S2213-2600(20)30165-X.
8. Phua J, Weng L, Ling L, Egi M, Lim C-M, Divatia JV, et al. Intensive care management of coronavirus disease 2019 (COVID-19): challenges and recommendations. *Lancet Respir Med*. 2020;8:506–17. doi:10.1016/S2213-2600(20)30161-2.
9. Breitbach S, Sterzing B, Magallanes C, Tug S, Simon P. Direct measurement of cell-free DNA from serially collected capillary plasma during incremental exercise. *J Appl Physiol*. 2014;117:119–30. doi:10.1152/jappphysiol.00002.2014.
10. Breitbach S, Tug S, Simon P. Circulating cell-free DNA: an up-coming molecular marker in exercise physiology. *Sports Med*. 2012;42:565–86. doi:10.2165/11631380-000000000-00000.
11. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. *Cell*. 2016;164:57–68.
12. Sun K, Jiang P, Cheng SH, Cheng THT, Wong J, Wong VWS, et al. Orientation-aware plasma cell-free DNA fragmentation analysis in open chromatin regions informs tissue of origin. *Genome Res*. 2019;29:418–27. doi:10.1101/gr.242719.118.
13. Sun K, Jiang P, Chan KCA, Wong J, Cheng YKY, Liang RHS, et al. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc Natl Acad Sci*. 2015;112:E5503–12. doi:10.1073/pnas.1508736112.
14. Michal M, Simon P, Gori T, König J, Wild PS, Wiltink J, et al. Psychodynamic Motivation and Training program (PMT) for the secondary prevention in patients with stable coronary heart disease: study protocol for a randomized controlled trial of feasibility and effects. *Trials*. 2013;14:314. doi:10.1186/1745-6215-14-314.

15. Helmig S, Frühbeis C, Krämer-Albers E-M, Simon P, Tug S. Release of bulk cell free DNA during physical exercise occurs independent of extracellular vesicles. *Eur J Appl Physiol.* 2015;115.
16. Tug S, Helmig S, Menke J, Zahn D, Kubiak T, Schwarting A, et al. Correlation between cell free DNA levels and medical evaluation of disease progression in systemic lupus erythematosus patients. *Cell Immunol.* 2014;292.
17. Zuo Y, Yalavarthi S, Shi H, Gockman K, Zuo M, Madison JA, et al. Neutrophil extracellular traps in COVID-19. *JCI Insight.* 2020. doi:10.1172/jci.insight.138999.
18. Chen X. Time-series plasma cell-free DNA analysis reveals disease severity of COVID19 patients. 2020.
19. Andargie TE, Tsuji N, Seifuddin F, Jang MK, Yuen PST, Kong H, et al. Cell-free DNA maps COVID-19 tissue injury and risk of death, and can cause tissue injury. *JCI Insight.* 2021. doi:10.1172/jci.insight.147610.
20. Zhang N. Circulating rather than alveolar extracellular DNA levels predict outcomes in influenza: a cohort study. 2020.
21. https://influenza.rki.de/Wochenberichte/2021_2022/2022-10.pdf.
22. <https://influenza.rki.de/wochenberichte.aspx>.
23. Jeong H, Lee J, Kim J, Choen S, Sohn KM, Kim Y-S, et al. Self-Assessment Questionnaire for Efficient and Safe Evaluation of Patients with Mild COVID-19. *Infect Chemother.* 2020;52:212. doi:10.3947/ic.2020.52.2.212.
24. Breitbach S, Sterzing B, Magallanes C, Tug S, Simon P. Direct measurement of cell-free DNA from serially collected capillary plasma during incremental exercise. *J Appl Physiol.* 2014;117.
25. (NICE) LNI for H and CE. COVID-19 rapid guideline: managing the long-term effects of COVID-19. *NICE Guidel.* 2020;188.
26. Singh K, Mittal S, Gollapudi S, Butzmann A, Kumar J, Ohgami RS. A meta-analysis of SARS-CoV-2 patients identifies the combinatorial significance of D-dimer, C-reactive protein, lymphocyte, and neutrophil values as a predictor of disease severity. *Int J Lab Hematol.* 2021;43:324–8. doi:10.1111/ijlh.13354.
27. Suardi LR, Pallotto C, Esperti S, Tazzioli E, Baragli F, Salomoni E, et al. Risk factors for non-invasive/invasive ventilatory support in patients with COVID-19 pneumonia: A retrospective study within a multidisciplinary approach. *Int J Infect Dis.* 2020;100:258–63. doi:10.1016/j.ijid.2020.09.012.

- 1
2
3 28. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk Factors Associated With Acute Respiratory
4 Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China.
5 JAMA Intern Med. 2020;180:934. doi:10.1001/jamainternmed.2020.0994.
6
7
8
9 29. Petrilli CM, Jones SA, Yang J, Rajagopalan H, O'Donnell L, Chernyak Y, et al. Factors associated with
10 hospital admission and critical illness among 5279 people with coronavirus disease 2019 in New York City:
11 prospective cohort study. BMJ. 2020;:m1966. doi:10.1136/bmj.m1966.
12
13
14 30. Shenoy N, Luchtel R, Gulani P. Considerations for target oxygen saturation in COVID-19 patients: are
15 we under-shooting? BMC Med. 2020;18:260. doi:10.1186/s12916-020-01735-2.
16
17
18 31. Strauß R, Ewig S, Richter K, König T, Heller G, Bauer TT. The Prognostic Significance of Respiratory
19 Rate in Patients With Pneumonia. Dtsch Arzteblatt Online. 2014. doi:10.3238/arztebl.2014.0503.
20
21
22 32. Marshall JC, Murthy S, Diaz J, Adhikari NK, Angus DC, Arabi YM, et al. A minimal common outcome
23 measure set for COVID-19 clinical research. Lancet Infect Dis. 2020;20:e192–7. doi:10.1016/S1473-
24 3099(20)30483-7.
25
26
27
28
29
30

31 **Acknowledgements**

32 We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen.
33

34 **Author statement**

35 DD is the principal investigator; she conceived the study, obtained funding, led the proposal and protocol
36 development, and assisted in manuscript preparation and revision. EN contributed to the study concept and
37 design and assisted in manuscript preparation and revision. JidS assisted in manuscript revision. EG
38 contributed to the study concept and design and assisted in manuscript preparation. PS contributed to the
39 acquisition and analysis of qualitative data and the development of the intervention and assisted in manuscript
40 preparation and revision. SB contributed to the study concept and design and drafted the manuscript. All
41 authors read and approved the final manuscript.
42
43
44
45
46
47
48
49
50
51

52 **Funding**

53 This research received no specific grant from any funding agency in the public, commercial or not-for-profit
54 sectors. This study is funded entirely by the University of Essen and the University of Mainz.
55
56
57
58
59

60 **Conflicts of interests**

1
2
3 The authors declare that they have no conflicts of interests.
4

5 **Consent for publication**

6
7
8 Not required.
9

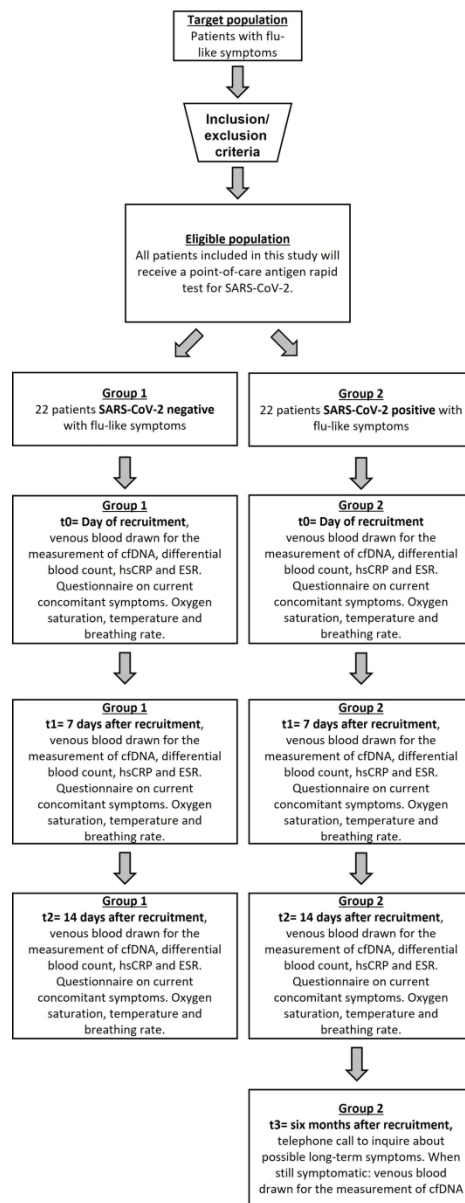
10 11 **Data Statement**

12 The study principal investigator and the co-investigators will have access to the full study data and materials.

13
14
15 The authors will be willing to share the individual-level study data after completion and publication of primary
16
17 and secondary analyses.
18
19

20 21 22 **Figure Legends**

23
24
25 Figure 1: Flowchart of the study design.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



122x316mm (300 x 300 DPI)

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.

1
2 **Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE
3 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
4 <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

For peer review only

6/bmjopen-2021-015864 on 16 June 2022. Downloaded from <http://bmjopen.bmj.com/> on April 17, 2024 by guest. Protected by copyright.

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2021-058647.R2
Article Type:	Protocol
Date Submitted by the Author:	19-May-2022
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 Schmittgen, 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,
Primary Subject Heading:	General practice / Family practice
Secondary Subject Heading:	Infectious diseases
Keywords:	MOLECULAR BIOLOGY, Cell biology < NATURAL SCIENCE DISCIPLINES, GENERAL MEDICINE (see Internal Medicine), COVID-19

SCHOLARONE™
Manuscripts

1
2
3 **cfDNA as a surrogate marker for COVID-19 severity in patients with**
4 **influenza-like symptoms with and without SARS-CoV-2 infections in**
5 **general practice: A study protocol for a prospective cohort study**
6
7
8
9

10
11
12
13 Dorothea Dehnen¹, Elmo Neuberger², Jürgen in der Schmitt¹, Ekaterini Giagkou¹, Perikles Simon², Suzan
14 Botzenhardt^{1*}
15

16
17
18
19 ¹Institute of General Practice, Medical Faculty, University of Duisburg-Essen, Essen, Germany

20
21 ²Department of Sports Medicine, Rehabilitation and Disease Prevention, Johannes Gutenberg University of
22 Mainz, Mainz, Germany
23

24
25
26 ***Corresponding author**

27 Suzan Botzenhardt, PhD

28 Institute for General Practice

29 University Hospital Essen

30 Hufelandstr. 55

31 45147 Essen / Germany

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

33 Email: suzan.botzenhardt@uk-essen.de

34
35
36
37
38
39
40
41
42 Word count: 3916 words
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 rate (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.

1
2
3 One marker that could play an important role in this determination is cell-free DNA (cfDNA), which is usually
4 released from cells by apoptosis, necrosis, NETosis, as well as active secretion [9, 10]. It comprises a high
5 variability of fragmented molecules that contain valuable information about gene expression and the
6 nucleosome pattern in relation to their tissue of origin [11–13]. Numerous studies have already demonstrated
7 that cfDNA levels are associated with various diseases and have been used in various research areas, such
8 as oncology, non-invasive prenatal diagnosis, organ transplantation, autoimmune diseases, trauma, coronary
9 heart disease and diabetes [11–16].

10
11 Recent studies have investigated the role of cfDNA as a potential marker for therapeutic targets of SARS-
12 CoV-2 in order to develop new therapeutic strategies for the disease [17]. In their study, Chen et al. profiled
13 and analysed for the first-time plasma cell-free DNA (cfDNA) of mild and severe COVID-19 patients. They
14 found that in comparison between mild and severe COVID-19 patients, Interleukin-37 signalling was one of
15 the most relevant pathways. Their data thus revealed potential tissue involvement, provided insights into
16 mechanism on COVID-19 progression, and highlighted utility of cfDNA as a non-invasive biomarker for disease
17 severity inspections [18]. In a further study, Andargie et al. showed that cfDNA levels correlated positively with
18 COVID-19 disease severity, C-reactive protein and D-dimer, and that the cfDNA profile at admission identified
19 patients who subsequently required intensive care or died during hospitalization. They conclude that cfDNA
20 could be used as a potential diagnostic biomarker to map sources of injury and as a prognostic biomarker to
21 predict COVID-19 trajectory and outcome by providing mechanistic information about COVID-19-induced
22 tissue injury [19]. However, in this study, cfDNA levels were not measured in patients before they presented
23 at the hospital. Our study will instead focus on patients who visit their GP's office with mild flu-like symptoms.
24 In addition to the studies on cfDNA levels and SARS-CoV-2, further studies have measured the impact of
25 influenza on cfDNA levels. Again, it was shown that patients had significantly increased cfDNA levels [19, 20].
26 The aim of our study is to determine if cfDNA levels differ in patients with mild or moderate flu symptoms when
27 either SARS-CoV-2 infection or infection with another common respiratory pathogen is present or if these
28 concentrations are similar to each other.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53

54 **Methods**

56 **Design**

57 This is a single-centre, two-arm, parallel cohort study with a 1:1 allocation ratio to be conducted between
58
59
60

1
2
3 August 2021 and April 2022. 22 patients with flu-like symptoms without a SARS-CoV-2 infection and
4
5 22 patients with flu-like symptoms with a SARS-CoV-2 infection will be included (n=44, age over 18 years)
6
7 (see Figure 1). Due to our study design, no randomisation is needed. Thus, no blinding will be performed.
8
9
10

11 ***insert Figure 1 here***
12
13
14
15

16 **Patients**

17 **Setting of the study and characteristics of participants**

18
19 This single-centre study will be conducted at the University Hospital in Essen. Patients who visit their general
20
21 practice in Mülheim an der Ruhr with flu-like symptoms will be asked to complete a screening questionnaire to
22
23 check if they met the inclusion criteria. If they meet the eligibility criteria, they will be included in the study.
24
25 Written informed consent will be obtained by the principal investigator from all patients willing to participate in
26
27 the study.
28
29
30

31 **Inclusion and exclusion criteria**

32
33 All persons enrolled in the study must provide full written informed consent and are required to complete a
34
35 baseline screening questionnaire to assess their eligibility.
36
37

38 Inclusion criteria are:

- 39 1. Age 18-99 years,
- 40 41 2. Consent given by the patient or legal representative for blood draw, oropharyngeal or nasopharyngeal
42 43 swab for a rapid SARS-CoV-2 antigen test and subsequent qPCR, if applicable,
- 44 45 3. Sufficient knowledge of the German language to understand the study content and instructions.
46 47

48 Exclusion criteria are:

- 49 50 1. Severe acute or chronic illness with known elevated cfDNA levels due to the underlying disease, e.g.:
51 52 a. Tumour disease
53 54 b. Severe renal insufficiency
55 56 c. Severe/moderate inflammatory diseases
57 58 d. Autoimmune diseases
59 60 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 $\leq 11.6\%$ (95% CI 8.1–20.3), and intermediate precision $\leq 12.1\%$ (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 t₀, t₁ and t₂). 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,

1
2
3 minor bleeding can occur during nasopharyngeal swabbing; serious injuries do not occur if the procedure is
4 performed correctly. The investigator will assess the severity of each adverse event and will report all serious
5 and non-serious adverse events in the electronic case report form. The investigator will also assess the causal
6 relationship of the serious adverse events to the trial intervention. Termination criteria have not been defined,
7 as it does not seem reasonable for the planned study with a short survey period.
8
9
10
11
12

13 **Sample size calculation**

14
15
16 With respect to the group with flu-like symptoms without a SARS-CoV-2 infection, we expect –with a relatively
17 high variance– a twofold increase in cfDNA concentrations compared to healthy subjects. A group comparable
18 to this can be found, for example, in the group of chronic inflammatory and currently non-acute diseases, such
19 as systemic lupus erythematosus. In one study, we showed that lupus patients had a mean cfDNA level of
20 44.7 ng/mL with a standard deviation of 53.5 ng/mL [16]. We hypothesize that a SARS-CoV-2 infection will
21 increase the levels by 100% once more compared with the cohort with influenza-like symptoms without a
22 SARS-CoV-2 infection. Furthermore, we want to be sure enough to obtain group sizes of at least
23 22 participants in each intervention group (total n=44) and to adjust for any dropouts during the study. Sample
24 size is planned by a two-sample t-test on a two-sided significance level of $\alpha=5\%$ to achieve a power of more
25 than 80%. We therefore plan to include a total of around 35 patients of comparable age and sex in both the
26 group with a positive and the group with a negative test.
27
28
29
30
31
32
33
34
35
36

37 Recruitment will take place in the general practice in Mülheim an der Ruhr via the respective principal
38 physician. We plan to include a total of 44 patients until April 2022.
39
40
41

42 **Plans to promote participant retention and complete follow-up**

43
44 Participants will benefit from the study as they will know immediately whether a SARS-CoV-2 infection is
45 present via point-of-care diagnostics (Roche SARS-CoV-2 Rapid Antigen Test: sensitivity: 96.52%, specificity:
46 99.68%) which will be subsequently validated by RT-qPCR. During the study, the patients will be cared for in
47 their home environment by their general practitioner. If participants express a desire to withdraw from the
48 study, they will be asked to complete an “end-of-study” visit. Data collected up to the time of withdrawal will
49 remain in the trial database and be included in data analysis, unless otherwise indicated by the participant.
50
51
52
53
54
55
56

57 **Data management**

58
59 Trial data will be collected in the electronic case report form by the principal investigator at the Institute of
60

1
2
3 General Practice at the University Hospital in Essen. Source documents, defined as any original document or
4 object making it possible to prove the existence or accuracy of data or facts recorded during the research, will
5 be kept by the principal physician according to the regulations in force. All questionnaire data will be entered
6
7 twice by two different persons to ensure the dual control principle. Using a software tool, a third person will
8
9 check the agreement between the two datasets resulting from the double entry. In cases where entries deviate
10
11 from one another, the third person will determine the correct entry by looking at the questionnaire. In cases
12
13 where the questionnaire answers are ambiguous, two persons will decide what should be entered by
14
15 discussion until a consensus is reached. All data concerning participant information will be stored in locked file
16
17 cabinets accessible only by the principal investigator. All collected data will be pseudonymised and will
18
19 therefore be traceable only by means of a code. All files containing names or other personal identifiers, such
20
21 as the informed consent forms, will be stored separately from the data containing this code number.
22
23
24

25 **Statistical methods**

26 **Statistical methods for primary and secondary outcomes**

27
28 The statistical analysis of the data will be performed with the statistical program SPSS, R or SAS using a
29
30 pseudonymised dataset. A correlation of the cfDNA concentration with the presence or absence of a SARS-
31
32 CoV-2 infection, as well as a correlation of these results with the data obtained from the questionnaires will be
33
34 determined. Descriptive statistics will summarise all study variables.
35
36

37
38 All data will be tested for normal distribution before and after log transformation using the Kolmogorov-Smirnov
39
40 test with the Lilliefors correction. If the assumption of normally distributed data cannot be rejected, arithmetic
41
42 group means \pm standard deviations will be calculated. If the normality test fails in at least one of the study
43
44 groups compared, all data will be expressed as group medians with inter-quartile ranges given in parentheses.
45
46 Between-group comparisons of primary and secondary outcomes will be performed at t_0 and at two
47
48 consecutive time points independently (t_1 , t_2) using unpaired Student's t-tests (comparing two groups, normally
49
50 distributed) or the Mann-Whitney U test (comparing two groups, not normally distributed) or analysis of
51
52 variance (three groups) and chi-square tests for numerical and categorical data, respectively. P values will be
53
54 considered statistically significant if $p \leq 0.05$. We plan to perform a subgroup analysis for the concomitant
55
56 symptoms and the cfDNA levels at t_0 , t_1 and t_2 in the event of differences within each arm.

57
58 Statistical analyses will be carried out according to the intention-to-treat approach and therefore will include all
59
60 participants. The extent of missing data will be analysed. We will explore missing data patterns and determine

1
2
3 the type of missing data. We will use multiple imputation to substitute missing values.
4
5
6

7 **Patient and Public Involvement**

8
9 No patients were involved in the development of the research questions. The results of the temperature
10 measurement, the oxygen saturation and breathing rate of each individual patient will be communicated to the
11 patient directly after the examination by the attending physician. The laboratory and clinical parameters and
12 the levels of the cfDNA will be disseminated after they have been measured.
13
14
15
16

17 **Declarations**

18 **Ethics and dissemination**

19
20 Ethical approval was issued on 2021/03/01 by the Ethics Committee Essen under the number 21-9916-BO.
21 Findings will be disseminated initially to the impacted community, then by publication in scientific journals and
22 on international congresses.
23
24

25 **Registration:** The study is registered with DRKS under the number DRKS00024722. Informed consent will be
26 obtained from all participants in this study.
27
28
29

30 **References**

- 31
32
33
34
35
36
37 1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia
38 in China, 2019. *N Engl J Med.* 2020;382:727–33. doi:10.1056/NEJMoa2001017.
39
40
41 2. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting
42 Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New
43 York City Area. *JAMA.* 2020;323:2052. doi:10.1001/jama.2020.6775.
44
45
46 3. Tian H, Liu Y, Li Y, Wu C-H, Chen B, Kraemer MUG, et al. An investigation of transmission control
47 measures during the first 50 days of the COVID-19 epidemic in China. *Science (80-).* 2020;368:638–42.
48
49
50
51
52
53
54
55 4. Young BE, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, et al. Epidemiologic Features and Clinical
56 Course of Patients Infected With SARS-CoV-2 in Singapore. *JAMA.* 2020;323:1488.
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

5. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. 2020;395:1054–62. doi:10.1016/S0140-6736(20)30566-3.
6. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, et al. Baseline Characteristics and Outcomes of 1591 Patients Infected With SARS-CoV-2 Admitted to ICUs of the Lombardy Region, Italy. *JAMA*. 2020;323:1574. doi:10.1001/jama.2020.5394.
7. Vincent J-L, Taccone FS. Understanding pathways to death in patients with COVID-19. *Lancet Respir Med*. 2020;8:430–2. doi:10.1016/S2213-2600(20)30165-X.
8. Phua J, Weng L, Ling L, Egi M, Lim C-M, Divatia JV, et al. Intensive care management of coronavirus disease 2019 (COVID-19): challenges and recommendations. *Lancet Respir Med*. 2020;8:506–17. doi:10.1016/S2213-2600(20)30161-2.
9. Breitbach S, Sterzing B, Magallanes C, Tug S, Simon P. Direct measurement of cell-free DNA from serially collected capillary plasma during incremental exercise. *J Appl Physiol*. 2014;117:119–30. doi:10.1152/jappphysiol.00002.2014.
10. Breitbach S, Tug S, Simon P. Circulating cell-free DNA: an up-coming molecular marker in exercise physiology. *Sports Med*. 2012;42:565–86. doi:10.2165/11631380-000000000-00000.
11. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. *Cell*. 2016;164:57–68.
12. Sun K, Jiang P, Cheng SH, Cheng THT, Wong J, Wong VWS, et al. Orientation-aware plasma cell-free DNA fragmentation analysis in open chromatin regions informs tissue of origin. *Genome Res*. 2019;29:418–27. doi:10.1101/gr.242719.118.
13. Sun K, Jiang P, Chan KCA, Wong J, Cheng YKY, Liang RHS, et al. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc Natl Acad Sci*. 2015;112:E5503–12. doi:10.1073/pnas.1508736112.
14. Michal M, Simon P, Gori T, König J, Wild PS, Wiltink J, et al. Psychodynamic Motivation and Training program (PMT) for the secondary prevention in patients with stable coronary heart disease: study protocol for a randomized controlled trial of feasibility and effects. *Trials*. 2013;14:314. doi:10.1186/1745-6215-14-314.

15. Helmig S, Frühbeis C, Krämer-Albers E-M, Simon P, Tug S. Release of bulk cell free DNA during physical exercise occurs independent of extracellular vesicles. *Eur J Appl Physiol.* 2015;115.
16. Tug S, Helmig S, Menke J, Zahn D, Kubiak T, Schwarting A, et al. Correlation between cell free DNA levels and medical evaluation of disease progression in systemic lupus erythematosus patients. *Cell Immunol.* 2014;292.
17. Zuo Y, Yalavarthi S, Shi H, Gockman K, Zuo M, Madison JA, et al. Neutrophil extracellular traps in COVID-19. *JCI Insight.* 2020. doi:10.1172/jci.insight.138999.
18. Chen X. Time-series plasma cell-free DNA analysis reveals disease severity of COVID19 patients. 2020.
19. Andargie TE, Tsuji N, Seifuddin F, Jang MK, Yuen PST, Kong H, et al. Cell-free DNA maps COVID-19 tissue injury and risk of death, and can cause tissue injury. *JCI Insight.* 2021. doi:10.1172/jci.insight.147610.
20. Zhang N. Circulating rather than alveolar extracellular DNA levels predict outcomes in influenza: a cohort study. 2020.
21. https://influenza.rki.de/Wochenberichte/2021_2022/2022-10.pdf.
22. <https://influenza.rki.de/wochenberichte.aspx>.
23. Jeong H, Lee J, Kim J, Choen S, Sohn KM, Kim Y-S, et al. Self-Assessment Questionnaire for Efficient and Safe Evaluation of Patients with Mild COVID-19. *Infect Chemother.* 2020;52:212. doi:10.3947/ic.2020.52.2.212.
24. Breitbach S, Sterzing B, Magallanes C, Tug S, Simon P. Direct measurement of cell-free DNA from serially collected capillary plasma during incremental exercise. *J Appl Physiol.* 2014;117.
25. (NICE) LNI for H and CE. COVID-19 rapid guideline: managing the long-term effects of COVID-19. *NICE Guidel.* 2020;188.
26. Singh K, Mittal S, Gollapudi S, Butzmann A, Kumar J, Ohgami RS. A meta-analysis of SARS-CoV-2 patients identifies the combinatorial significance of D-dimer, C-reactive protein, lymphocyte, and neutrophil values as a predictor of disease severity. *Int J Lab Hematol.* 2021;43:324–8. doi:10.1111/ijlh.13354.
27. Suardi LR, Pallotto C, Esperti S, Tazzioli E, Baragli F, Salomoni E, et al. Risk factors for non-invasive/invasive ventilatory support in patients with COVID-19 pneumonia: A retrospective study within a multidisciplinary approach. *Int J Infect Dis.* 2020;100:258–63. doi:10.1016/j.ijid.2020.09.012.

- 1
2
3 28. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk Factors Associated With Acute Respiratory
4 Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China.
5 JAMA Intern Med. 2020;180:934. doi:10.1001/jamainternmed.2020.0994.
6
7
8
9 29. Petrilli CM, Jones SA, Yang J, Rajagopalan H, O'Donnell L, Chernyak Y, et al. Factors associated with
10 hospital admission and critical illness among 5279 people with coronavirus disease 2019 in New York City:
11 prospective cohort study. BMJ. 2020;:m1966. doi:10.1136/bmj.m1966.
12
13
14 30. Shenoy N, Luchtel R, Gulani P. Considerations for target oxygen saturation in COVID-19 patients: are
15 we under-shooting? BMC Med. 2020;18:260. doi:10.1186/s12916-020-01735-2.
16
17
18 31. Strauß R, Ewig S, Richter K, König T, Heller G, Bauer TT. The Prognostic Significance of Respiratory
19 Rate in Patients With Pneumonia. Dtsch Arzteblatt Online. 2014. doi:10.3238/arztebl.2014.0503.
20
21
22 32. Marshall JC, Murthy S, Diaz J, Adhikari NK, Angus DC, Arabi YM, et al. A minimal common outcome
23 measure set for COVID-19 clinical research. Lancet Infect Dis. 2020;20:e192–7. doi:10.1016/S1473-
24 3099(20)30483-7.
25
26
27
28
29
30

31 **Acknowledgements**

32 We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen.
33

34 **Author statement**

35 DD is the principal investigator; she conceived the study, obtained funding, led the proposal and protocol
36 development, and assisted in manuscript preparation and revision. EN contributed to the study concept and
37 design and assisted in manuscript preparation and revision. JidS assisted in manuscript revision. EG
38 contributed to the study concept and design and assisted in manuscript preparation. PS contributed to the
39 acquisition and analysis of qualitative data and the development of the intervention and assisted in manuscript
40 preparation and revision. SB contributed to the study concept and design and drafted the manuscript. All
41 authors read and approved the final manuscript.
42
43
44
45
46
47
48
49
50
51

52 **Funding**

53 This research received no specific grant from any funding agency in the public, commercial or not-for-profit
54 sectors. This study is funded entirely by the University of Essen and the University of Mainz.
55
56
57
58
59

60 **Conflicts of interests**

1
2
3 The authors declare that they have no conflicts of interests.
4

5 **Consent for publication**

6
7
8 Not required.
9

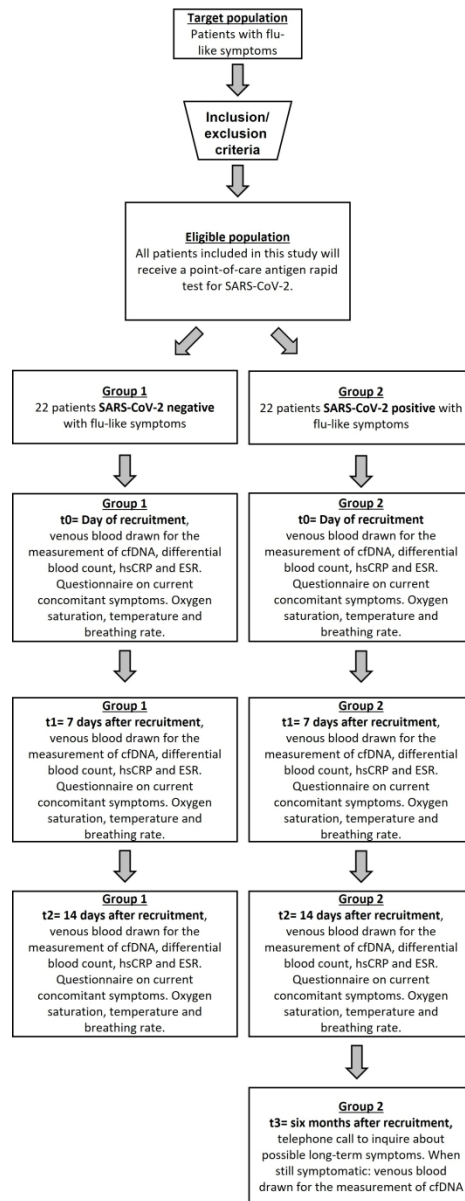
10 11 **Data Statement**

12 The study principal investigator and the co-investigators will have access to the full study data and materials.

13
14
15 The authors will be willing to share the individual-level study data after completion and publication of primary
16
17 and secondary analyses.
18
19

20 21 22 **Figure Legends**

23
24
25 Figure 1: Flowchart of the study design.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



122x316mm (330 x 330 DPI)

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.

1
2 **Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE
3 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
4 <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

For peer review only

6/bmjopen-2021-015864 on 16 June 2022. Downloaded from <http://bmjopen.bmj.com/> on April 17, 2024 by guest. Protected by copyright.