BMJ Open

Translational deep phenotyping of deaths related to the COVID-19 pandemic: protocol for a prospective observational autopsy study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-049083
Article Type:	Protocol
Date Submitted by the Author:	20-Jan-2021
Complete List of Authors:	Henningsen, Mikkel Jon; University of Copenhagen Faculty of Health and Medical Sciences, Forensic Pathology Khatam-Lashgari, Apameh; University of Copenhagen Faculty of Health and Medical Sciences, Forensic Pathology Jacobsen, Christina; University of Copenhagen Faculty of Health and Medical Sciences, Forensic Pathology Olsen, Kristine Boisen; University of Copenhagen Faculty of Health and Medical Sciences Brøchner, Christian Beltoft; Rigshospitalet, Pathology Banner, Jytte; University of Copenhagen Faculty of Health and Medical Sciences
Keywords:	COVID-19, Forensic pathology < PATHOLOGY, Adult pathology < PATHOLOGY, Computed tomography < RADIOLOGY & IMAGING, IMMUNOLOGY, CARDIOLOGY

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Title

Translational deep phenotyping of deaths related to the COVID-19 pandemic: protocol for a prospective observational autopsy study

Authors

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ABSTRACT

Introduction

The coronavirus disease 2019 (COVID-19) pandemic is an international emergency with an extreme socio-economic impact and a high mortality and disease burden. The evolving COVID-19 outbreak is neither fully understood nor fully pictured. Therefore, there is a need to enhance (1) the mapping of pathophysiological pathways by approaching translational deep phenotyping and (2) the envision of transmission, cell adherence, virulence, immune response, organ changes and impact of genetics in order to provide a more comprehensive explanation for public health outcomes and mortality risk associated with this pandemic.

Methods and analysis

An autopsy algorithm has been created to cover all cases undergoing both clinical and forensic autopsy in eastern Denmark as long as the epidemic exists in Denmark. The algorithm describes advanced tissue sampling and a translational analytic follow-up for deep phenotype mapping. The translational approach covers registry data, advanced imaging, gross autopsy findings, microscopic organ changes, post-mortem biochemical investigation, microbiological profiling and immunological status at the time of death, and in future research projects, genetic and epigenetic profiling.

Ethics and dissemination

This study has been approved by the Regional Ethics Committee of the Region of Greater Copenhagen (No: H-20078436) and the Danish Data Protection Agency (No: 2002-54-1080). Next of kin gave informed consent. The study results will be published in peer-reviewed journals.

Trial registration number

This study is purely observational and, as such, does not meet the criteria of the International Committee of

Medical Journal Editors for clinical trials; thus, there is no need for registration in databases such as clinicaltrials.gov. To facilitate cooperation in research and avoid unnecessary duplicate work, we nevertheless wish to publish our protocol.

STRENGHTS AND LIMITATIONS OF THIS STUDY

- A large-scale autopsy study on COVID-19-related deaths.
- Comprehensive biobank and extensive registry data as umbrella for planned and future sub-projects.
- The internal control group partly compensating for the observational design.
- Prospective and systematised data collection.

INTRODUCTION

In late 2019 in Wuhan (Hubei, China), pneumonia cases of unknown aetiology appeared.[1] The cause was later identified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)—a single-stranded, RNA-enveloped, zoonotic *Betacoronavirus*. It is the seventh coronavirus discovered and the third, along with SARS-CoV[2] and Middle Eastern respiratory syndrome (MERS-CoV),[3] that causes severe symptoms in human beings. The other coronaviruses—CoV-OC43, CoV-HKu1, CoV-229E and CoV-NL63—merely result in the common cold.[4] Coronaviruses mutate frequently, and many mutations of SARS-CoV-2 have already been registered.[5, 6] Some mutations are more aggressive than others, which suggests that ongoing phylogenetic mapping of new strains is important.[7] A regularly updated map is available at https://nextstrain.org/ncov.

Current knowledge of the pathophysiology of SARS-CoV-2 is largely based on previous experience with the SARS-CoV[8] and MERS-CoV epidemics.[9] Autopsy studies on SARS-CoV-2-related deaths are emerging, but except a few,[10-12] most are case series (e.g. [13, 14]).

SARS-CoV-2 enters through body orifices and binds via its spike protein to angiotensin-converting enzyme 2 (ACE2) receptors located on different cell types, including type II alveolar cells, respiratory epithelial cells,[4, 6-8] myocardial cells,[15] cells in the oesophagus and ileum, and cells in the kidneys and bladder, which may explain the extra-pulmonic symptoms observed in patients suffering from COVID-19.[16] In those with chronic heart conditions, ACE2 expression might be increased,[17] as well as after myocardial injury.[18] Conversely, Oudit et al. demonstrated in a murine model that SARS-CoV infection led to a decrease in myocardial ACE2 mRNA expression, which was possibly cytokine-mediated.[15]

SARS-CoV-2 is capable of human-to-human transmission with a reproductive rate (R_0) of 2.2.[19] The infection may present either symptomatically or asymptomatically, with early indications that a large portion—including children[20]—presents asymptomatically and serves as a carrier facilitating asymptomatic transmission[21] and hence posing a substantial hindrance to curbing the pandemic. In those symptomatic, the infection may be mild, moderate or severe.[22] Data suggest that patients hospitalised with COVID-19 are middle-aged or older and predominantly male.[23, 24] Common symptoms include fever, dry cough, myalgia, fatigue, dyspnoea, weight loss[1] and loss of smell, which is commonly seen in viral infections,[25] although perhaps through different mechanisms. The patient group with the most severe symptoms is characterised by age older than 60 years[26] and at least two to three comorbidities[1]—the most common being cardiovascular diseases (CVD), including hypertension,[27] diabetes mellitus and respiratory disease.[1, 24, 28] The comorbidities might pose a risk for patients with severe COVID-19 compared to non-severe.[24, 28]

Major complications in patients with the most severe course of disease include sepsis, acute respiratory distress syndrome (ARDS), acute cardiac and kidney injury, arrhythmia and shock, as well as secondary infection and ultimately death.[1, 24, 29] The occurrence of ARDS is well known from the SARS and MERS epidemics.[30] The severity of disease pathophysiology is not fully understood, but in all coronavirus-related

diseases with severe ARDS, a modification of the immunological reaction to the virus infection in severe cases is common.[14, 30] An increase in pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and IL-12; antiviral T helper 1 (Th1) cytokine interferon (IFN)-γ[30]; and Th17[31]—as well as a decrease in anti-inflammatory IL-10—has been noted.[30, 31] Also, higher concentrations of cytokines have been registered in ARDS caused by SARS-CoV-2,[32] and it is believed that a cytokine storm is associated with disease severity.[33] Autopsy studies are suggested to characterise and elucidate the role of Th1 and Th2 responses in the pathogenesis of ARDS in COVID-19.[32] On the basis of Cameron et al.'s studies during the SARS epidemic, it has been indicated that a malfunction exists in the switch from innate to adaptive immunity in patients suffering from severe SARS.[33, 34]

The importance of CVD as a risk factor for increased morbidity and mortality during COVID-19 is becoming progressively apparent.[17, 27, 35] Two pathways for myocardial injury have been suggested: (1) cytokine storm[17] (or secondary haemophagocytic lymphohistiocytosis) and (2) viral myocarditis (or stress cardiomyopathy). The pathogenesis might involve ACE2 receptors in myocardial cells in combination with respiratory dysfunction and hypoxaemia caused by ARDS.[17] Also, an unbalanced response of Th1 and Th2 cells might play a role.[30] During the Toronto SARS outbreak, the SARS-CoV viral RNA was detected in 35% of the autopsied hearts, and in these patients, the infection was associated with a more aggressive course of illness.[15] Myocardial injury was evident from the rising levels of cardiac troponin I in those who could not survive.[29]

Clinically, medical imaging—specifically chest computed tomography (CT)—has gained importance as a diagnostic tool, as changes on chest CT are characteristic.[36] Typical lesions associated with viral pneumonia are ground-glass opacities, mixed ground-glass opacities and consolidation, vascular enlargement in the lesions, and traction bronchiectasis. The lesions are typically located peripherally.[1, 36] Also, in cases in which reverse transcription polymerase chain reaction (RT-PCR) testing was initially false negative, chest CT has served as a diagnostic tool.[37]

Internationally, SARS-Cov-2 is classified into Hazard Group 3 and in Denmark into Hazard Group 2.[38] In this respect, the handling of those who die of a possible SARS-CoV-2 infection with or without COVID-19 is possible following the standard procedures at Danish autopsy facilities. As other countries have been hesitant to perform autopsies because of concerns regarding transmission to healthcare workers, the present study is even more necessary.[10, 12] Autopsy studies provide insights into the consequences of SARS-CoV-2 infection and the course of disease in COVID-19, which are essential for correct classification of deaths, as demonstrated both in COVID-19-suspected deaths and in general.[12, 39] Autopsy studies enable better treatment of patients (e.g. adjusting anticoagulative therapy for COVID-19[12]) and help understand the pathogenesis of novel diseases.[40] Most of these studies on SARS-CoV-2-related deaths are either small,[41-43] subject to bias and restrictive in their investigations[11] or lack biochemical, virological, bacteriological and genetic analysis; nor do they store tissue[12] for further studies. Therefore, this study provides a much-needed comprehensive umbrella for deep and diverse investigations.

Rationale for study

Autopsy studies enable extensive and unique examination and tissue sampling, which is not possible in living patients. To our knowledge, no large-scale autopsy studies were initiated during the SARS-CoV or MERS-CoV epidemics, and few in the current SARS-CoV-2 pandemic have been conducted.[12, 43] Further, differences in sampling and reporting of autopsy case series[13, 14] make their meta-analysis difficult. Historically, autopsy studies have provided novel insights. This study, therefore, fills a substantial knowledge gap.

Aims and objective

It is hypothesised that an optimised molecular autopsy of those who die of COVID-19 may map the importance of somatic disease and organ changes, the cause of death and underlying comorbidities, the effect of pharmaceuticals on disease severity, the impact of immunological mechanisms, and virological and bacteriological status. Future sub-projects would investigate genetic and epigenetic profiles. The objective is to identify high-risk profiles for SARS-CoV-2-related deaths and prevent these in the future.

Research question

This observational study collects tissue and data for a biobank aimed at answering the following research questions: (1) Which somatic diseases are present in SARS-CoV-2-related deaths? (2) Which organ changes occur? (3) What is the cause of death? (4) What are the underlying comorbidities? (5) What pharmaceuticals are present in COVID-19-related deaths, and how does their presence correspond to organ changes? 6) What is the impact of immunological mechanisms? (7) What are the virological, bacteriological and immune status of the deceased in relation to COVID-19? (8) Who is at risk of death from COVID-19?

Time frame

Inclusion began 1 May 2020. As of December 2020, 40 cases had been included. The inclusion is going to continue as long as the epidemic exists in Denmark, and an increase in cases is expected in early 2021. Tentative analysis will be initiated upon reaching 50 included cases, which is expected in early 2021. Fifty cases are significantly more compared to most autopsy studies, yet significantly fewer than the 80 by Edler et al.[12] Later studies may focus on long-term effects in COVID-19 survivors who die because of other reasons. Deaths within 1 week of vaccination will be autopsied similarly to those described in this protocol, and tissue will also be stored in a biobank.

METHODS AND ANALYSIS

Study setting

This is a prospective, autopsy-based, observational cohort study based at the Department of Forensic Medicine, University of Copenhagen, Denmark. The study is being conducted in collaboration with the Department of Pathology at Rigshospitalet, Copenhagen.

Cases may be included from other forensic institutes and pathology departments across Denmark. They are referred either among deceased hospitalised patients or at the medico-legal inquest. Included cases where no SARS-CoV-2 is demonstrated on virological examination by Statens Serum Institut (SSI) will serve as internal controls. Figure 1 illustrates the inclusion flow chart.

Additional data

Additional registry data will be acquired from national health registries, including—but not limited to—prescribed pharmaceuticals and earlier diagnoses.

Imaging and autopsy

Post-mortem CT (PMCT) is performed in accordance with the Danish Accreditation and Metrology Fund (DANAK) and ISO/IEC17020:2012 standards on all cases handled at forensic departments in Denmark. The Department of Forensic Medicine, University of Copenhagen, may also perform post-mortem magnetic resonance imaging of the brain and heart in select cases. Standard autopsy is performed in line with the ISO 17020 standard, with an additional COVID19-specific algorithm specifying extensive tissue sampling. Each centre handles tissue samples for microscopy according to local standards and arrangements, and samples for virological and bacteriological examination are stored locally until analysis.

Documentation

Standardised and pro-necessaire photo-documentation and standardised written report forms are used for both gross and radiological findings.

Sampling

The specific algorithm dictates tissue sampling, location, sidedness and so on. Fluids collected for chemical analysis are collected in tubes both with (urine and intra-vitreous liquid) and without (peripheral blood, urine, intra-vitreous liquid, gall, stomach contents and heart blood) additives, and solids for chemical analysis include the muscle, liver, brain and hair. For virological examination, fluids (spinal fluid, pleural fluid, urine, heart blood, or serum of heart blood), tissues (brain, tonsil, right upper and left lower lobes of the lungs, heart muscle, small intestine, bladder wall, or kidneys) and throat swabs are stored in tubes without additives. Additional swabs from the throat, middle ear and sinuses are stored in tubes with phosphate-buffered saline. Samples for bacteriological analysis of liquids (heart blood), tissues (brain, right upper and left lower lobes of the lungs, heart muscle, heart valves, small intestine and kidneys) and throat swabs are kept in tubes without additives. Samples are kept at −80°C until PCR analysis. Tissue for histopathological analysis is fixed in 4% formalin and then embedded in paraffin. Additional tissue is stored at −80°C. Samples include the spinal cord, olfactory bulb, orbital frontal cortex, insula, thalamus, pons, cerebellum, medial occipital cortex, medulla oblongata, hippocampus, olfactory epithelium, peroneal nerve, tibialis anterior muscle, conjunctivae, tonsil, soft palate, uvula, sublingual gland, tongue (anteriorly and posteriorly), centrally and peripherally from the upper and lower lobes of both lungs, heart (anteriorly, laterally and posteriorly from the right and left ventricle walls), septum and papillary muscle, aortic arch, thoracic aorta, abdominal aorta, oesophagus, stomach, duodenum, jejunum, ascending colon, descending colon, liver, spleen, pancreas, adrenal gland, kidneys, lymph node, bone marrow, thyroid gland, pituitary gland, testis/ovary and prostate/uterus. (See table 1 in the Appendix.) Additional tissue may be sampled on a case-to-case basis (e.g. upon discovery of a tumour).

Analysis

Gross autopsy findings are identified by either board-certified surgical pathologists or board-certified forensic pathologists in collaboration with junior doctors. In case of doubt, a second board-certified forensic pathologist assists, totalling the presence of three doctors. Autopsy practice follows guidelines as set forth by Recommendation no. R 3 of the Committee of Ministers to Member States on the harmonisation of medico-legal autopsy rules (99) and authoritative textbooks such as *The Coroner's Autopsy* by B. Knight and *Autopsy Diagnosis and Technique* by O. Saphir.[44-47] Board-certified surgical pathologists perform histopathological analysis.

All the forensic chemistry departments at the three forensic institutes in Denmark perform chemical analysis in accordance with DANAK and ISO 17020 standards. Future genetic analysis is performed in the Forensic Genetics section at the Department of Forensic Medicine, University of Copenhagen, in accordance with DANAK and ISO 17025 standards.

A junior and a senior forensic pathologist interpret post-mortem images for non-research purposes. For later research purposes, images are interpreted de novo by researchers blinded to autopsy findings.

Data storage

Text and image data are stored locally on closed servers in various secure electronic systems, which are also used in routine work. Data will be extracted into a database stored on secure, closed servers, where all cases are assigned a unique project identification number to ensure pseudo-anonymisation and blinding.

Statistical analysis

This is an umbrella study for several current sub-projects and would enable future projects to utilise the data sampled. The statistical programmes, methods and sample sizes will vary between projects and will be described in publications to come.

Cohort description

In this study, SARS-CoV-2-related deceased are included in two ways. In Denmark, the police decide whether a medico-legal inquest is necessary. At the medico-legal inquest, the police are advised by either a medical health officer or a forensic pathologist, and then they decide whether to perform a forensic autopsy or not in accordance with the Danish Health Act. The Department of Forensic Pathology, University of Copenhagen, and the Department of Pathology, Rigshospitalet, Copenhagen, include all cases that meet the stated criteria. Additional cases may be referred from other departments of forensic pathology or pathology, and thus, this study potentially covers all of Denmark.

All cases with a history of current or prior COVID-19, PMCT indicative of COVID-19 (e.g. ground-glass opacities, crazy paving, consolidation, arcade sign and traction bronchiectasis), or ante-mortem positive COVID-19 test are going to be included. A negative test does not result in exclusion because of the unknown false-negative rate of the tests currently employed.

All relevant hospital departments have received written information about this study and are encouraged to request autopsy in those dying from COVID-19. Clinical departments (e.g. lung medicine, infectious diseases and intensive care units [ICUs]) may request an autopsy from the departments of pathology that apply the same inclusion criteria as that for police-requested cases, except no inclusion based on PMCT findings. PMCT is still performed, if included.

Cases with extensive putrefaction, those too overweight for PMCT, where consent cannot be obtained, and where the COVID-19-specific autopsy algorithm is incompatible with the purpose of the autopsy (e.g. homicide investigation) are excluded.

Patient and public involvement

Neither patients nor next of kin were involved in the design of this study, though they may be in the specific subprojects.

ETHICS AND DISSEMINATION

In Denmark, research on the deceased requires informed consent from next of kin. For autopsies requested by clinical departments, consent to research and autopsy is given simultaneously. For police-requested forensic autopsies, next of kin are contacted through letter no sooner than 1 month after autopsy, and if there is no response, then by telephone. The same approach has been successfully used in a previous national study, the SURVIVE study,[48] and it is believed to be the least emotionally disturbing to next of kin. The obtained consent rate in the SURVIVE study was more than 90.[48] The current study has been approved by the Regional Ethics Committee of the Region of Greater Copenhagen (No: H-20078436) and the Danish Data Protection Agency (No: 2002-54-1080).

All data are available upon request to the steering group on the condition that the intended research is sound and that legal matters, such as General Data Protection Regulation (GDPR), are addressed properly. Results will be published in peer-reviewed journals.

DISCUSSION

Patients recruited from hospitals will most likely differ from those not hospitalised. Likewise, those recruited specifically from ICUs will most likely differ from other patients. Cases recruited from hospitals will likely have died from COVID-19. On the contrary, police-requested autopsies will provide examples of COVID-19-related deaths or pathological changes in people who did not manage to seek help, had a quicker course of disease, or died from other causes, such as accidents.

This is the first large autopsy study with a planned, systematic and focused registration and sampling for pathological changes and virological, bacteriological, chemical and genetic analysis of COVID-19-related deaths. Autopsy investigations carry inherent advantages and disadvantages to the examination of the living.

Like all observational studies, this study is subject to selection bias, confounding and measurement errors. Cases suspected to be infected with SARS-CoV-2 may prove not to be. This makes the actual sample in this study smaller, but its strength is that these cases may serve as internal controls. Measurement error and misclassification are always a possibility, as is confounding. Information from police and hospital records may contain information vulnerable to recall bias, interviewer bias and confirmation bias.

In both forensic pathology and surgical pathology in Denmark, two pathologists always inspect the organs and co-sign the work in accordance with the standard procedures. It is expected that this standard practice would lessen the amount of measurement error and misclassification in the current study. Further, the timing of investigation and sampling should have little effect, as all deceased are kept in a morgue from death to autopsy.

Confounding will be handled through statistical analysis and knowledge that causation cannot be incurred from the findings of this study.

Multi-centre studies carry the benefits of larger and faster recruiting, results that are more generalisable, and smaller bias in sampling, but they require strict adhesion to protocol to avoid unsought heterogeneity. The current study benefits from interdisciplinary cooperation in that forensic pathologists, surgical pathologists, neuropathologists, epidemiologists, virologists, otolaryngologists and chemists all contribute within their field of expertise.

A major issue in the SARS-CoV-2 pandemic is to determine the cause of death from current SARS-CoV-2 infection against death from the consequences of SARS-CoV-2 infection against death from other causes while infected with SARS-CoV-2. A recent study by Edler et al. proposed categorising deaths as (1) definite COVID-19-related deaths, (2) probable COVID-19-related deaths, (3) possible COVID-19-related deaths, or (4) SARS-CoV-2 detected but unrelated to the cause of death.[12] This categorisation may prove relevant to investigate preventive measures, abandon unnecessary measures, steer pharmaceutical treatment, and guide society in future SARS-CoV-2 waves and future pandemics.

Contributor statement

JB and CJ conceived the study and secured external funding through grants. JB is the chief investigator and guarantor. JB and KBO coordinated with the involved parties. JB, MJH and AK-L secured approval from the Ethics Committee for the research projects and the Data Protection Agency for the bio bank. JB, CJ, KBO, MJH, AK-L and CBB contributed to the formation of the protocol. MJH and AK-L wrote the first draft, all contributed to the writing of the manuscript, and all approved the final version.

Competing interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: all authors had financial support from Lundbeck Foundation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Acknowledgements
Professor Niels Lynnerup should be acknowledged for his role in conceiving the study and securing external funding.

Funding
This study is supported by the Lundbeck Foundation with 2.5 million DKK (Ca. 300,000 GBP) (grant: R319-2020-896). Funds are managed by the University of Copenhagen.

Legend for figures
Figure 1. Inclusion and consent flow-chart

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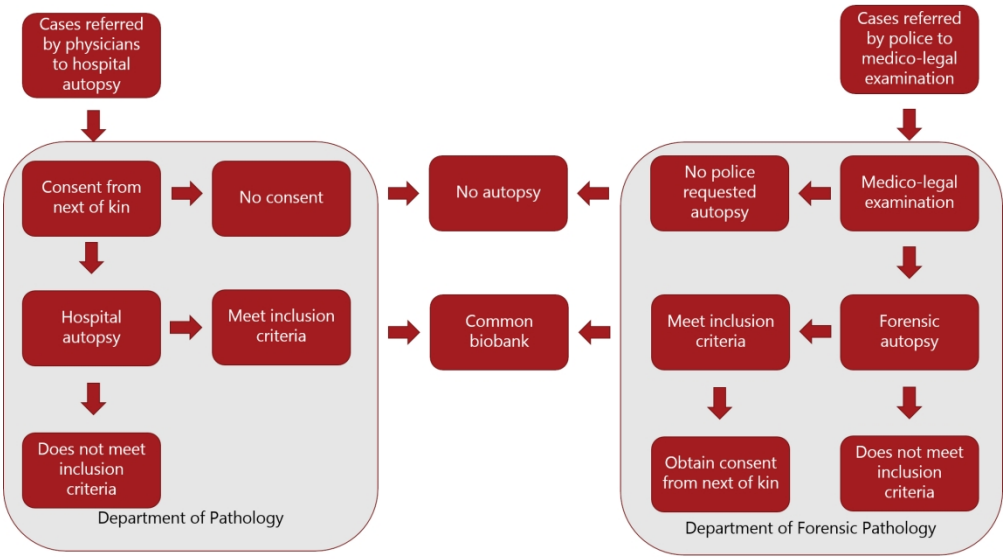

Figure 1: Flowchart demonstrating inclusion

190x106mm (300 x 300 DPI)

Appendix

Table 1 Samples for biobank

Sample	Histopathology	−80°C storage	Chemical analysis	Bacteriological analysis	Virological analysis
Spinal cord	+				
Olfactory bulb	+	+			
Orbital frontal cortex	+	+	+	+	+
Insula	+	+			
Thalamus	+	+			
Pons	+	+			
Cerebellum	+	+			
Medial occipital cortex	+	+			
Medulla oblongata	+				
Hippocampus	+				
Olfactory epithelium	+				
Peroneal nerve	+	+			
Tibialis anterior muscle	+	+			
Conjunctivae	+				
Tonsil	+	+			+
Soft palate	+	+			
Uvula	+	+			
Sublingual gland	+	+			
Tongue anteriorly	+	+			
Tongue posteriorly	+	+			
Lung, upper lobe pleural	+	+		+	+
Lung, upper lobe central	+	+			
Lung, lower lobe pleural	+	+		+	+
Lung, lower lobe central	+	+			
Heart, LV anteriorly	+	+			
Heart, LV laterally	+				
Heart, LV posteriorly	+	+			
Heart, RV anteriorly	+	+			
Heart, RV laterally	+				
Heart, RV posteriorly	+	+			
Heart septum	+	+		+	+
Heart papillary muscle	+				
Heart valves				+	
Aorta arch	+	+			
Aorta thoracic	+	+			
Aorta abdominal	+	+			
Oesophagus	+	+			
Stomach	+	+			
Duodenum	+	+			
Jejunum	+	+		+	+

Ascending colon	+	+			
Descending colon	+	+			
Liver	+	+	+		
Spleen	+	+			
Pancreas	+				
Adrenal gland*	+				
Kidneys	+	+		+	+
Lymph node	+	+			
Bone marrow	+				
Thyroid gland*	+				
Pituitary gland	+				
Testis/ovary*	+				
Prostate/uterus*	+				
Spinal fluid					+
Vitreous liquid			+		
Pharyngeal swab				+	+
Stomach contents			+		
Urine/bladder wall			+		+
Pleural liquid		+			
Pericardial liquid		+			
Heart blood		+			
Heart blood serum					
Peripheral blood			+	+	+

* Only police-requested autopsies. LV, left ventricular; RV, right ventricular.
'+' means sample taken. 'Blank' means no sample taken. Chemical and virological analyses are performed on all cases. Histopathology and bacteriological samples are stored for later analysis. –80°C samples are stored for later analysis.

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract Observational, in title and abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found Summary of background, design and approvals. Protocol means no data yet.
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported Done
Objectives	3	State specific objectives, including any prespecified hypotheses Done
Methods		
Study design	4	Present key elements of study design early in the paper Done
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection Settings, locations,dates, recruitment and data collection described. No follow up as it is an autopsy study.
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up Done. No follow up, as included persons are deceased. *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls Persons included who test negative for COVID-19 serve as internal controls, described in article. *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable Umbrella for several sub projects. These will each describe in more detail.
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 Done.
Bias	9	Describe any efforts to address potential sources of bias Done
Study size	10	Explain how the study size was arrived at Done
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable,

		describe which groupings were chosen and why Not relevant
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed Internal controls. Unknown whether case or control at inclusion. No loss to follow up. *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses None performed.

Continued on next page

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 40 included at time of submission
		(b) Give reasons for non-participation at each stage Done – i.e. no consent.
		(c) Consider use of a flow diagram Included
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders Not relevant for this protocol
		(b) Indicate number of participants with missing data for each variable of interest Not known at protocol stage
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time Unknown at this point as several planned and future sub projects will utilize data gathered.
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
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 Not known at protocol stage
		(*b*) Report category boundaries when continuous variables were categorized Not known at protocol stage
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period Not known at protocol stage
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses Not known at protocol stage
Discussion		
Key results	18	Summarise key results with reference to study objectives Not known at protocol stage
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias Done
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence Not known at protocol stage
Generalisability	21	Discuss the generalisability (external validity) of the study results Done
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

Done

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

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

BMJ Open

Translational deep phenotyping of deaths related to the COVID-19 pandemic: protocol for a prospective observational autopsy study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-049083.R1
Article Type:	Protocol
Date Submitted by the Author:	15-Jun-2021
Complete List of Authors:	Henningsen, Mikkel Jon; University of Copenhagen Faculty of Health and Medical Sciences, Department of Forensic Pathology, Section of Forensic Pathology Khatam-Lashgari, Apameh; University of Copenhagen Faculty of Health and Medical Sciences, Department of Forensic Medicine, Section of Forensic Pathology Olsen, Kristine Boisen; University of Copenhagen Faculty of Health and Medical Sciences, Department of Forensic Medicine, Section of Forensic Pathology Jacobsen, Christina; University of Copenhagen Faculty of Health and Medical Sciences, Department of Forensic Pathology, Section of Forensic Pathology Brøchner, Christian Beltoft; Rigshospitalet, Department of Pathology Banner, Jytte; University of Copenhagen Faculty of Health and Medical Sciences, Department of Forensic Medicine, Section of Forensic Pathology
Primary Subject Heading:	Infectious diseases
Secondary Subject Heading:	Legal and forensic medicine, Pathology, Radiology and imaging
Keywords:	COVID-19, Forensic pathology < PATHOLOGY, Adult pathology < PATHOLOGY, Computed tomography < RADIOLOGY & IMAGING, IMMUNOLOGY, CARDIOLOGY

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Title

Translational deep phenotyping of deaths related to the COVID-19 pandemic: protocol for a prospective observational autopsy study

Authors

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ABSTRACT

Introduction

The coronavirus disease 2019 (COVID-19) pandemic is an international emergency with an extreme socio-economic impact and a high mortality and disease burden. The COVID-19 outbreak is neither fully understood nor fully pictured. Autopsy studies can help understand the pathogenesis of COVID-19 and has already resulted in better treatment of patients. Structured and systematic autopsy of COVID-19 related deaths will enhance the mapping of pathophysiological pathways, not possible in the living. Furthermore, it provides an opportunity to envision factors translationally for the purpose of disease prevention in this and future pandemics. This is the protocol for an autopsy study that offers an umbrella for deep and diverse investigations of COVID-19 related deaths, including a systematic investigation of "long" COVID-19 by means of extensive and systematic tissue sampling.

Methods and analysis

A COVID-19 specific autopsy algorithm has been created to cover all cases undergoing clinical or forensic autopsy in Denmark. The algorithm describes advanced tissue sampling and a translational analytic follow-up for deep phenotyping. The translational approach covers registry data, post-mortem imaging, gross autopsy findings, microscopic organ changes, post-mortem toxicology, post-mortem biochemical investigation, microbiological profiling and immunological status at the time of death, and future research projects covering genetics and epigenetics on an organ level.

Ethics and dissemination

This study has been approved by the Regional Ethics Committee of the Region of Greater Copenhagen (No: H-20078436) and the Danish Data Protection Agency (No: 2002-54-1080). Next of kin gave informed consent to research. The study results will be published in peer-reviewed journals.

Trial registration number

This study is purely observational and, as such, does not meet the criteria of the International Committee of Medical Journal Editors for clinical trials; thus, there is no need for registration in a database of research trials, such as clinicaltrials. To facilitate cooperation in research, provide transparency on case recruitment for publications to come and to avoid unnecessary duplicate work, we nevertheless wish to publish our protocol.

STRENGHTS AND LIMITATIONS OF THIS STUDY

- A standardised, prospective autopsy study on COVID-19-related deaths with systematised data collection.
- A multi-disciplinary, translational approach elucidating changes from protein level to whole human.
- A comprehensive bio bank and extensive registry data as an umbrella for planned and future research.
- The internal control group partly compensates for the observational design.
- Limited by a selected sample of COVID-19 diseased only including the ones undergoing autopsy.

INTRODUCTION

In late 2019 in Wuhan, China an outbreak of pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged,[1] and later evolved into the global coronavirus disease 2019 (COVID-19) pandemic that has claimed millions of lives. Along with SARS-CoV[2] and MERS-CoV,[3] it is the third coronavirus that causes severe symptoms in humans. Coronaviruses mutate frequently, and many variants of SARS-CoV-2, each with its own characteristics, have already been registered.[4 5]

Historically, autopsy studies have provided novel insights on epidemic disease.[6] Important knowledge of the pathophysiology of coronavirus was gained both from autopsy studies during the SARS[7] and MERS epidemics[8] and currently from autopsy studies on COVID-19 related deaths.[9-12] The importance of autopsy studies in the current pandemic is already reflected in better treatment of patients, e.g. adjusting anti-coagulative therapy for COVID-19.[10] Beside a few cases in some autopsy studies on COVID-19,[13-16] only two case-series[17 18] and a study by Romanova et al.[19] include out-of-hospital deaths. Autopsies of hospital deaths demonstrate hospitalized fatal cases and late disease stages influenced by treatment and forensic autopsies provide an overview of different stages of disease

Much is known about the pathophysiology of SARS-CoV-2 but unanswered questions still remain. SARS-CoV-2 binds via its spike protein to angiotensin-converting enzyme 2 (ACE2) receptors located on different cell types, including type II alveolar cells, respiratory epithelial cells,[5 7 20 21] myocardial cells,[22] cells in the oesophagus and ileum, cells in the kidneys and bladder, and endothelial cells,[23] which may explain the extra-pulmonic symptoms observed in patients suffering from COVID-19.[24] The transmembrane protease serine 2 (TMPRSS2) is also suspected to play a vital role.

It is well known that old age, co-morbidities such as cardio-vascular disease (CVD), diabetes, chronic lung disease, chronic kidney disease, cancer, and obesity increase the disease burden,[1 25-33] and severe COVID-19 disease include sepsis, acute respiratory distress syndrome (ARDS), acute cardiac and kidney injury, arrhythmia, as well as secondary infection and ultimately death.[1 27 34] The importance of CVD as a risk factor for increased morbidity and mortality during COVID-19 has become progressively apparent,[29 31 32] but the exact pathogenesis is not fully understood. In the 2003 Toronto SARS outbreak, SARS-CoV viral RNA was detected in 35% of the autopsied hearts, and in these patients, the infection was associated with a more aggressive course of illness.[22] Myocardial injury was evident from rising levels of cardiac troponin I in those

who did not survive,[34] and in the current pandemic, inflammation and micro-thrombi has been detected in the coronary arteries.[35] ARDS is well known from the SARS and MERS epidemics,[36 37] and the ongoing COVID-19 pandemic. The involvement of other organs and the severity of COVID-19 are still not fully understood and secondary effects mediated by a modification of the immunological reaction are assumed to contribute to the severity of COVID-19.[1 36 38-40]

An emerging problem is so called "long" COVID-19, or post-acute COVID-19, which is defined as persisting symptoms and/or long-term complications, including neurologic symptoms with disturbed senses, more than four weeks after symptom debut.[41] Retrospective studies on initial COVID-19 survivors have demonstrated excess death and disease burden up to several months after hospital discharge.[42 43] In England, Ayoubkhani et al. found that in the months after discharge a great number of patients (47,780 people) who had been hospitalized with COVID-19 died at an eight times greater rate than matched controls. In an American setting, Al-Aly et al. found that patients who had suffered from COVID-19 experienced increased burden of respiratory-, neurologic-, mental-, metabolic-, cardiovascular-, musculo-skeletal-, and gastro-intestinal disease, an increased use of non-opioid analgesics, opioids, anti-depressants, anxiolytics and benzodiazepines and had increased risk of death.

Examination of the brain has been avoided for safety reasons in many autopsy studies on COVID-19,[44-51] which leaves a knowledge gap regarding central nervous system involvement. Though a large number of autopsy studies have been published on COVID-19, a large proportion is on less than three deceased[17 44 45 52-62] and some are restricted to leaving organs "in-situ" for gross examination and tissue sampling,[44 53] or rely on biopsies only.[38 63-77] Studies with complete autopsies of three to 100 deceased have been performed,[9-12 47-50 78-90] but also some of these were restricted to "in situ" organ examination.[9 50]

Internationally, SARS-Cov-2 is classified into Hazard Group 3 and due to fear of transmission from deceased to autopsy personnel, several guidelines on COVID-19 autopsies were published in the early stages of the pandemic by e.g. Hanley et al. and Santurro et al.[91 92] In Denmark SARS-Cov-2 is classified into Hazard Group 2.[91] In this respect, the handling of those who die of a possible SARS-CoV-2 infection with or without COVID-19 disease is possible following the standard procedures at Danish autopsy facilities, including post-mortem imaging, and furthermore allows for a comprehensive autopsy study.[10 93]

Post-mortem imaging has emerged as a promising tool for performing non-invasive "autopsy",[94] and may be of value in bio-hazardous cases. Only a few autopsy studies have included post-mortem imaging.[95]

Rationale for study

Autopsy studies enable extensive and unique examination and tissue sampling, which is not possible in living patients. No autopsy studies with extensive tissue sampling were initiated during neither the SARS-CoV nor MERS-CoV epidemics,[6] and only a few studies in the current SARS-CoV-2 pandemic include more than 25 deceased.[10-12 48 51 77 81] To our knowledge, no autopsy study on "long" COVID-19 has been performed.

This paper describes the protocol for an ongoing multi-centre autopsy study of COVID-19 related deaths, and presents some of the planned and future research projects. The COVID-19 autopsies constitute a framework of complete, systematic autopsy, with multi-modal post-mortem imaging, extensive pre-defined tissue sampling, including bio banking at -80°C, toxicological-, microbiological- and molecular analyses combined with genetic examination. Furthermore, the extensive Danish registries of disease, medication, and socio-economic status allow for consideration of factors beyond those present at autopsy, which provides the opportunity for deep and diverse investigations of COVID-19 related deaths and a systematic investigation of "long" COVID-19 by means of autopsy.

We hypothesise that combining registry data with immediate autopsy findings, advanced molecular, pharmaceutical, immunological, microbiological, genetic and epigenetic investigations in those who die of

COVID-19 or suffer from "long" COVID-19 will help understand the pathogenesis of COVID-19, which is the rationale for conducting the here described study.

Aims and objective

The overall aim is to reduce death and disease burden in COVID-19 and future pandemics. The overall objectives to reach this aim is to identify high-risk profiles for COVID-19 related deaths. Several research projects are already defined and have the following aims:

Project 1: Post-mortem imaging, aims to register imaging findings in COVID-19 related deaths and correlate them to autopsy findings and ante-mortem imaging.

Project 2: Macroscopic and microscopic changes, aims to contribute to basic understanding of pathological and histopathological pre-existing and COVID-19 related changes in all organs, and will constitute a basis for comparison to imaging findings and furthermore relate to advanced molecular investigations

Project 3: Chemical and toxicological analysis, aims to both identify potential harmful or beneficial drugs and medications present in COVID-19 related deaths, and to determine to what extent medication is distributed inreach the lung tissue. Furthermore, it aims to phenotype risk profiles.

Project 4: Loss of smell, aims to identify how the smell epithelium, the olfactory nerve, the olfactory nerve bulb, and the taste buds are affected by the invasion of the virus, and to determine whether ACE2 receptors and the transmembrane protease serine 2 (TMPRSS2) are present in the olfactory pathway or in supporting cells.

Project 5: Advanced microbiological examination, aims to improve the understanding of infection, detect mutation rate of SARS-CoV-2, validate the immunochemical assays for detection of SARS-CoV-2 antibodies for use on autopsy samples, and examine ACE2 receptor expression, the significance of the morphology of the paranasal sinuses and whether this correlates to the microbiome.

Project 6: Aberrant signalling, aims to establish the effects of SARS-CoV-2 on specific organs, e.g. the liver, and to elucidate the role of aberrant ACE2/Angiotensin II signalling.

Research question

This observational study collects tissue and data for a bio bank aiming at answering what happens at gene, protein, cell, tissue and organ level in the individual human infected with SARS-CoV-2. More specifically, immediate autopsy findings combined with deep and advanced molecular investigation will aim at answering the following research questions: 1) Which co-morbidities, autopsy findings and ante-mortem diagnosis' are present in COVID-19 related deaths? 2) What is the cause of death? 3) How does post-mortem imaging correspond to autopsy findings? 4) What pharmaceuticals are present in COVID-19-related deaths, and how does their presence correspond to organ changes? 5) What is the impact of immunological mechanisms? 6) What are the virological, bacteriological and immune status of the deceased in relation to COVID-19? 7) Who is at risk of death from COVID-19? 8) What is the underlying role of genetics and epigenetics? 9) When is death caused by COVID-19, accelerated by COVID-19 or un-related to SARS-CoV-2 infection?

The first two research questions, covered by sub-project 2 and 4, have already been investigated by others. The repetition in this study serves to correlate the findings using the herein proposed advanced methods in each deceased individual.

Time frame

Inclusion began May 1st, 2020. As of December 2020, 40 cases had been included, and 80 by May 2021. The inclusion will continue as long as the epidemic exists in Denmark, and an additional six months to capture

"long" COVID-19 cases. A decrease in cases is expected from mid-2021. Tentative analysis will be initiated in mid-2021. The first 40 cases were all included on the basis of confirmed or suspected acute COVID-19.

METHODS AND ANALYSIS

Study setting

This is a prospective, autopsy-based, observational cohort study based at the Section of Forensic Pathology, Department of Forensic Medicine, University of Copenhagen, Denmark. The study is being conducted in collaboration with the Department of Pathology at Rigshospitalet, Copenhagen.

Cases may be included from other the two forensic institutes in Denmark and from pathology departments in eastern Denmark. All forensic institutes and the department of pathology at Rigshospitalet employ the same COVID-19 specific autopsy algorithm. Cases are recruited either from deceased hospitalised patients or at the medico-legal inquest.

Additional data

Additional registry data will be acquired from national health registries, including, but not limited to, The Register of Medicinal Product Statistics (LSR – Lægemiddelstatistikregisteret), which contains data on all sales of prescription medicine and over-the-counter medicine reported from Danish pharmacies, [96] The National Patient Register (LPR – Landspatientregisteret), which contains data on utilization of secondary health care and includes admissions, ambulatory visits and visits to emergency rooms),[97] The National Health Insurance Service Register (SSR – Sygesikringsregisteret), which concerns utilization of primary health care including dentists, chiropractors, physiotherapists, psychologists and podiatrists, The Attainment Register,[98] which gives insight into the highest education attained for each person and is updated once a year, and the Danish registers on personal labour market affiliation from Statistics Denmark, which contains employment status on each Danish citizen, which is also updated every year.

Imaging and autopsy

Post-mortem computed tomography (PMCT) is performed in accordance with internal procedures on all cases handled at the forensic departments in Denmark. The institutes are accredited by the Danish Accreditation and Metrology Fund (DANAK) and ISO/IEC17020:2012. The Department of Forensic Medicine, University of Copenhagen, may also perform post-mortem magnetic resonance imaging (PMMRI) of the brain and heart in cases suspected of COVID-19. PMMRI is superior to PMCT for soft tissue visualisation, especially of the central nervous system (CNS)[99] and the heart.[100] PMMRI is routinely used at the University of Copenhagen in cases with suspected CNS or cardiac disease as it allows in-situ diagnosis. It is too time-consuming to employ on all regular cases.

Autopsy is performed according to international standards,[101 102] with an additional COVID19-specific algorithm specifying extensive tissue sampling. Board-certified clinical pathologists perform histopathological analysis.

Documentation

Standardised and pro-necessaire photo-documentation and standardised written report forms are used for both gross and radiological findings.

Sampling

The COVID-19 specific algorithm dictates tissue sampling, location, sidedness and so on. Fluids collected for chemical and toxicological analysis are collected in tubes both with (urine and intra-vitreous liquid) and without (peripheral blood, urine, intra-vitreous liquid, gall, stomach contents and heart blood) additives, and

solids for chemical and toxicological analysis include muscle, liver, brain and hair. For a complete overview of sampled tissue, see table 1 in the appendix. Additional tissue may be sampled on a case-to-case basis (e.g. upon discovery of a tumour).

Analysis

It is standard procedure to sample and store tissue at autopsy, as it is not possible to acquire these at a later point. The tissue samples are stored to enable cost effective ad hoc analysis and later re-analysis, should police investigation or clinical questions prompt such. An extended autopsy algorithm has been developed for handling of all cases with suspected or confirmed current or prior (death within six months of symptoms) COVID-19. This algorithm serves diagnostic and work safety purposes, but also serves a research purpose. All autopsies are requested by either police or clinical hospital departments.

The comprehensive tissue sampling in this protocol is for research purposes and is analysed in several research projects, as briefly detailed here.

Project 1: Post-mortem imaging. Post-mortem images, PMCT and PMMRI, will be interpreted de novo by researchers blinded to autopsy findings and reported in structured forms. Quantitative tools for scoring organ changes in post-mortem imaging will be developed and the imaging findings will be correlated to autopsy findings and histopathological findings.

Project 2: Macroscopic and microscopic changes. Standard histopathological analyses of tissue samples will be performed by conventional bright field light microscopy of standard hematoxylin and eosin (H&E) stained slides supported by various special stains (e.g., PAS, PAS-D, Alcian Blue -Van Gieson) and immunohistochemical markers (e.g., inflammatory markers anti-CD3, anti-CD20, anti-CD68; complement deposits such as anti-C4d). The examinations will be supported by other special stains and specific antibodies, immunofluorescence analysis of expression patterns and molecular analysis including PCR-analysis, in situ hybridization and potentially multiplex immunofluorescence and Nanostring GeoMx Deep Spatial Profiling of regions of interest. A uniform way of describing changes will be carried out across organs and related to gross findings and patient data undertaken by specialist in clinical pathology and molecular pathology. A non-exhaustive list includes morphological changes, detection of virus and virus-related changes, inflammation, vascular changes including thrombosis and development of micro-thrombi and fibrotic changes.

Project 3: Chemical and toxicological analysis. The chemical and toxicological examinations will be performed by the forensic Chemistry Department at The Forensic Institute, University of Copenhagen, with established methods such as time-of-flight mass spectrometry, liquid chromatography and specific liquid chromatography tandem mass spectrometry. Sample analysis will include lung tissue, blood and serous fluids. Risk profiles will be determined through metabolomic analysis and clinical biochemical investigation of C-reactive protein (CRP), cortisol and biomarkers for e.g. diabetes and hormones.

Project 4: Loss of smell. The well-known long-term effects of COVID-19 disease on the sense of smell will be investigated by histopathological analysis as detailed for sub-project 2 to determine the expression of ACE2 receptors and the transmembrane protease serine 2 (TMPRSS2) and supported by PCR analysis to identify potential presence of SARS-CoV-2 in the tissue.

Project 5: Advanced microbiological examination. The Department of Microbiology and Infection Control, Statens Serum Institut (SSI,) will perform virological analysis with three commercially available serological tests which will be evaluated for their sensitivity and specificity to detect SARS-CoV-2. To detect the presence of SARS-CoV-2 in tissues, specific PCR will be performed followed by whole-genome sequencing of virus for SARS-CoV-2 positive isolates, microbiome analysis, and multiplex RT-PCR-based assay for seventeen different respiratory tract viruses. Bacteria will be detected on growth media as in routine practice. Levels of antibodies in relevant fluids will be assessed.

Project 6: Aberrant signalling. The effects of SARS-CoV-2 and aberrant ACE2/Angiotensin II signalling in specific organs, e.g. the liver, will be explored with targeted transcriptomic analysis (RNAseq), metabolomic analyses and fluorescence in situ hybridization (FISH) to investigate the immune cell and metabolomic landscapes of SARS-CoV-2 infection, and also to investigate the mechanism of renin-angiotensin system and ACE2/Angiotensin II-induced liver damage. Furthermore, the SARS-CoV-2-induced changes to the transcriptome will be examined.

Data storage

All electronic data, files and images, are stored on a local closed network without connection to the internet. The project data are stored in a database and processed on the same local closed network. Data are managed with software also used for routine work. All cases are assigned a unique project identification number

Statistical analysis

This is an umbrella study for several ongoing research projects. The bio bank will enable future projects to utilise the data sampled. The statistical programmes, methods and sample sizes will vary between projects and will be described in detail in publications to come. In brief, descriptive statistics will be used to quantify typical findings in COVID-19 related deaths, divided into relevant groups of e.g. in-hospital death, with intensive unit care, acute COVID-19, "long" COVID-19 etc. and differences between groups will be analysed with McNemar's test, Chi-square test and Student's t-test. For continuous covariates, regression models will be employed. P-values of <0.05 are considered significant. Analysis will be restricted to cases with complete data for the given sub-project. As missing data most likely will be a result of investigator error rather than reasons within the deceased subject, we assume minimal bias from this practice, although it may reduce the effective sample size.

For evaluating the effects of pharmaceuticals and drugs, we will perform multivariate analysis adjusted for sex, age and known diseases, and comparison made to findings in matched non-COVID-19 related deaths to elucidate the role of medications in COVID-19 related deaths.

For evaluating the congruence between imaging and autopsy as well as histopathological findings, we will calculate sensitivity and specificity for post-mortem CT and MRI, and to assess intra-observer and inter-observer agreement we will calculate Cohen's kappa.

Cohort description

In this study, deceased are included in two ways. In Denmark, all deaths are reported to the police when a criminal act, suicide or accident caused death, when a person is found dead, when death was sudden and not medically expected, when work-related disease is suspected to have caused death, when death may have been a result of error in medical treatment or disease prevention, when a person dies in custody and when it cannot be ruled out that that the police may have an interest in the death.

At medico-legal inquest, the police are advised by either a medical health officer or a forensic pathologist, in accordance with the Danish Health Act, and decide whether or not a forensic autopsy is required. By law, the police always request forensic autopsy when a criminal offense may have contributed to death (or later suspicion of such may arise), when manner of death cannot be determined at medico-legal inquest and police affairs deem autopsy necessary, and when illicit drugs are suspected to have caused death.

Inclusion criteria for the research project and bio bank are at least one of the following: Confirmed or probable history of current or prior COVID-19, positive ante-mortem test, or PMCT findings indicative of COVID-19 (e.g. ground-glass opacities, crazy paving, consolidation, arcade sign and traction bronchiectasis). Negative ante-mortem testing does not results in exclusion, as neither sensitivity nor specificity of these test are perfect.

The Department of Forensic Pathology, University of Copenhagen, and the Department of Pathology, Rigshospitalet, Copenhagen, include all cases that meet the stated criteria. Additional cases from eastern Denmark may be referred from other departments of clinical pathology to Rigshospitalet for autopsy. All forensic departments in Denmark employ the described COVID-19 autopsy algorithm, and this study therefore potentially covers all of Denmark. Clinical departments (e.g. lung medicine, infectious diseases and intensive care units (ICU)) may request an autopsy from the departments of pathology that apply the same inclusion criteria as that for police-requested cases, except there is no inclusion based on PMCT findings. PMCT is then performed, if included.

Included cases in which neither a confirmed history of COVID-19 disease nor SARS-CoV-2 demonstrated on post-mortem virological examination by SSI will serve as internal controls. Figure 1 illustrates the inclusion flow chart.

Prior COVID-19 is defined as death within six months of first symptoms, as studies by Ayoubkhani et al. and Al-Aly et al. on initial COVID-19 survivors demonstrate excess death and disease burden up to several months after hospital discharge.[42 43]

Cases with extensive putrefaction, too large for PMCT, where consent cannot be obtained, and where the COVID-19-specific autopsy algorithm is incompatible with the purpose of the autopsy (e.g. homicide investigation) are excluded. In forensic pathology, a deceased may be discovered at any given time after death. The interval between death and autopsy is potentially unknown. For hospital deaths, the deceased is moved to the morgue after the doctor confirms the presence of rigor mortis or livor, though practical circumstances may delay this process.

In order to determine "extensive putrefaction" objectively and reproducibly, we defined this as a radiological alteration index (RAI) of >50 (scale from 0 to 100) and applied analysis as described by Egger et al.[103] The RAI is a semi quantitative estimation of the extent of post-mortem gas formation in seven anatomical locations as graded from one to three on PMCT, and has been demonstrated to correlate well with the grade of external putrefaction.[104]

Patient and public involvement

Neither patients nor next of kin were involved in the design of this study.

ETHICS AND DISSEMINATION

In Denmark, research on the deceased requires informed consent from next of kin. For autopsies requested by clinical departments, consent to research and autopsy is given simultaneously. For police-requested forensic autopsies, police obtain either consent to autopsy from next of kin or obtain a court order mandating autopsy. For research purposes on forensic autopsies, next of kin are contacted through letter at the earliest one month after autopsy, and if there is no response, then by telephone. The same approach has been successfully used in a previous national study, the SURVIVE study,[105] and it is believed to be the least emotionally disturbing to next of kin. The obtained consent rate in the SURVIVE study was more than 90% of the included cases.[105] The current study has been approved by the Regional Ethics Committee of the Region of Greater Copenhagen (No: H-20078436) and the Danish Data Protection Agency (No: 2002-54-1080).

All data are available upon request to the steering group on the condition that the intended research is sound and that legal matters, such as General Data Protection Regulation (GDPR), are addressed properly. Results will be published in peer-reviewed journals.

DISCUSSION

COVID-19 is an ongoing global crisis, and has already sparked many autopsy research projects. Summaries of these studies are provided in systematic reviews by Maiese et al., Satturwar et al., Peiris et al. and Borczuk.[95 106-108] The tissue sampling and auxiliary investigations performed have been extensive, but also limited by restrictions on how to perform a safe autopsy in suspected COVID-19 related deaths. Given a novel and deadly disease, it was necessary to report findings as soon as possible. This results in many smaller studies with either a few selected investigations or selected reporting.

In autopsy studies, all relevant tissue must be sampled and stored to answer current and future research questions. Matschke et al. had 110 potentially eligible cases, but only sufficient samples from 43 cases to perform their study on the brain in COVID-19.[109] This highlights why prospective, systematic sampling is necessary to provide a broader understanding of the pathogenesis of COVID-19.

Most autopsy studies have been performed on hospitalized patients, but a few studies[13-16 19] and case-series[17 18] include out-of-hospital deaths. The current study will recruit both clinical and forensic cases of individuals deceased up to six months after COVID-19 symptoms. Our cases therefore include both hospitalizations with and without intensive unit care and out-of-hospital deaths, and therefore reflect a heterogeneous population both in terms of age, sex, overall health and circumstances of life and death. Patients recruited from hospitals will most likely differ from those not hospitalised and likewise, those recruited specifically from ICUs will most likely differ from other patients. Cases recruited from hospitals will likely have died from COVID-19. Police-requested autopsies will provide examples of COVID-19-related deaths or pathological changes in individuals who did not manage to seek help, had a quicker course of disease, or died from other causes. Collectively, the heterogeneity of the study population provides an opportunity to document COVID-19 at all stages of disease, and thus give a fuller picture, as well as an opportunity to provide details on the long-term health consequences, including "long" COVID-19 disease. Conversely, statistical inference is more difficult, as each group may be small. This issue will be handled based on results of statistical analysis.

A major issue in the SARS-CoV-2 pandemic is to determine the cause of death from current SARS-CoV-2 infection against death from the consequences of SARS-CoV-2 infection against death from other causes while infected with SARS-CoV-2. Edler et al. proposed categorising deaths as (1) definite COVID-19-related deaths, (2) probable COVID-19-related deaths, (3) possible COVID-19-related deaths, or (4) SARS-CoV-2 detected but unrelated to the cause of death.[10] This categorisation is used in many industrialized countries and may prove relevant to investigate preventive measures, abandon unnecessary measures, steer pharmaceutical treatment, and guide society in both this and future pandemics. To facilitate comparison between our and other studies, this categorization will also be adopted in this study.

To our knowledge, no autopsy studies have investigated "long" COVID-19, as they naturally have recruited cases of individuals deceased from acute COVID-19. As death and disease is increased in the months after initial infection, we also include cases that die within six months of first COVID-19 symptoms. Some "long" COVID-19 symptoms are related to the CNS. Matschke et al. have performed a neuropathological study on acute COVID-19 related deaths,[109] but the CNS remains less well examined than other organs, such as the lungs.[95] Our COVID-19 specific autopsy algorithm include extensive sampling of brain tissue and neuro-pathological special investigation, and we hope our study will contribute to this area of research.

In all fields of medicine, multi-centre studies carry the benefits of larger samples, faster recruiting, more generalizable results, and smaller bias in sampling, but they require strict adhesion to protocol to avoid unsought heterogeneity. These benefits are reflected by an exponential growth in multi-centre studies published on PubMed during the last 20 years. The current study, along with those by e.g. Carsana et al., Sonzogni et al., Borczuk et al., and Basso et al. are examples of multi-centre autopsy studies,[12 35 48 76] that benefit from interdisciplinary cooperation in that e.g. forensic pathologists, clinical pathologists, neuropathologists, epidemiologists, virologists, otolaryngologists and chemists all contribute within their field

of expertise to provide a translational understanding of COVID-19 from gene via organ to deceased to benefit the living.

PMCT has been sparsely investigated. Of the first 28 autopsy studies published, only four included PMCT.[95] In the living, computed tomography serves as a diagnostic tool in COVID-19,[110 111] but post-mortem imaging is different to clinical radiology,[112] and more research on the congruence between post-mortem imaging findings and autopsy is needed.

Like all observational studies, this study is subject to selection bias, confounding and measurement errors. Cases suspected to be infected with SARS-CoV-2 may prove not to be. This makes the actual sample in this study smaller, but its strength is that these cases may serve as internal controls. Measurement error and misclassification are always a possibility, as is confounding. Information from police and hospital records may contain information vulnerable to recall bias, interviewer bias and confirmation bias.

In both forensic pathology and clinical pathology in Denmark, two pathologists always inspect the organs and co-sign the work in accordance with the standard procedures. It is expected that this standard practice would lessen the amount of measurement error and misclassification in the current study. Further, the timing of investigation and sampling should have little effect, as all deceased are kept in a morgue from death to autopsy.

Confounding will be handled through statistical analysis.

The here described study and bio bank may seem superfluous given that the current COVID-19 pandemic seems to be lessening during spring 2021, with vaccine coverage improving by the hour. But this is only true for some parts of the world and the risk of future pandemics and epidemics with variants of SARS-CoV-2 remains. Growing populations living closely, humans living close to animals, extensive national and international travelling, and climate change are all factors that will likely lead to future pandemics.[113] Both MERS and SARS were coronaviruses, and bats harbour more than 91 variants of coronaviruses.[113] This study and bio bank offer an opportunity to understand the pathogenesis of COVID-19 – and the knowledge gained will help combat COVID-19 in e.g. South America, Africa, and Asia and hopefully future pandemics and epidemics with variants of coronavirus, as knowledge gained in one pandemic may help combat the next.[6] There is no reason not to learn as much as possible about SARS-CoV-2 and COVID-19 related deaths in order to potentially improve diagnosis and treatment in future viral pandemics.

Contributor statement

JB and CJ conceived the study and secured external funding through grants. JB is the chief investigator and guarantor. JB and KBO coordinated with the involved parties. JB, MJH and AK-L secured approval from the Ethics Committee for the research projects and the Data Protection Agency for the bio bank. JB, CJ, KBO, MJH, AK-L and CBB contributed to the formation of the protocol. MJH and AK-L wrote the first draft, all contributed to the writing of the manuscript, and all approved the final version.

Competing interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: all authors had financial support from Lundbeck Foundation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Acknowledgements

Professor Niels Lynnerup should be acknowledged for his role in conceiving the study and securing external funding. Niels Walentin should be acknowledged for his contribution on electronic data handling and storage.

Funding

This study is supported by the Lundbeck Foundation with 2.5 million DKK (Ca. 300,000 GBP) (grant: R319-2020-896). Funds are managed by the University of Copenhagen.

Legend for figures

Figure 1. Inclusion and consent flow-chart

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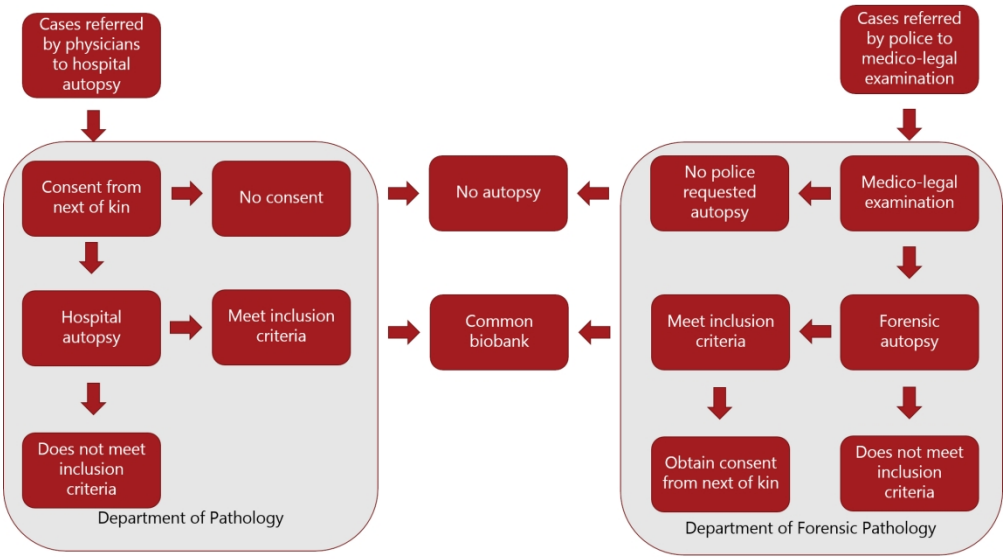

Figure 1: Flowchart demonstrating inclusion

190x106mm (300 x 300 DPI)

Appendix

Table 1 Samples for bio bank

Sample	Histopathology	−80°C storage	Chemical analysis	Bacteriological analysis	Virological analysis
Spinal cord	+				
Olfactory bulb	+	+			
Orbital frontal cortex	+	+	+	+	+
Insula	+	+			
Thalamus	+	+			
Pons	+	+			
Cerebellum	+	+			
Medial occipital cortex	+	+			
Medulla oblongata	+				
Hippocampus	+				
Olfactory epithelium	+				
Peroneal nerve	+	+			
Tibialis anterior muscle	+	+			
Conjunctivae	+				
Tonsil	+	+			+
Soft palate	+	+			
Uvula	+	+			
Sublingual gland	+	+			
Tongue anteriorly	+	+			
Tongue posteriorly	+	+			
Lung, upper lobe pleural	+	+		+	+
Lung, upper lobe central	+	+			
Lung, lower lobe pleural	+	+		+	+
Lung, lower lobe central	+	+			
Heart, LV anteriorly	+	+			
Heart, LV laterally	+				
Heart, LV posteriorly	+	+			
Heart, RV anteriorly	+	+			
Heart, RV laterally	+				
Heart, RV posteriorly	+	+			
Heart septum	+	+		+	+
Heart papillary muscle	+				
Heart valves				+	
Aorta arch	+	+			
Aorta thoracic	+	+			
Aorta abdominal	+	+			
Oesophagus	+	+			
Stomach	+	+			
Duodenum	+	+			
Jejunum	+	+		+	+

Ascending colon	+	+			
Descending colon	+	+			
Liver	+	+	+		
Spleen	+	+			
Pancreas	+				
Adrenal gland*	+				
Kidneys	+	+		+	+
Lymph node	+	+			
Bone marrow	+				
Thyroid gland*	+				
Pituitary gland	+				
Testis/ovary*	+				
Prostate/uterus*	+				
Spinal fluid					+
Vitreous liquid			+		
Pharyngeal swab				+	+
Stomach contents			+		
Urine/bladder wall			+		+
Pleural liquid		+			
Pericardial liquid		+			
Heart blood		+			
Heart blood serum					
Peripheral blood			+	+	+

* Only police-requested autopsies. LV, left ventricular; RV, right ventricular.

'+' means sample taken. 'Blank' means no sample taken. Chemical and virological analyses are performed on all cases. Histopathology and bacteriological samples are stored for later analysis. –80°C samples are stored for later analysis.

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	Line
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2 - 3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	35 – 41
Introduction			
Background /rationale	2	Explain the scientific background and rationale for the investigation being reported	59 – 133
Objectives	3	State specific objectives, including any prespecified hypotheses	135 - 171
Methods			
Study design	4	Present key elements of study design early in the paper	180 →
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	180 - 187 174 - 177
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	299 - 333
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	322 - 324
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	189 - 199 201 - 212 218 – 224 234 – 273 411 - 421 Table 1 in Appendix
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	189 – 199 201 - 212 234 – 273
Bias	9	Describe any efforts to address potential sources of bias	411 - 421
Study size	10	Explain how the study size was arrived at	181
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	281 - 296 369 - 381
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	281 - 296
		(*b*) Describe any methods used to examine subgroups and interactions	281 - 296
		(*c*) Explain how missing data were addressed	288 - 2290
		(*d*) If applicable, explain how loss to follow-up was addressed	n.a.
		(*e*) Describe any sensitivity analyses	n.a.
Results			**n.a.**
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	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive	14*	(a) Give characteristics of study participants (eg demographic, clinical, social)	

data		and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	Report numbers of outcome events or summary measures over time	
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	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	n.a.
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	57 370 – 381 411 - 421
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.
Generalisability	21	Discuss the generalisability (external validity) of the study results	370 - 381
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	452 - 454

*Give information separately for exposed and unexposed groups.

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