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ACCELERATED ARTICLE

SGLT2-Inhibition reverts urinary peptide changes associated with severe COVID-19: An in-silico proof-of-principle of proteomics-based drug repurposing

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Abstract

Severe COVID-19 is reflected by significant changes in urine peptides. Based on this observation, a clinical test predicting COVID-19 severity, CoV50, was developed and registered as in vitro diagnostic in Germany. We have hypothesized that molecular changes displayed by CoV50, likely reflective of endothelial damage, may be reversed by specific drugs. Such an impact by a drug could indicate potential benefits in the context of COVID-19. To test this hypothesis, urinary peptide data from patients without COVID-19 prior to and after drug treatment were collected from the human urinary proteome database. The drugs chosen were selected based on availability of sufficient number of participants in the dataset ($n > 20$) and potential value of drug therapies in the treatment of COVID-19 based on reports in the literature. In these participants without COVID-19, spironolactone did not demonstrate a significant impact on CoV50 scoring. Empagliflozin treatment resulted in a significant change in CoV50 scoring, indicative of a potential therapeutic benefit. The study serves as a proof-of-principle for a drug repurposing approach based on human urinary peptide signatures. The results support the initiation of a randomized control trial testing a potential positive effect of empagliflozin for severe COVID-19, possibly via endothelial protective mechanisms.

KEYWORDS
COVID-19, drug repurposing, peptides, proteomics, SGLT2 inhibition, urine

The current COVID-19 pandemic has generated multiple challenges for clinicians and patients. One major issue is that the severity of the disease course cannot be predicted with certainty at early disease stages. While most patients experience a moderate course of disease, comparable with common cold, a fraction of infected patients develop severe symptoms, leading to elevated oxygen requirements, mechanical ventilation, organ failure and death in a significant proportion of individuals [1]. Risk factors for severe COVID-19 are, among others, age and comorbidities such as diabetes, obesity and chronic kidney disease [2]. While these risk factors help with prognosis, they are not necessarily linked with molecular pathophysiology. In addition, these risk factors are generally not modifiable, limiting their value in guiding therapeutic interventions such as anti-viral therapies and those targeting immune response dysregulation.

In a recent study we investigated urine peptides for their association with severe COVID-19 [3]. We demonstrated that patients with severe COVID-19 course and patients with mild/moderate COVID-19 have a substantially different urinary peptide pattern (UPP). The most

Abbreviations: CE-MS, capillary electrophoresis coupled to mass spectrometry; CMap, Connectivity Map; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; RAAS, renin-angiotensin-aldosterone system; SGLT2, sodium-glucose transport protein 2; UPP, urinary peptide pattern; WHO, World Health Organization

prominent changes were detected in collagen fragments, specifically a reduction of collagen alpha-1(I) chain and collagen alpha-1(III) chain, and an increase in collagen alpha-2(I) chain fragments. In addition, a significant reduction of the abundance of CD99 antigen and polymeric immunoglobulin receptor and an increase in alpha-1-antitrypsin derived peptides were observed [4,5]. Based on these differences, a signature comprised of 50 urinary peptides termed CoV50 was developed [4], representing a molecular signature associated with disease severity [4].

Due to the urgent need presented by the pandemic, an interim assessment of the study was initiated and recently published [4]. The data demonstrated that CoV50 enables prediction of COVID-19 severity, and consequently early intervention [4]. Based on the data, the CoV50 test was approved on the basis of the Directive 98/79/EC on in vitro diagnostic devices by the Federal Institute for Drugs and Medical Devices (BfArM), the responsible German authority (registration no: DE/CA09/0829/IVD/007). The CoV50 test is currently being used to predict COVID-19 course, aiming at improving outcome with early therapeutic intervention.

A detailed investigation of the urine peptides significantly affected in patients that subsequently develop severe COVID-19 (defined here as World Health Organization [WHO] grade 6 or higher) indicated highly significant similarity to peptides altered in chronic and acute kidney disease [5]. The results also revealed that severe COVID-19 may be associated with endothelial damage, in agreement with the current literature [6]. As a consequence, therapy directed against SARS-CoV-2 may not deliver benefit if initiated at a later stage of the disease, when the endothelial layer is already damaged due to the virus infection. In fact, it has been shown that antiviral therapies display benefit only if initiated in the very early phases of SARS-CoV-2 infection [7,8]. Thus, it is plausible that interventions targeting endothelial injury induced by the viral infection, and not the SARS-CoV-2 virus per se, may be beneficial in the treatment/prevention of severe COVID-19.

Drug repurposing has gained significant interest as a means to boost drug development. This is mainly attributed to the opportunity to decrease overall cost of drug development and timelines [9]. One computational (in silico) approach that has been used by multiple groups [10] including us [11,12], is prediction of drug efficacy based on the molecular signature of drug impact [13]. A well-established solution in this respect is the Connectivity Map (CMap) analysis [14]. In CMap, molecular disease signatures are matched against the transcriptomic-based molecular signatures induced by drug treatment in vitro.

In this study, our aim was to determine if drugs can impact the severe COVID-19 signature (CoV50). The study builds on what is known about the molecular signature associated with severe COVID-19, and was based on access to urinary peptide data from interventional studies involving patients without COVID-19. Such an analysis was feasible since urinary peptide data were obtained using the same technology, and are thus highly comparable [15]. The approach chosen here is the first attempt to use human-derived urinary peptide data for drug repurposing.

Power calculations indicated that in order to detect a 50% change in the CoV50 scoring with a p-value < 0.05 and 80% power, datasets from a minimum of 17 subjects before and after treatment are required. We therefore only included studies involving at least 20 subjects. Investigation of the data in the urine proteome database [15] resulted in the identification and extraction of datasets from patients without COVID-19 undergoing treatment with the aldosterone antagonist spironolactone from the HOMAGE trial [16,17], and the sodium-glucose transport protein 2 (SGLT2)-inhibitor empagliflozin [18,19]. As a part of the HOMAGE trial [16,17], the placebo group was also investigated. The details on the studies and the patient datasets included are listed in Table 1.

The outline of this study is graphically depicted in Figure 1. To identify a potential drug benefit in the context of COVID-19, ideally patients with COVID-19 receiving drug or placebo should be investigated. Unfortunately, such data are generally not available. As an alternative, the impact of drugs on the CoV50 signature can be investigated based on data from subjects without COVID-19 before and after drug treatment. Samples from these patients are expected to score negative for CoV50, since they did not have COVID-19. A change of the CoV50 score in the positive direction (towards severe COVID-19) would be considered as a signal for a potential negative effect of the drug. A change of the CoV50 score in the negative direction (reverting the CoV50 signature) indicates a potential benefit.

Significance Statement

COVID-19 pandemic has imposed a heavy burden on society, health care and economics. Multiple drugs have been tested in the context of COVID-19. Some of these have demonstrated benefit only at early stages of disease. However, effective treatments for patients with severe COVID-19 are still lacking. Computational drug repurposing emerged as a promising approach to boost drug development. It allows predicting drug efficacy based on the molecular signature of drug impact, mainly using transcriptomics data from cell lines.

Recently, we demonstrated that urinary proteomics profiles differ significantly between patients with severe COVID-19 compared to those with mild/moderate disease. This resulted in the development of a molecular signature associated with COVID-19 severity (CoV50). The CoV50 signature may allow insight into the clinical course of COVID-19, thereby guiding therapeutic interventions.

Here we report, for the first time, the application of clinical proteomics data, obtained from clinical trial participants, using a drug repurposing approach. The CoV50 signature was used to examine if molecular changes associated with COVID-19 severity in patients without COVID-19 might be altered using existing drugs. Empagliflozin demonstrated a partial, yet significant reversion of the CoV50 signature, indicating a potential benefit in severe COVID-19.

TABLE 1 Characteristics of the subjects and datasets investigated in this study

Treatment	Number of datasets (subjects)	Age (±SD)	Male/Female	Mean CoV50 before treatment (± SD)	Mean CoV50 after treatment (± SD)	*p*-value (paired t-test)
Empagliflozin	80 (40)	24.2 (5.4)	20/20	−1.317 (± 0.534)	−1.520 (± 0.178)	0.005
Spironolactone	163 (163)	73.7 (6.1)	133/30	−1.661 (± 0.667)	−1.707 (± 0.635)	0.445
Placebo	167 (167)	73.8 (6.0)	122/45	−1.696 (± 0.723)	−1.626 (± 0.663)	0.203

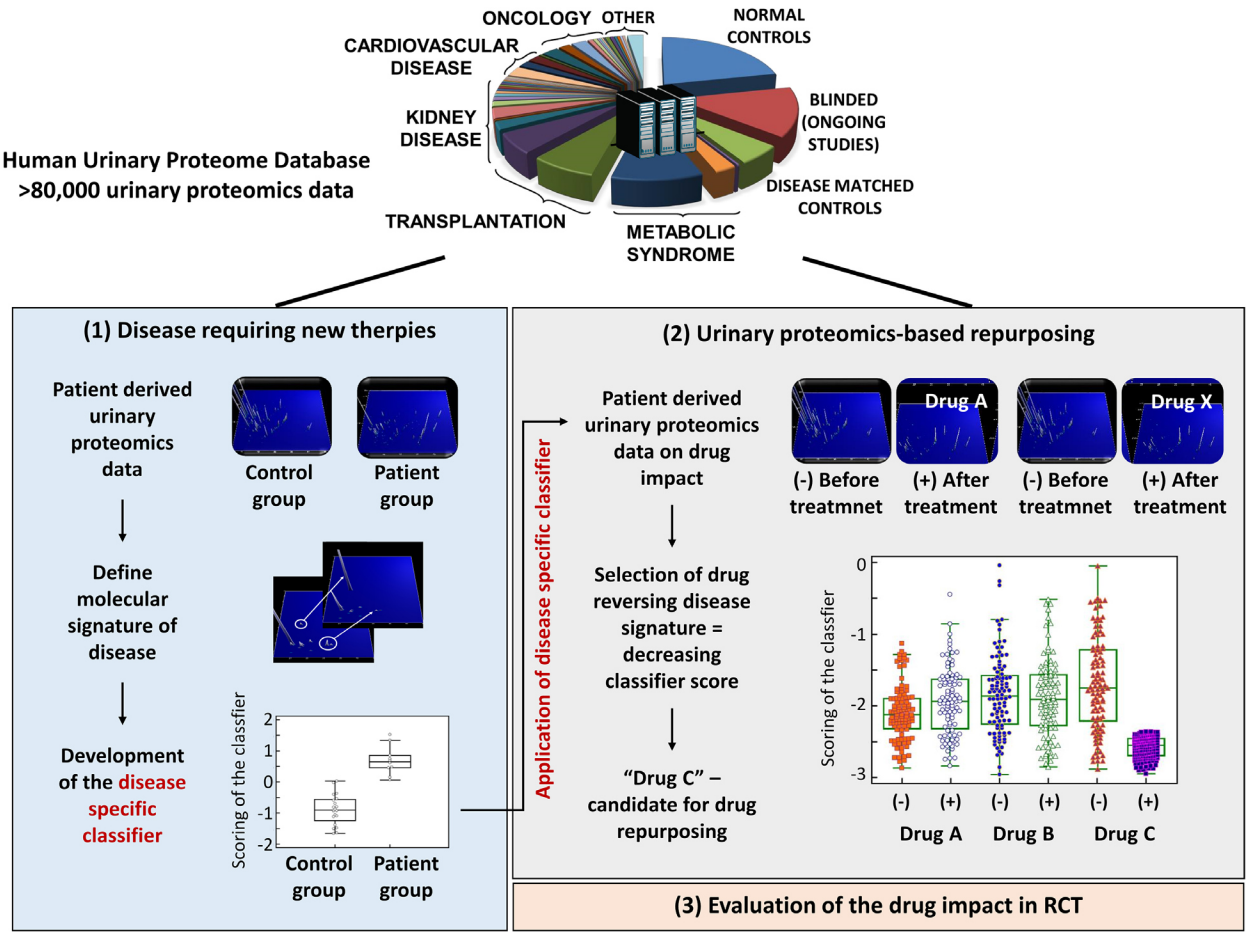

FIGURE 1 Schematic depiction of the study design. The underlying hypothesis of the study was that drugs that are able to revert a disease signature, here a signature based on urine peptides, may be beneficial in treating the respective disease. As such, we tested if a urinary peptide-based signature, CoV50, that is associated and predicts critical course of COVID-19 (left panel), is affected by drug treatment. For this purpose, data from COVID-19 free patients before and after treatment with specific drugs from the human urinary database were extracted and the impact of the drug treatment on the CoV50 scoring was investigated. RCT, randomised controlled trial

Within this study, the CoV50 signature was applied to samples from patients without COVID-19 collected at baseline (before treatment) and during follow-up (after treatment). The distribution of the CoV50 scoring in the baseline and follow-up samples is shown in Figure 2. While this was a study in a population without COVID-19, participants had detectable levels of urinary peptides included in the CoV50 signature. As expected, the study subjects generally scored negative for severe COVID-19. There was no significant difference in the CoV50 scoring in the placebo group before and after treatment. No significant difference was also detectable as a result of treatment with spironolactone. In contrast, empagliflozin treatment significantly reduced the level of the CoV50 score ($p = 0.04$) in a dataset of 160 samples [19] (80 before and 80 after treatment), from an average score of -1.76 (before treatment) to an average of -1.89 (after treatment).

A significant difference in the CoV50 scoring between the spironolactone and the empagliflozin cohorts was observed. This may be due to the significant difference in age (see Table 1), however, we have not detected an association of the CoV50 scoring with age. A more

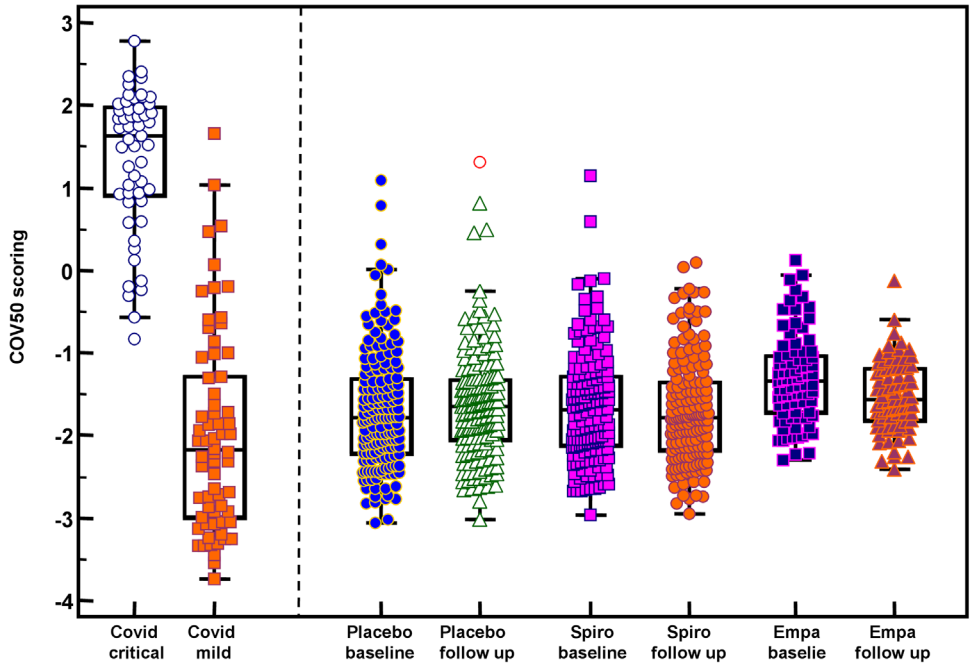

FIGURE 2 CoV50 scoring of the subjects investigated. For comparison, the distribution of CoV50 scoring of patients with mild (WHO grade 1–3) or critical (WHO grade 6–8) disease is presented on the left. Empa, empagliflozin; Spiro, spironolactone

likely explanation could be the different trial locations: the HOMAGE trial was conducted in Europe, while the empagliflozin cohort is from Canada.

Collectively, we investigated an impact of spironolactone and empagliflozin on the CoV50 scoring in patients without COVID-19 to assess a potential therapeutic benefit in severe COVID-19 treatment. We demonstrated that the molecular profile induced by severe COVID-19 opposes the changes induced by empagliflozin. This indicates that exposure to empagliflozin may revert, at least in part, the molecular changes in severe COVID-19.

Until now, most therapies investigated in COVID-19 in controlled trials comprise of biological therapies (monoclonal anti-virus directed [20] and anti-C5a antibodies [21] and polyclonal convalescent plasma [22]), inflammation inhibitors [23], small-molecule anti-virus therapies (remdesivir, lopinavir and ribavirin, hydroxychloroquine [8]) and steroids [24] in high and medium dosage schemes. Most of these drugs target the virus, and, as a result, are typically ineffective when applied at later stages of the disease. This indicates the urgent need for additional drugs that show efficacy at these stages. Several authors suggested that spironolactone may have potential positive impact in the context of COVID-19 [25]. Spironolactone, an aldosterone antagonist inhibiting the renin-angiotensin-aldosterone system (RAAS), is primarily used for management of heart failure, edematous conditions (nephrotic syndrome, ascites in cirrhotic adults), hypertension or hyperaldosteronism. Potential benefits from its use in COVID-19 patients were generally based on theoretical considerations. We could not identify a clinical trial testing a potential benefit of spironolactone. In the BISCUIT trial a limited number of patients treated with bromohexine and spironolactone was compared to placebo, and a significant reduction on the duration of COVID-19 was reported [26]. However, in the small cohort of 33 patients, the fractional impact of any one of the two drugs apparently has not been assessed. Consequently, the impact of spironolactone treatment on COVID-19 cannot be evaluated with sufficient certainty. Our data presented here do not indicate a potential benefit of spironolactone.

SGLT2 inhibitors have shown to affect pathways dysregulated in COVID-19 including inflammation [27,28] and endothelial dysfunction [29,30]. These drugs are primarily used for the treatment of type 2 diabetes, with protective effects in cardiovascular and kidney diseases [31]. SGLT2 inhibitors were also recently approved for treatment of patients with heart failure and reduced ejection fraction, and for treatment of chronic kidney disease. The impact of SGLT2 inhibition on COVID-19 course was investigated in the DARE-19 study [32,33]. Treatment with dapagliflozin resulted in a lower number of events of organ dysfunction or death in comparison to placebo [33]. Although the results did not reach the statistical significance, possibly due to the low number of events observed in the trial, they indicate potential beneficial effect of SGLT2 inhibitors in COVID-19. A trial investigating SGLT2 inhibitors for the prevention of death or organ failure in COVID-19 in high risk patients, based on CoV50 scoring, is expected to demonstrate benefit. Selection of high-risk patients would allow to increase the number of events in the trial (e.g. organ dysfunction or death), and thus reach an anticipated level of statistical power. COVID-19 patients with a CoV50 score above the cut-off (0.47) have a 7-fold increased risk of death compared to patients with the CoV50 signature score below the cut-off [4]. In another paper, Israelsen and colleagues reported similar 30-day mortality in diabetic patients with COVID-19 who were undergoing treatment with SGLT2 inhibitors or glucagon-

like peptide-1 agonists [34]. Since this was a cohort study based on the data retrieved from national registries, an impact of these drugs could not be assessed in comparison to the placebo group.

Potential benefits of SGLT2 inhibitors in severe COVID-19 are further supported by reports from animal studies on protective effects of SGLT2 inhibition in the context of endothelial dysfunction [30]. Since a major component of severe COVID-19 appears to be endothelial dysfunction, drugs that demonstrate protective effects in this context may display benefits in preventing/reducing severe COVID-19.

Several observational studies reported on the repurposing of established drugs like aspirin [35] and statins [36] to abolish COVID-19 clinical thrombo-embolic endpoints. However, proteomic data are usually not available from these observational studies. As demonstrated in this study, it should be feasible to identify non-COVID-19 investigations with, for example, aspirin and statins, and in-silico model the consequences of such treatments, based on urinary proteomics/peptidomics.

Our study has shortcomings. First, it is a retrospective analysis of previously collected data. Second, the impact of the different drugs on the CoV50 signature cannot be interpreted as sure predictors of efficacy in COVID-19. This analysis was conducted in COVID-19 free patients and the CoV50 scores, as expected, did not indicate severe disease. In addition, changes in the UPP as a result of the intervention likely represent an indirect effect of the drugs, and no conclusion can be made with confidence on the mechanism of action or specific drug targets. Considering the complexity of disease at the molecular level, it is expected that development of drugs able to reverse the molecular signature, rather than focusing on a single target, will deliver more effective therapeutics. The observed significant decrease of the CoV50 score following treatment suggests that SGLT2 inhibitors may confer some protection from severe COVID-19 course. A significant impact of SGLT2 inhibition on the course of COVID-19 would need to be demonstrated in a properly powered prospective clinical trial.

In conclusion, the study and data presented here are a first indication that proteomics data from clinical trial participants can be exploited in a drug repurposing approach. A substantial benefit of the approach shown here may be that it is based on patient data, not on cell lines or animal models, and consequently may better depict pathophysiology in patients, which are by far more complex than single cells.

1 | EXPERIMENTAL PROCEDURES

For this study urinary proteome data obtained by capillary electrophoresis coupled to mass spectrometry (CE-MS) and stored in the human urine proteome database [15] were assessed. This database currently contains urinary proteome/peptidome information on >80,000 samples, assessing a dataspace comprised total of >100,000 peptides and low molecular weight proteins [15]. Information on the peptide sequencing from liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and CE-MS/MS analysis, and anonymized clinical information of participants enrolled in the studies are also included. The CE-MS technology applied has been described in detail including reproducibility, repeatability, procedures for sample preparation, data evaluation and normalization [4,37,38]. CE-MS analysis was performed with a P/ACE MDQ CE (Beckman Coulter, USA) coupled to a micro-TOF-MS (Bruker Daltonic, Germany). Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaFinder software [4]. Only signals with charge>1 observed in a minimum of three consecutive spectra with a signal-to-noise ratio of at least four were considered. The generated peak list included polypeptides characterised by mass and migration time. Mass and migration time were calibrated using internal standards by applying global and local linear regression, respectively [4]. Signals of 29 abundant peptides derived from collagens, generally not affected by disease, were used as internal standards for calibration of signal intensity using linear regression. This procedure was shown to be an easy and reliable method to address both analytical and dilution variances in a single calibration step [38]. The final peak list was comprised of polypeptides described by its calibrated molecular mass (Da), calibrated CE migration time (min) and normalized signal intensity. More detailed information on the peptide sequencing was described elsewhere [39]. All underlying studies were conducted conforming with regulations on the protection of individuals participating in medical research and in accordance with the principles of the Declaration of Helsinki (2013) and received ethical approval by the responsible institutional review boards. Written informed consent was obtained from all participants at the time of sampling. All datasets received were anonymized. Specifically, for all samples from COVID-19 patients the Ethics Committee of the German-Saxonian Board of Physicians, Dresden, Germany (number EK-BR-88/20.1) and the Institutional Review Boards of the recruiting sites provided ethical clearance [4]. The protocol was registered with the German Register for Clinical Studies (www.drks.de), number DRKS00022495, which is interconnected with the WHO International Clinical Trial Registry Platform (www.who.int/clinical-trials-registry-platform). For the samples from patients undergoing empagliflozin treatment, all patients consented to the study, which was approved by the University of Toronto University Health Network with approval #11-0213 [19]. All samples from subjects undergoing spironolactone treatment and placebo controls were from the HOMAGE trial, a prospective, randomized, open-label, blinded-endpoint, multicentre design, in which people at increased risk of developing HF were randomly assigned to receive either spironolactone or standard of care ("control")–not receiving spironolactone or other mineralocorticoid receptor antagonists (ClinicalTrials.gov identifier: NCT02556450). The study was approved by all relevant ethics committees and regulatory bodies [17].

Distribution of the CoV50 scoring was evaluated using MedCalc (version 12.1.0.0, MedCalc Software, Mariakerke, Belgium; https://www.medcalc.org/). For analysis of differences in the scoring between baseline and drug treatment, paired T testing was applied, statistical significance was assumed at $p < 0.05$.

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CONFLICT OF INTERESTS

Harald Mischak is the founder and co-owner of Mosaiques Diagnostics (Hannover, Germany). Agnieszka Latosinska and Justyna Siwy are employed by Mosaiques Diagnostics.

DATA AVAILABILITY STATEMENT

The CE-MS raw data are available on Zenodo: https://doi.org/10.5281/zenodo.5464140.

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