RESEARCH

Unquantifiably low aldosterone concentrations are prevalent in hospitalised COVID-19 patients but may not be revealed by chemiluminescent immunoassay

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Abstract

Objective: Previous studies have reported conflicting findings regarding aldosterone levels in patients hospitalised with COVID-19. We therefore used the gold-standard technique of liquid chromatography–tandem mass spectrometry (LCMSMS) to address this uncertainty.
Design: All patients admitted to Cambridge University Hospitals with COVID-19 between 10 March 2020 and 13 May 2021, and in whom a stored blood sample was available for analysis, were eligible for inclusion.
Methods: Aldosterone was measured by LCMSMS and by immunoassay; cortisol and renin were determined by immunoassay.
Results: Using LCMSMS, aldosterone was below the limit of detection (<70 pmol/L) in 74 (58.7%) patients. Importantly, this finding was discordant with results obtained using a commonly employed clinical immunoassay (Diasorin LIAISON®), which over-estimated aldosterone compared to the LCMSMS assay (intercept 14.1 (95% CI −34.4 to 54.1) + slope 3.16 (95% CI 2.09–4.15) pmol/L). The magnitude of this discrepancy did not clearly correlate with markers of kidney or liver function. Solvent extraction prior to immunoassay improved the agreement between methods (intercept −14.9 (95% CI −31.9 to −4.3) and slope 1.0 (95% CI 0.89–1.02) pmol/L) suggesting the presence of a water-soluble metabolite causing interference in the direct immunoassay. We also replicated a previous finding that blood cortisol concentrations were often increased, with increased mortality in the group with serum cortisol levels > 744 nmol/L (P = 0.005).
Conclusion: When measured by LCMSMS, aldosterone was found to be profoundly low in a significant proportion of patients with COVID-19 at the time of hospital admission. This has likely not been detected previously due to high levels of interference with immunoassays in patients with COVID-19, and this merits further prospective investigation.

Key Words
- COVID-19
- SARS-CoV-2
- renin-angiotensin-aldosterone system

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Introduction

As of February 2022, the United Kingdom had recorded in excess of 18.8 million cases of COVID-19, with at least 161,000 cases resulting in death (1). The speed of onset and severity of the pandemic has spurred a coordinated response from the global biomedical community on a scale not previously seen. This includes attempts to better understand the pathogenesis of the disease, to identify factors that can be used to predict risk and disease trajectory in individual patients, and to deliver preventative and curative interventions for the world's population.

Given the key role of angiotensin-converting enzyme 2 (ACE2) in facilitating the entry of SARS-CoV-2 virus particles into the lung (alveolar epithelial type II cells), gastrointestinal tract (luminal intestinal epithelial cells) and other tissues (2, 3), exploration of the potential effects on the renin–angiotensin–aldosterone system (RAAS) is of interest in understanding the pathogenesis of COVID-19. ACE2 inhibits RAAS activation by converting angiotensin II (AngII) to angiotensin 1–7 (Ang 1–7). Ang 1–7 exerts anti-inflammatory, anti-oxidative and vasodilatory effects via binding to the Mas receptor (4). AngII binds AngII receptor type 1 which then exerts pro-inflammatory, pro-oxidative and vasoconstrictive effects (5). It may also contribute to pro-fibrotic effects, hypercoagulability and immunothrombosis by inducing tissue factor and plasminogen activator inhibitor-1 expression by endothelial cells. AngII further binds to the angiotensin I receptor on the adrenal glands, stimulating the release of the mineralocorticoid aldosterone.

SARS-CoV-2 has the potential to activate RAAS and the secretion of aldosterone, by preventing this ACE2-Ang 1–7-mediated RAAS inhibition. The uninhibited AngII may then play a role in the pathogenesis of observed hypertension (6), inflammation, immunothrombosis and possible fibrosis in COVID-19.

Whilst elevated serum cortisol has been identified as a marker of poor prognosis in COVID-19 patients (7), the evidence regarding RAAS activation is less clear. Early studies found evidence of increased RAAS activation (8, 9, 10), but subsequent reports suggested no association (11). Similarly, there is conflicting evidence regarding serum aldosterone levels, with both increased concentrations (12) and no changes reported (11). In addition, there have been several case reports of hyporeninemic hypoaldosteronism (13). A low aldosterone/renin ratio has also recently been suggested as predictive of increased severity (14).

To the best of our knowledge, all published studies measuring aldosterone in COVID-19 patients have used non-extraction immunoassays. These methods lack specificity and are prone to interference, for example, the polar aldosterone metabolite aldosterone-18-glucuronide has been shown to cross-react in a non-extraction assay commonly used in clinical laboratories (15, 16). This is more apparent in patients with renal failure as hydrophilic metabolites accumulate. Mass spectrometric methods for aldosterone are now increasingly available in clinical laboratories and do not suffer this interference. Whilst the clinical effectiveness of non-extraction immunoassays in the diagnosis of primary hyperaldosteronism is still contested (17), mass spectrometric methods are metrologically superior and are more likely to represent the biologically active aldosterone fraction.

During the pandemic, we observed low aldosterone levels in a number of patients, which we had not anticipated. Motivated by this observation, in this study, we used a tandem mass spectrometric method to estimate serum aldosterone concentration in patients admitted to the hospital with SARS-CoV-2 infection. We correlate these serum aldosterone results with clinical outcomes and compare results from the tandem mass spectrometric method with re-measurements using immunoassay methods. We also evaluate the association between high cortisol concentrations and 28-day survival, as previously described by Tan *et al*. (7).

Methods

Study population and data collection

All patients admitted (or discharged from the Emergency Department) to Cambridge University Hospitals (CUH), UK, who had a positive diagnostic test for SARS-CoV-2, between 10 March 2020 and 13 May 2021 were eligible for inclusion. Diagnostic testing for SARS-CoV-2 at the hospital used either a real-time RT-PCR of the RdRp gene from a nasopharyngeal swab or the SAMBA II point-of-care test (18).

We made use of blood samples drawn from a biobank at CUH, which was established early in the pandemic to store, where available, blood samples from patients with COVID-19. We retrospectively measured aldosterone, cortisol and renin levels on the available stored samples from the biobank. All patients with at least one stored sample from around the time of their first positive SARS-CoV-2 result (within 72 h) were included. Within this time window, the earliest available sample was used. Samples were excluded from our analysis if the blood sample was collected after the patient had already

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received glucocorticoid or mineralocorticoid therapy (e.g. dexamethasone, fludrocortisone, hydrocortisone, methylprednisolone, prednisolone). Following the findings of the RECOVERY study, dexamethasone became part of the standard treatment for patients with COVID-19 receiving supplemental oxygen (19).

Clinical data for all patients presenting to hospital with COVID-19 were extracted from the hospital's electronic health records system (including age, gender, BMI, comorbidities, observations, routine laboratory results and outcomes).

Laboratory procedures

Aldosterone was measured using a liquid chromatography–tandem mass spectrometric method (LCMSMS) adapted from Hinchliffe and colleagues (20), with a lower limit of quantitation (LLOQ) of 70 pmol/L. The assay in the clinical laboratory is accredited by UKAS (https://www.ukas.com/) to the ISO 15189 standard. Serum cortisol was measured using Siemens Centaur direct competitive chemiluminescent immunoassay (CLIA) (LLOQ 25 nmol/L). On a subset of patients where appropriately stored plasma samples were available (samples not chilled prior to centrifugation and plasma stored frozen), we also measured plasma renin concentration, using the Diasorin LIAISON® immunometric CLIA method (LLOQ 1.96 mU/L).

To explore potential differences between aldosterone as determined by LCMSMS vs immunoassay, aldosterone was re-measured in a subset of patients, where sample volume permitted, using the Diasorin LIAISON® direct competitive CLIA (LLOQ 1.91 ng/dL).

Statistical analyses

The association between aldosterone, cortisol and renin in paired samples was assessed by linear regression, imputing the value 35 pmol/L for aldosterone values below the LLOQ of 70 pmol/L, which is statistically equivalent to assuming that these values are uniformly distributed between 0 and the LLOQ (21).

To assess the association between high cortisol concentrations (>744 nmol/L) and outcomes (7), a Kaplan–Meier estimate of mortality within 28 days of a positive SARS-CoV-2 test, stratified by the first cortisol concentration following the positive test (within 72 h), was used. Tan *et al.* selected the threshold of 744 nmol/L as the value that maximises the log-rank statistic, i.e. the difference between survival curves. The selection of the cortisol threshold that optimises the difference between survival curves in our cohort was also replicated and the difference in survival curves was assessed by a log-rank test. Similarly, the difference in survival curves of cohorts with high or low aldosterone concentrations was investigated.

Scatter plots, with Passing–Bablok regression lines, and Bland–Altmann plots were used to assess the agreement between the LCMSMS and CLIA methods. To explore the potential association between bias in the CLIA results with renal function, we examined associations with estimated glomerular filtration rate (eGFR) and creatinine clearance (Cockroft-Gault) using linear regression that is robust to outliers (22).

Follow-up analysis

The CLIA was repeated following solvent extraction of serum to remove potential aqueous interference from patient blood samples that would not be detected by the LCMSMS method. In brief, 350 μL of patient sample was mixed with 2250 μL of methyl tertiary-butyl ether (MTBE) by vortex for 15 min. The lower aqueous layer was frozen and the upper MTBE layer decanted and evaporated to dryness at 60°C under nitrogen flow. The sample was reconstituted in 350 μL of steroid-free serum (DRG Instruments GmbH, Marburg, Germany). Extraction efficiency was calculated using a cohort of 15 anonymised COVID-19 negative control subjects (data not shown). Extraction efficiency was shown to be proportional to LCMSMS aldosterone concentration, and the COVID-19 samples were corrected using this mean percentage extraction recovery of 38%.

Results

Study population

Two hundered thirty-one patients who tested positive for SARS-CoV-2 had at least 1 measurement of aldosterone, cortisol or renin within 72 h of their first positive SARS-CoV-2 test. Of these, 95 patients received glucocorticoid therapy and 2 patients received mineralocorticoid therapy prior to the collection of the blood sample, leaving 134 patients in the study cohort (126 with an aldosterone measurement available; 88 with measured cortisol and 50 with measured renin).

Eighty patients (59%) were male and the median age was 64 years (IQR 46, 88). Of the patients, 118 (88%) were admitted to the hospital and 16 (12%) were discharged

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from the Emergency Department. Fifteen patients (11.2%) were admitted to the intensive care unit (ICU) and the in-hospital mortality was 13.4%. Other baseline parameters and markers of severity are described in Table 1. The patients in our study had similar age, gender, ethnicity, BMI and severity markers to the overall population of SARS-CoV-2 patients presenting to the hospital (Supplementary Table 1, see section on supplementary materials given at the end of this article), although these patients had lower interleukin-6 (median 8.1 vs 11.9); were less likely to be admitted to ICU (11.2% vs 18.9%); and were more likely to have diabetes (32.8% vs 20.7%) or other endocrine diseases (14.2% vs 8.7%).

Biochemical markers

The distribution of aldosterone, cortisol and renin is visualised by histograms in Fig. 1. The aldosterone concentrations after the positive test in Fig. 1A are remarkably low, with the concentration of aldosterone below the LLOQ (70 pmol/L) in 58.7% of the patients (74 patients). Analysis of cortisol levels found elevated levels, with 20.5% of patients having results greater than 744 nmol/L, the level proposed by Tan *et al*. to maximise the difference between patient survival curves (Fig. 1B). Figure 1C depicts the renin concentrations, which were predominantly low despite the low aldosterone concentration.

There was evidence that higher aldosterone concentrations were associated with both higher cortisol (beta = 0.316, 95% CI 0.06–0.57, $P = 0.02$) and higher renin concentrations (beta = 0.062, 95% CI 0.025–0.099, $P < 0.00001$), as shown in Fig. 2. Stratification of the cohort according to whether aldosterone was above or below the LLOQ did not reveal any significant differences in clinical characteristics (Supplementary Table 2) or in the survival curves (Supplementary Fig. 2). There was no obvious correlation between aldosterone level and renal function (Supplementary Fig. 3).

Association of cortisol levels and mortality

The survival probability of patients with a high concentration of cortisol (above 744 nmol/L) was lower than for those with a low concentration of cortisol (below 744 nmol/L). As shown in Fig. 3, there was a marked difference in 28-day mortality, with 44% of patients dying in the high cortisol group, compared to 11% of patients in the low cortisol group. Whilst the 95% CI around the survival curves overlap over the entire time frame,

Table 1 Patient characteristics following their first positive SARS-CoV-2 test.

Characteristics	
Sample size (*n*)	134
Age at admission, median (IQR) (range)	64 (46, 88) (20–102)
Gender (male), *n* (%)	80 (59%)
Ethnicity, *n* (%)	
White	90 (67.2%)
Black	1 (0.7%)
Asian	13 (9.7%)
Other	3 (2.2%)
Not specified/prefer not to say	27 (20.1%)
Body mass index, median (IQR), kg/m^2	27.8 (23.5, 32.3)
Observations, median (IQR)	
Heart rate, beats/min	86 (74, 95)
Temperature, °C	37.1 (36.6, 37.7)
Respiratory rate, breaths/min	19 (17, 22)
Oxygen saturation (SpO_2), %	96 (94, 98)
Mean arterial pressure, mmHg	90 (82, 99)
Blood tests, median (IQR)	
C-reactive protein, mg/L	51 (19, 107)
White cell count, 10^9/L	6.2 (5, 8.9)
Sodium, mmol/L	137.8 (135.6, 140.0)
Potassium, mmol/L	4.0 (3.8, 4.5)
Neutrophils, 10^9/L	4.7 (3.4, 7.0)
Lymphocytes, 10^9/L	1.0 (0.7, 1.5)
Interleukin-6, pg/mL	8.1 (3.6, 18.5)
Urea, mmol/L	5.8 (4.2, 8.8)
Creatinine, µmol/L	72 (62, 93)
D-dimer, ng/mL	221 (142, 461)
Troponin, ng/L	8.5 (3.0, 24.7)
pH value	7.40 (7.37, 7.44)
Medical history, *n* (%)[a]	
Heart disease	24 (17.9%)
Hypertension	48 (35.8%)
Beta blockers at admission[b]	27 (20.2%)
Diabetes	44 (32.8%)
Endocrine disease (other than diabetes)	19 (14.2%)
Stroke	3 (2.2%)
Dementia	10 (7.5%)
Asthma	20 (14.9%)
Respiratory disease (other than asthma)	17 (12.7%)
Chronic kidney disease	9 (6.7%)
Chronic liver disease	10 (7.5%)
Malignancy non-haematological	18 (13.4%)
Malignancy haematological	4 (3.0%)
Immunocompromised	2 (1.5%)
Treatments and outcomes, *n* (%)	
In-hospital deaths	18 (13.4%)
Admitted to ICU	15 (11.2%)
Invasive mechanical ventilation	12 (8.9%)
Renal replacement therapy	10 (7.5%)

[a]See Supplementary Table 3 for ICD-10 code lists. [b]Bisoprolol, atenolol, propranolol and carvedilol.

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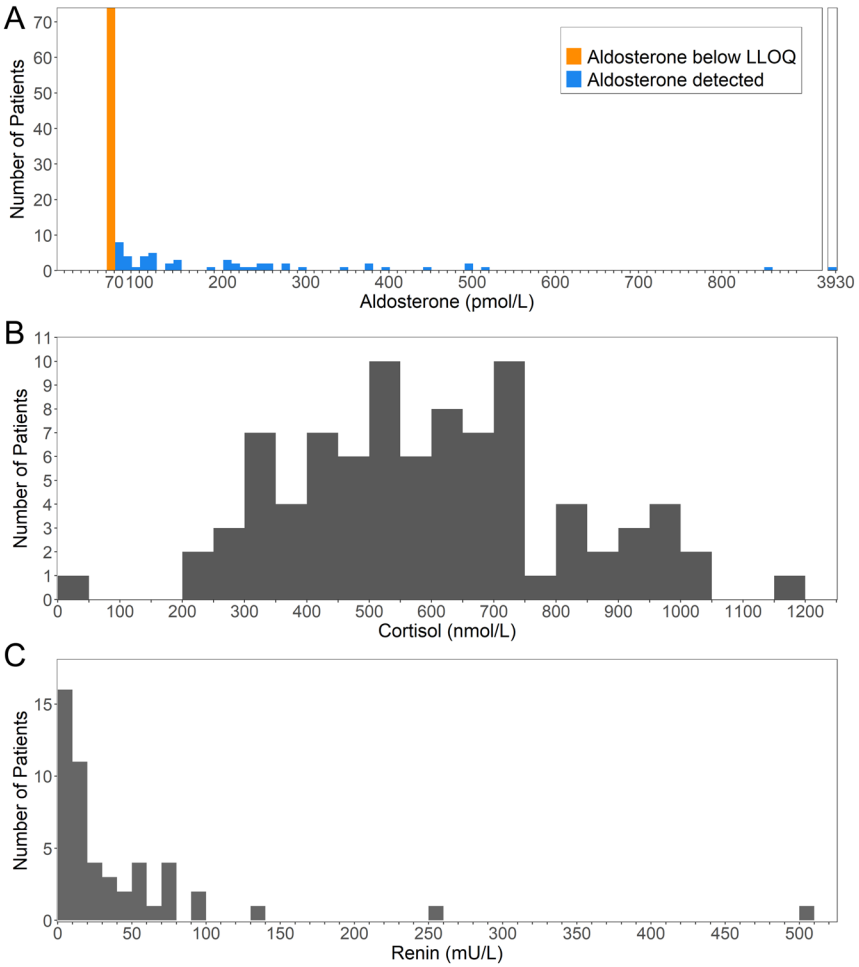

Figure 1
Histograms of the first available result: (A) aldosterone, measured using LCMSMS, (B) cortisol and (C) renin.

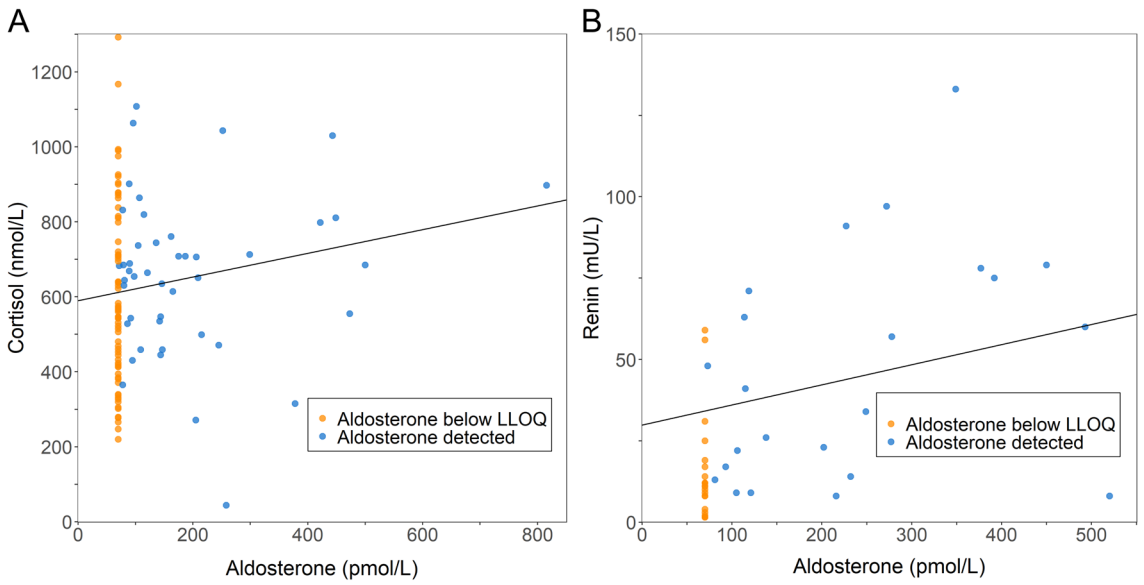

Figure 2
Scatter plots of paired aldosterone, cortisol and renin results, with linear regression lines shown. (A) Aldosterone (measured using LCMSMS) against cortisol. (B) Aldosterone (LCMSMS) against renin (two extreme outliers are outside the plotting region; see Supplementary Fig. 1 for zoomed-out version).

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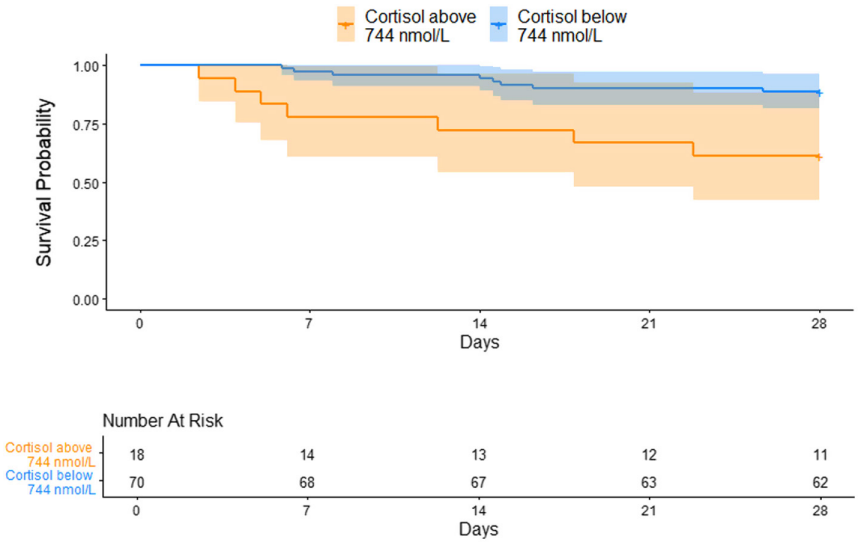

Figure 3
Kaplan–Meier survival plot for 28-day survival in steroid naive patients after first positive test, stratified by the first cortisol result after the test. The 95% CIs are shown by shading.

the log-rank test comparison of the complete survival curves indicates a significant difference between the two groups ($P = 0.005$). The average length of hospital stay in the high cortisol group (19.1 days) was longer than in the low cortisol group (11.2 days). The cortisol threshold maximising the log-rank test for the difference of survival curves in our cohort was 801 nmol/L.

LCMSMS and CLIA method comparison for aldosterone

The considerable disparity between our LCMSMS aldosterone results and previously published studies (12, 11), which used immunoassay, is explained by Fig. 4. This shows that re-measurements of aldosterone

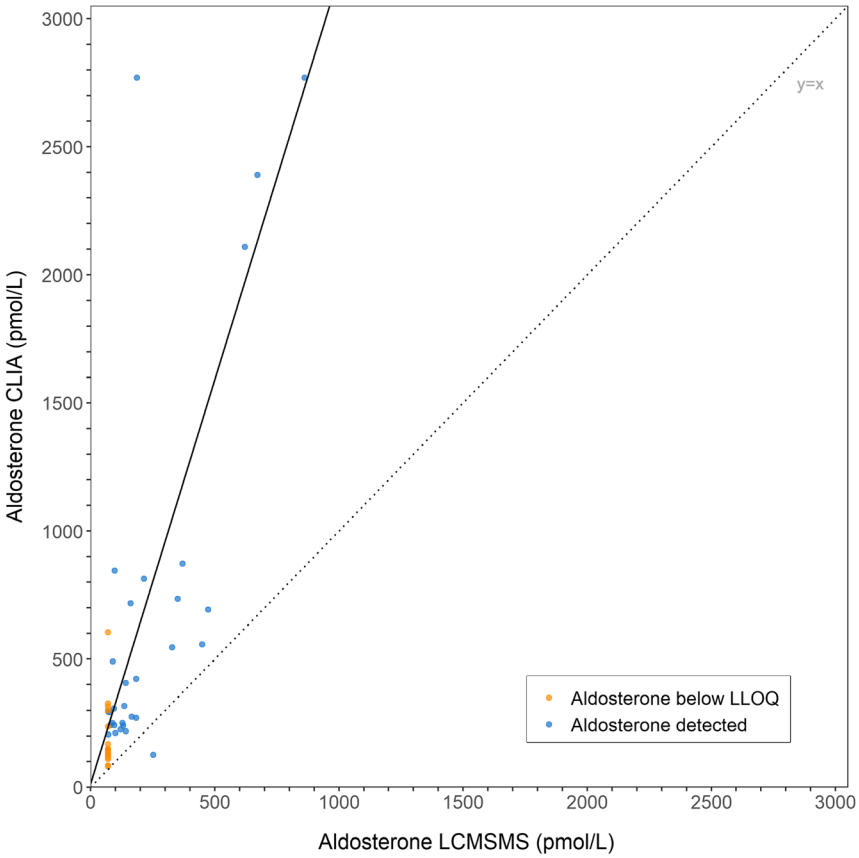

Figure 4
Comparison of aldosterone measured by LCMSMS and CLIA with Passing–Bablok regression line (solid black) shown. The dotted grey line indicates the y = x identity line.

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by CLIA displayed significant proportional positive bias compared to the LCMSMS results: CLIA (pmol/L) = 14.1 + 3.16 × LCMSMS (pmol/L), with intercept 95% CI −34.4 to 54.1 and slope 95% CI 2.09–4.15. The Bland–Altmann plot (Supplementary Fig. 4) further highlights the large mean difference between the methods (319.5 pmol/L), wide limits of agreement, and suggests the differences increase as the average of the LCMSMS and CLIA measurements increases. No clear explanation for the difference between LCMSMS and CLIA results was observed in markers of renal or liver function (Supplementary Fig. 5).

The agreement between methods was considerably improved following solvent extraction of serum, as shown in Supplementary Fig. 6: Extracted CLIA = -14.9 + 1.0 × LCMSMS (intercept 95% CI −31.9 to −4.3 and slope 95% CI 0.9–1.0), with reduced random noise (Pearson $R^2 = 0.97$ cf. 0.60), suggesting interference in the immunoassay by a water-soluble metabolite.

Discussion

Given the role of ACE2 and the RAAS in COVID-19 (6), the finding of low aldosterone concentration is surprising and in conflict with previous studies that have deployed immunoassays to measure aldosterone in COVID-19 patients. Whilst there are case reports of hyporeninemic hypoaldosteronism in COVID-19 (13), these patients do not generally present with clinical features of hypoaldosteronism, suggesting that despite the lack of correlation between serum aldosterone and cortisol concentration, sufficient mineralocorticoid activity is present to avoid decompensation. Other mechanisms to consider include activation of epithelial sodium channels in the distal nephrons by AngII as opposed to aldosterone, increasing sodium reabsorption and volume expansion (at the cost of potassium and hydrogen ion excretion), which may then provide further negative feedback for RAAS, with associated hyporeninemia and hypoaldosteronism while AngII over-expression continues to be facilitated by ACE2 inhibition.

Our data do reproduce the findings of Tan *et al.* (7) that serum cortisol is elevated in COVID-19 patients and is a negative prognostic indicator. In addition to activating the glucocorticoid receptor, cortisol is also an effective ligand for the mineralocorticoid receptor (MR). In normal physiology, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) inactivates cortisol to cortisone thus preventing crosstalk at this receptor. However, when cortisol levels are significantly raised, 11β-HSD2 is saturated and activation of MR ensues. Accordingly, a possible explanation of the counter-intuitive observation of low serum aldosterone concentration without clinically apparent hypoaldosteronism is that the high serum cortisol concentrations found in these patients have sufficient mineralocorticoid activity to suppress RAAS. However, the weak positive correlation between aldosterone and cortisol in these patients does not fully support this finding and a more complex explanation is required. An alternative explanation could be that the polar aldosterone metabolites have activity at the MR as well as cross-reacting in the non-extraction immunoassay. However, previous studies suggest that if aldosterone metabolites do bind to the MR, they do so with much reduced affinity (23). It is also possible that a cross-reacting steroid could generate falsely high cortisol values in the cortisol CLIA assay. A CLIA for cortisol was chosen for this study to consolidate the data of Tan *et al.* (7) and also due to sample volume constraints. An unidentified steroid that could interfere with both the cortisol and aldosterone CLIAs and bind to the MR is a possible unifying hypothesis for these data; this would be a topic for further study.

The high prevalence of undetectably low aldosterone concentration in patients with COVID-19 has not been previously reported, but prior studies have reported results from immunoassay rather than mass spectroscopy (14, 24). The limited agreement in aldosterone results between CLIA and LCSMS is evident. Whilst this poor correlation has been previously established (25, 26, 27), and we have noted a similar degree of discordance in our own laboratory in samples taken from patients without COVID-19 (unpublished data), the effect in COVID-19 patients is exaggerated. Solvent extraction significantly improves the correlation between methods suggesting a water-soluble metabolite may be cross-reacting in the direct immunoassay; this has also been previously demonstrated using extraction immunoassay (15). Aldosterone-18-glucuronide (17), the principal metabolite of aldosterone, is a likely candidate. Increases in this metabolite are unlikely to be a direct effect of COVID-19 infection but may reflect secondary organ dysfunction due to systemic illness. Whilst renal impairment has been shown indirectly to increase aldosterone glucuronide (28), a correlation between eGFR and the discrepancy between the two assays was not demonstrable in this cohort.

To our knowledge, this study is the first to report aldosterone levels in COVID-19 patients as measured by LCMSMS, along with re-measurements using CLIA.

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A weakness of our study is that, whilst it appears that our sample is representative of the broader cohort of hospitalised COVID-19 patients in terms of clinical characteristics at admission, our sample is a convenience sample of patients with suitable available samples in the hospital's biobank, rather than a prospectively collected cohort. This also meant we were unable to measure renin in all patients, due to the more specific sample requirements. The previously described correlation between the aldosterone method discrepancy and eGFR (16) was not demonstrable in this cohort, and marked discordance was also evident in some patients with well-preserved eGFR. This may be due to another mechanism contributing to the excess polar metabolite in this cohort but could also be due to the limited power of the study to detect a renal threshold effect or due to the shortcomings of eGFR as a marker of renal function (29). Nonetheless, we believe our findings to be important and hope they will spur further research on the role of RAAS in COVID-19. For instance, it remains to be seen how aldosterone levels vary during the clinical course of patients with COVID-19, and whether serial measurements could have value as a biomarker.

In conclusion, these data demonstrate that aldosterone cannot be accurately estimated in serum from patients with SARS-CoV-2 infection using direct competitive immunoassay due to the presence of a water-soluble interference. When measured using gold-standard LCMSMS, serum aldosterone is found to be remarkably low in most patients with COVID-19. The mechanism of this reduction remains obscure with no obvious correlation with glucocorticoid status or kidney function.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EC-22-0190.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethics

The study was approved by a UK Health Research Authority ethics committee (20/WM/0125). Patient consent was waived because the de-identified data presented here were collected in line with routine clinical practice at the study hospital; there was no requirement for informed consent.

Data availability statement

The data that support the findings of this study are available from the corresponding authors, but restrictions apply to the availability of these data, which were used under license for the current study and so are not publicly available. Data are, however, available from the authors upon reasonable request, subject to permission being obtained from Cambridge University Hospitals.

Approval of final submission

All authors approved of the final version of this script to be published and are accountable for the work presented.

Author contribution statement

Conception: M G, D J H and J P conceived the research project and designed the study. Data collection: D J H, M W, S L C and R J B G extracted and curated the dataset. D J H and K T provided the laboratory analysis. Analysis tools and data interpretation: M W, R J B G and D J H analysed and contributed to the selection and creation of analysis tools. M G, J P, S L C and D J H interpreted the data and results. Implementation: M W, R J B G and D J H performed the implementation and analysis of the proposed method. Draft writing: D J H, M W, S L C and R J B G wrote the initial draft. All authors contributed to substantively revising the article for important intellectual content.

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