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Circulating ACE2 level and zinc/albumin ratio as potential biomarkers for a precision medicine approach to COVID-19

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ABSTRACT

Highly mutable influenza is successfully countered based on individual susceptibility and similar precision-like medicine approach should be effective against SARS-COV-2. Among predictive markers to bring precision medicine to COVID-19, circulating ACE2 has potential features being upregulated in both severe COVID-19 and predisposing comorbidities. Spike SARS-CoVs were shown to induce ADAM17-mediated shedding of enzymatic active ACE2, thus accounting for its increased activity that has also been suggested to induce positive feedback loops leading to COVID-19-like manifestations. For this reason, pre-existing ACE2 activity and inhibition of ACE2/ADAM17 zinc-metalloproteases through zinc chelating agents have been proposed to predict COVID-19 outcome before infection and to protect from COVID-19, respectively. Since most diagnostic laboratories are not equipped for enzymatic activity determination, other potential predictive markers of disease progression exploitable by diagnostic laboratories were explored.

Concentrations of circulating albumin, zinc, ACE2 protein and its activity were investigated in healthy, diabetic (COVID-19-susceptible) and SARS-CoV-2-negative COVID-19 individuals.

ACE2 both protein levels and activity significantly increased in COVID-19 and diabetic patients. Abnormal high levels of ACE2 characterised a subgroup (16–19%) of diabetics, while COVID-19 patients were characterised by significantly higher zinc/albumin ratios, pointing to a relative increase of albumin-unbound zinc species, such as free zinc ones.

Data on circulating ACE2 levels are in line with the hypothesis that they can drive susceptibility to COVID-19 and elevated zinc/albumin ratios support the therapeutic use of zinc chelating inhibitors of ACE2/ADAM17 zinc-metalloproteases in a targeted therapy for COVID-19.

Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; LCFA, long-chain fatty acid; U, unit; Zn, zinc; Alb, albumin; BCG, Bromocresol green; SD, standard deviation; ROC, receiver operating characteristic; AUC, area under the ROC curve; O.R., odds ratio; CI, confidence interval; HRG, histidine-rich glycoprotein; HDL, high-density lipoprotein cholesterol; PLA-2, phospholipase A2; RTCs, randomised controlled trials; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; MCA, 7-methoxycoumarin-4-acetic acid; 5-Br-PAPS, [(2-5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfo-propylamino) phenol]; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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1. Introduction

The wide-spread mortality and morbidity associated with the COVID-19 pandemic have driven the development of several immunological and pharmacological strategies to counter the pathological effects of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Among them, mass vaccination strategy and some early pharmacological treatments have been shown to exert some protective effects against COVID-19 (Consolaro et al., 2022; Cosentino et al., 2022; Fazio et al., 2021; Perico et al., 2023; Suter et al., 2021; Zamai and Rocchi, 2022).

In order to face viral infections, two main medical approaches have been pursued so far, i.e. mass and "precision-like" medicine. Mass vaccination for poliovirus eradication is an example of "mass medicine" approach, while personalised drug/vaccine treatment based on individual characteristics of susceptibility to influenza infection exemplifies a precision-like medicine approach. When vaccination is able to permanently block viral infection/transmission activating a "sterilizing immunity" (as in the case of polio), mass vaccination is the first choice option because it can stop the repeated health and economic damages caused by recurrent infections. However, herd immunity through mass vaccination can be achieved only in the presence of viruses with low genetic variability. Indeed, we have never tried to reach herd immunity through a mass vaccination strategy against highly mutable RNA viruses such as influenza; rather vaccination based on yearly prevalent virus variant is recommended for protection of a vulnerable minority (people with advanced age, immunodeficiency and multiple comorbidities). On the other hand, most (unvaccinated) influenza patients are treated with nonsteroidal anti-inflammatory drugs (NSAIDs) and/or with other tailored treatments depending on individual drug hypersensitivity and disease severity. This precision-like medicine approach applied to highly mutable influenza virus has been proved effective. Similarly to flu but unlike poliovirus, SARS-CoV-2 is a rapidly mutable RNA virus, suggesting that a precision-like medicine approach may be more effective than a mass medicine one.

Although SARS-CoV-2 is infecting a huge number of people around the world, only a minority of them develops severe symptoms threatening life, mainly aged people with comorbidities (such as cardiopathies, hypertension and diabetes). Instead, a large majority experiences different types of mild symptoms or no symptoms at all, suggesting that severe COVID-19 mainly depends on host predisposition rather than on SARS-COV-2 virulence. Therefore, the discovery of host biomarkers linked to the development of severe forms of COVID-19 is welcome to both predict people's susceptibility and individually guide preventive or symptomatic treatment plans with vaccines or drugs, respectively.

Among other blood markers, systemic ACE2 activity following SARS-CoV-2 infection has been found persistently elevated and correlated with COVID-19 outcome (Fagyas et al., 2022; Nagy et al., 2021; Patel, S.K. et al., 2021; Pucci et al., 2021; Reindl-Schwaighofer et al., 2021; Valle Martins et al., 2021; van Lier et al., 2021). A possible mechanistic explanation for this finding is that SARS-CoV infections have been shown to induce ADAM17-mediated shedding of an enzymatic active ACE2 from surface membrane of cells, such as endothelial cells (Glowacka et al., 2010; Haga et al., 2008; Lambert et al., 2005; Yeung et al., 2021). Notably, the establishment of positive feedback loops stemmed from systemic hyperactivity of pathways downstream of ACE2 (i.e. angiotensin (1–7)/Mas receptor and angiotensin (1–9)/angiotensin type 2 receptor) has been suggested to be consistent with COVID-19 clinical manifestations and enzymatic inhibitors and zinc-chelators interfering with enzymatic activities of ACE2 and/or ADAM17 zinc-metalloproteases have been proposed for COVID-19 treatment (Lartey et al., 2022; Montanari et al., 2021; Yeung et al., 2021; Zamai, 2020, 2021, 2023; Zanza et al., 2021). Since circulating ACE2 activity at hospital admission was able to predict mortality (Fagyas et al., 2022) and comorbidities associated with severe COVID-19 (such as diabetes, cardiopathies, hypertension, dyslipidaemia, male gender and advanced age) were characterised by pre-existing high levels of circulating ACE2 activity (Anguiano et al., 2015; Epelman et al., 2009; Ortiz-Perez et al., 2013; Ramchand et al., 2018; Soro-Paavonen et al., 2012; Uri et al., 2014, 2016; Walters et al., 2017), basal levels of systemic ACE2 activity in uninfected subjects are expected to individually predict COVID-19 outcome in case of SARS-CoV-2 infection. Unfortunately, evaluation of enzymatic activities such as that of ACE2 zinc-metalloprotease is not usually performed in routine diagnostics and the development of alternative markers easily and routinely determinable by diagnostic laboratories are needed for population screening programs. In this regard, elevated levels of circulating ACE2 protein were also associated with COVID-19 risk factors (such as male sex, cardiovascular disease, diabetes, metabolic syndrome and older age) (Kornilov et al., 2020; Narula et al., 2020; Sama et al., 2020; Wallentin et al., 2020) and with worse COVID-19 outcome in hospitalized patients (Kragstrup et al., 2021; Lundstrom et al., 2021; Reindl-Schwaighofer et al., 2021; van Lier et al., 2021), suggesting that pre-existing levels of ACE2 protein could be an alternative marker to predict COVID-19 progression/severity before infection. Indeed, levels of circulating ACE2 protein at hospital admission have been shown to predict COVID-19 outcome (Kragstrup et al., 2021), further suggesting an ACE2-mediated underlying mechanism that links COVID-19 risk factors and severe outcome. However, a correlation between circulating ACE2 protein and ACE2 enzymatic activities was not always found in COVID-19 patients (Reindl-Schwaighofer et al., 2021), suggesting only a partial correspondence between ACE2 protein and its activity. In this regard, ACE2 autoantibodies and/or spike protein "masking" of circulating ACE2 protein may affect its detection during SARS-CoV-2 infection and/or plasma factors inhibiting (an unknown small cationic molecule, Lew et al., 2008) and/or activating (such as free zinc and chloride ions, and SARS-CoV-2 spike binding, Anguiano et al., 2015; Kiseleva et al., 2021; Lu and Sun, 2020; Towler et al., 2004) ACE2 may interfere with its activity independently of its protein amount. Interestingly, concentrations of long-chain fatty acid (LCFA) in plasma can affect free zinc (an ACE2 activator) concentrations because they induce allosteric release of zinc ions from albumin (Coverdale et al., 2019; Lu et al., 2012), the major reservoir/carrier of plasma zinc that is typically present at low concentrations in severe COVID-19 (Herlekar et al., 2021; Huang et al., 2020; Zamai, 2020; Zamai and Rocchi, 2022). However, plasma free zinc can be easily internalised by endothelial cells for their zinc-dependent cellular functions (such as enzymatic activities), while reducing total circulating zinc (mostly represented by albumin-bound fraction). On the other hand, the increase of circulating ACE2 (carrying zinc

ions) in severe COVID-19 (and diabetes) might compensate the reduction of albumin-bound fraction, ultimately shifting circulating zinc species from albumin-bound to albumin-unbound ones. In this regard, ratio between circulating zinc and albumin has been hypothesised to be a surrogate marker of circulating ACE2 and a possible predictive marker of severe forms of COVID-19 (Montanari et al., 2021; Zamai, 2020).

Based on above considerations, we have evaluated circulating ACE2 activity and protein, together with serum albumin and zinc in healthy, COVID-19-susceptible diabetic and SARS-CoV-2-negative COVID-19 individuals. Altogether the results further support both the potential predictive value of ACE2 levels before infection and the therapeutic use of zinc chelating agents for severe COVID-19.

2. Material and methods

2.1. Subjects

Levels of circulating markers were determined in blood samples collected from hospitalized COVID-19 patients (SARS-CoV-2 positivity confirmed by polymerase chain reaction (PCR) test of a nasopharyngeal swab at admission) [$n = 25$, M = 12, F = 13, mean age 74 ± 14 (range 52–92)], diabetic patients [$n = 31$, M = 21, F = 10, mean age 63 ± 13 (range 21–86)], and healthy subjects selected from blood donors [$n = 31$, M = 25, F = 6, mean age 48 ± 10 (range 29–65)]. The collection of peripheral blood samples was done during the period between March 23 and July 28, 2021. Blood was collected in a single batch either in serum separator tubes for the analytical determination of albumin or zinc, or in lithium heparin tubes before being aliquoted into cryovials and stored at −80 °C until assayed for ACE2 protein or activity evaluation (Lew et al., 2008). Since the presence of SARS-CoV-2 might interfere with the correct evaluation of ACE2 protein (Montanari et al., 2021; Zamai, 2020), only SARS-CoV-2-negative subjects were considered among COVID-19 patients [$n = 24$, M = 12, F = 12, mean age 74 ± 14 (range 52–92)]. Blood samples were collected from each COVID-19 patient within one week from negativisation, which was confirmed by PCR analysis. COVID-19 patients (n = 24) were a heterogeneous group of COVID-19 convalescent patients hospitalized at "Ospedale di Comunità" of Fossombrone (PU, Italy) suffering from the following pre-existing pathologic conditions: type 2 diabetes (n = 6, 25%), hypertension (n = 2, 8.3%), cardiovascular disorders (n = 3, 12.5%), chronic obstructive pulmonary disease (n = 3, 12.5%), Alzheimer's disease (n = 1, 4.2%), chronic renal insufficiency (n = 1, 4.2%), glaucoma (n = 1, 4.2%), hypothyroidism (n = 1, 4.2%), bone trauma with acetabulum fracture (n = 1, 4.2%). Three patients (12,5%) died during hospitalisation. Diabetic patients [$n = 3$ (10%) with type I diabetes, $n = 28$ (90%) with type II diabetes, $n = 20$ (65%) treated with insulin, $n = 6$ (19%) with cardiovascular disorders and $n = 9$ (29%) under antihypertensive treatment] were from the Endocrinology and Diabetes Unit at the "Santa Maria della Misericordia" Hospital (Urbino, PU, Italy); healthy subjects were enrolled at the Blood Transfusion Centre from the same Urbino Hospital. All subjects gave their informed consent to participate in the study. The study was carried out in accordance with the Declaration of Helsinki for experiments involving humans after approval by the Internal Review Board of Azienda Sanitaria Unica Regionale - Area Vasta 1 ASUR Marche granted on February 25, 2021 (protocol number n. 25/02/2021).

2.2. ACE2 protein and activity

Plasma ACE2 protein levels were assessed by the ACE2 human ELISA kit from Adipogen Life Sciences (Liestal, Switzerland), following the Manufacturer's instructions. The assay was characterized by a capture antibody (polyclonal) specific for human ACE2 precoated onto the 96-well plate and by a detection biotinylated antibody (monoclonal) which recognized bound ACE2 protein. The limit of detection of the assay was 40 pg/ml. Heparinized plasma aliquots stored at −80 °C were thawed on ice and diluted 1:2 before analysis. Plasma ACE2 activity was evaluated in five μl sample by the fluorimetric ACE2 activity assay kit from Sigma-Aldrich (St. Louis, MO, USA), which utilizes the ability of an active ACE2 to cleave a synthetic 7-methoxycoumarin-4-acetic acid (MCA) based peptide substrate to release a free fluorophore (Anguiano et al., 2015). A standard curve was performed by adding increasing quantities of MCA, and ACE2 specificity of substrate cleavage was checked using the specific ACE2 inhibitor furnished by the Manufacturer (final concentration 100 μM). Fluorescence (ex/em 320/420 nm) was monitored for up to 2 h at room temperature as indicated by the Manufacturer. ACE2 activity was expressed as (U/L), where one unit (U) is the amount of enzyme that catalyses the release of 1 nmol of MCA per minute from the substrate under the assay conditions at room temperature.

2.3. Zinc and albumin

The quantitative determination of zinc (Zn) was carried out with direct colorimetric method using 5-Br-PAPS [(2-5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfo-propylamino) phenol] without deproteinization and the absorbance of zinc-5-Br-PAPS complex was measured at the wavelength of 570 nm (Sentinel Diagnostic kit).

The quantitative determination of albumin (Alb) was carried out with a colorimetric method using Bromocresol green (BCG) and the absorbance of the albumin-BCG complex was measured bichromatically (wavelength 600/800 nm) (Beckman Coulter AU System Albumin method).

2.4. Statistical analysis

Descriptive statistics have been reported as mean (standard deviation, SD) or median (interquartile range, Q1-Q3) as appropriate. Differences between healthy subjects and patient groups were analysed by Mann-Whitney *U* test (Fagyas et al., 2022; Lundstrom et al.,

2021). The ROUT method (with Q set to 1%) was applied to identify the outliers in each group of subjects for each parameter assessed and chi-square test was used to check if outlier number of ACE2 activity and protein levels was statistically different between control and diabetics or between insulin-dependent and insulin-independent diabetics. A receiver operating characteristic (ROC) plot had been obtained by calculating the sensitivity and specificity of every observed data value and by plotting the sensitivity against the false-positive fraction (1 - specificity). ROC curves were used to test the ability to correctly classify controls versus COVID-19s and controls versus diabetics using ACE2 activity and protein levels and Zn/Alb ratio. The area under the ROC curve (AUC) allows an evaluation of the global assessment of the performance of the test. Linear regression analyses, Pearson correlation coefficients and *p* values were used considering ACE2 protein or albumin (independent variables) and ACE2 activity or Zn (dependent variables) for each group (control, diabetics, COVID-19s). Multivariate logistic regression calculates the significance of the association of independent variables (sex, Zn, Alb, Zn/Alb ratio, ACE2 activity, ACE2 levels) with respect to a binary response variable (controls/diabetics and controls/COVID-19s). Nagelkeke R^2 value measures the proportion of the total variation of the dependent variable can be explained by independent variables in the current model. To compare ROC curves obtained from multivariate logistic analysis in their diagnostic accuracy, we compared the respective AUC according to the method of Hanley and McNeil (1983). All elaborations have been performed using Excel, GraphPad Prism 6.0, or SPSS 22.0. Two-tailed p-values <0.05 were considered statistically significant.

3. Results

3.1. Circulating ACE2 protein and activity evaluation

In order to verify previous results, circulating ACE2 protein and its activity were evaluated on frozen plasma samples collected from COVID-19, diabetic and healthy individuals.

ACE2 activity. As expected, ACE2 activity [expressed as median (Q1-Q3) was significantly higher in both diabetic [15.2 (12.0–20.5) U/L, $p < 0.05$] and COVID-19 [17.7 (13.4–24.4) U/L, $p < 0.01$] patients compared to healthy donors [12.7 (9.9–15.3) U/L] (Fig. 1). Instead, differences between COVID-19 and diabetic patients were not significant. Among the healthy controls, none of them were outliers exhibiting an abnormally high ACE2 activity (identified by the ROUT method, Q = 1%, see Fig. 1). Among diabetic patients, five (16,1%) were outliers (identified by the ROUT method, Q = 1%, $\chi^2 = 4.03$; $p = 0.026$, significant difference between diabetics and controls) exhibiting an abnormally high ACE2 activity (above 40 U/L, see Fig. 1), while most of them (83.9%, 26 out of 31) had an ACE2 activity distribution similar to healthy donors. The presence of this subgroup of diabetic outliers suggests a bimodal distribution that introduces an additional variance, which may account for reduced power of subsequent statistical analyses. Differently from diabetics, the heterogeneous group of COVID-19 patients exhibited an increase of ACE2 activity with a unimodal distribution (without outliers, see Fig. 1).

The diagnostic value of plasma ACE2 activity was further tested with a ROC curve for healthy donors vs. COVID-19 or diabetic patients (Fig. 2). The cut-off value donors vs. COVID-19 was 19.10 U/L [AUC (95% confidence interval): 0.752 (0.621–0.884, $p = 0.001$]. The cut-off value donors vs. diabetics was 16.06 U/L [AUC (95% confidence interval): 0.658 (0.523–0.794), $p = 0.032$]. Considering the cut-off of 19.10 U/L, 3 (9,7%) out of 31 donors, 10 (32,3%) out of 31 diabetic patients and 12 (50,0%) out of 24 COVID-19 patients exhibit an ACE2 activity above the cut-off value.

ACE2 protein. Similarly to ACE2 activity, circulating ACE2 protein [expressed as median (Q1-Q3)] was significantly higher in both diabetic [2.35 (1.85–3.63) ng/mL, $p < 0.05$] and COVID-19 [3.56 (1.77–4.23) ng/mL, $p < 0.05$] patients compared to healthy donors [1.89 (1.13–2.89) ng/mL] (Fig. 3). Instead, differences between COVID-19 and diabetic patients were not significant. Among the healthy controls, one (3.2%) was outlier exhibiting an abnormally high ACE2 protein concentration (above 9.6 ng/ml, identified by the

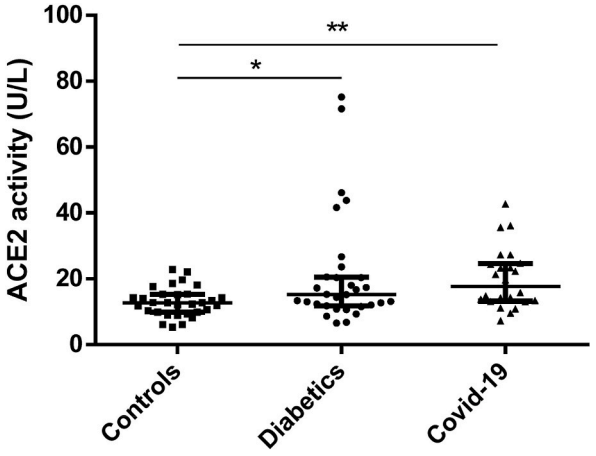

Fig. 1. Circulating ACE2 activity for healthy controls (n = 31), diabetics (n = 31), and COVID-19 patients (n = 24). Bars indicate median and interquartile range. *p < 0.05, **p < 0.01.

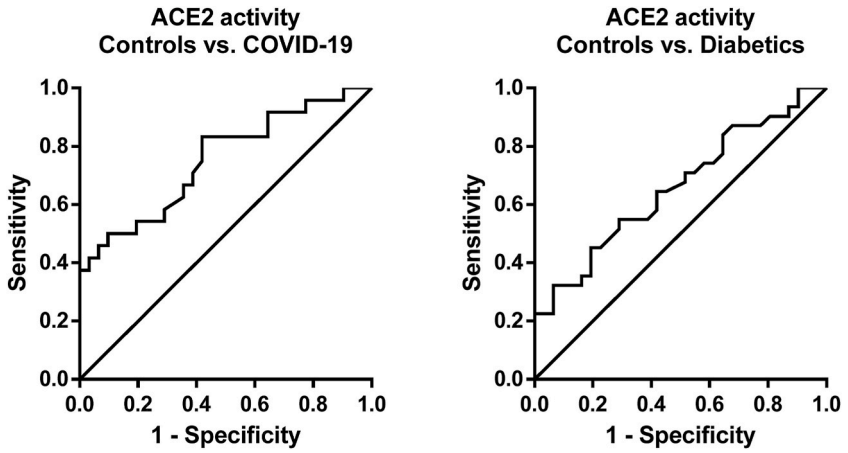

Fig. 2. ROC curve analysis for ACE2 activity for healthy donors vs. COVID-19 (left panel) or diabetic patients (right panel).

ROUT method, Q = 1%, see Fig. 3). Among the diabetic patients, six [19.4%, all of them suffering of type 2 diabetes, five (83%) treated with insulin and only one of them (17%) with cardiovascular disorders or under antihypertensive treatment] were outliers (identified by the ROUT method, Q = 1%, $\chi^2 = 5.43$; p = 0.011, significant difference between controls and diabetics) exhibiting an abnormally high ACE2 protein concentration (above 8.2 ng/ml, see Fig. 3), while most of them (80.6%, 25 out of 31) had a distribution of ACE2 protein concentrations similar to healthy donors, suggesting a bimodal distribution. Interestingly, among the 20 insulin-dependent patients, 5 expressed abnormal high ACE2 levels (25%), while only 1 (9%) out of 11 insulin-independent patients expressed it at high levels ($\chi^2 = 8.72$; p = 0.003, significant difference between insulin-dependent and insulin-independent diabetics). Among these six outliers, there were all the five outliers presenting an abnormally high ACE2 activity, indicating that there was a subgroup of diabetics characterised by very high levels of ACE2. On the other hand, the presence of this subgroup of diabetic outliers introduces an additional variance that may account for lack of significance in subsequent statistical analyses (see later). Differently from diabetics, COVID-19 patients showed an increase of ACE2 levels with a unimodal distribution (without outliers, see Fig. 3).

The diagnostic value of plasma ACE2 protein levels was further tested with a ROC curve for healthy donors vs. COVID-19 or diabetic patients (Fig. 4). The cut-off values were: controls vs. COVID-19 patients, 3.38 ng/mL [AUC (95% confidence interval): 0.672 (0.526–0.819), p = 0.0299] and controls vs. diabetic patients, 2.205 ng/mL [AUC (95% confidence interval): 0.613 (0.471–0.756), p = 0.1250]. Considering the cut-off of 3.38 ng/mL, 5 (16,1%) out of 31 donors, 8 (25,8%) of 31 diabetic patients and 13 (54,2%) of 24 COVID-19 patients possess an ACE2 protein level above the cut-off value.

The linear regression analysis of ACE2 levels vs. ACE2 activity showed a significant positive correlation between the two variables for each group of subjects (Fig. 5), suggesting that in these three groups of subjects (in particular in diabetic and COVID-19 patients), ACE2 level can be used as surrogate marker of ACE2 activity. In searching of other surrogate markers of ACE2 activity, we have further assessed circulating levels of albumin, zinc and their ratio.

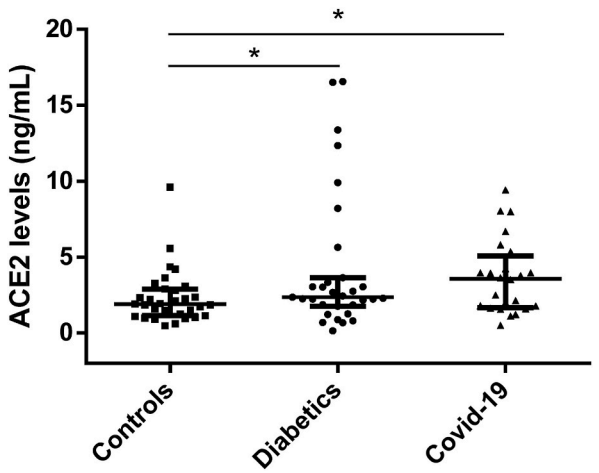

Fig. 3. Circulating ACE2 protein levels for healthy controls (n = 31), diabetics (n = 31), and COVID-19 patients (n = 24). Bars indicate median and interquartile range. *p < 0.05.

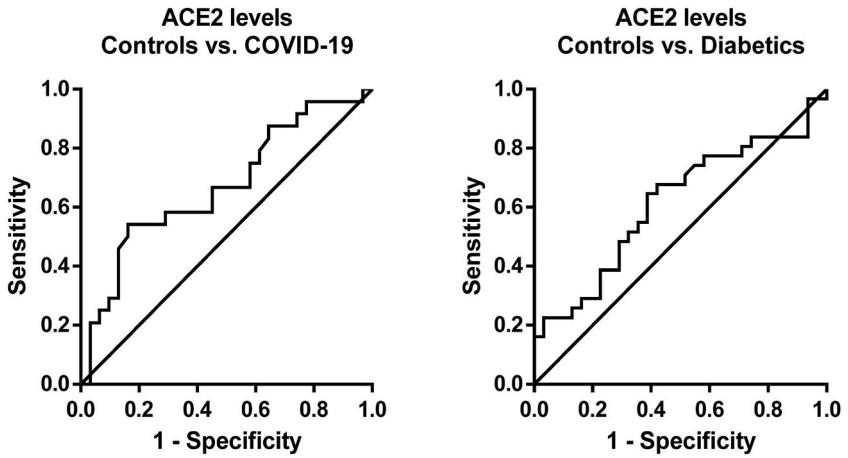

Fig. 4. ROC curve analysis for ACE2 protein levels for healthy donors vs. COVID-19 (left panel) or diabetic patients (right panel).

3.2. Circulating zinc and albumin evaluation

Total zinc concentration [expressed as median (Q1-Q3)] in serum of COVID-19 (SARS-CoV-2-negative) patients remained comparable to those of both healthy donors [82 (72–97) vs. 87 (72–99) μg/dL, p = ns] and diabetic patients [82 (72–97) vs. 70 (58–82) μg/dL, p = ns]. Instead, differences between diabetic patients and healthy donors were significant [70 (58–82) vs. 87 (72–99) μg/dL, p < 0.001] (Fig. 6). Compared to healthy donors, the decrease of serum zinc concentration was significant in (20) patients treated with insulin [70 (56–82) vs. 87 (72–99) μg/dL, p < 0.006] but not in those (11) insulin-independent [74 (58–91) vs. 87 (72–99) μg/dL, p = 0.214].

As expected, albumin levels [expressed as median (Q1-Q3)] were significantly lower in COVID-19 patients compared to both healthy donors [3.2 (2.9–3.3) vs. 4.3 (4.2–4.5) g/dL, p < 0.0001] and diabetic patients [3.2 (2.9–3.3) vs. 4.3 (4.1–4.5) g/dL, p < 0.0001], while they were similar between diabetic patients and healthy donors [4.3 (4.1–4.5) vs. 4.3 (4.2–4.5) g/dL, p = ns] (Fig. 7). Interestingly, the three dead patients had the lowest values of albumin (≤2.4 g/dL, and the oldest age) but not the highest values of ACE2 protein or activity (not shown), indicating that, in our cohort, albumin (but not ACE2) concentration may predict mortality.

To indirectly evaluate the "bioactive" albumin-unbound zinc, Zn/Alb ratio was considered. Interestingly, Zn/Alb ratio [expressed as median (Q1-Q3)] was significantly higher in COVID-19 patients compared to both healthy subjects [28.4 (23.2–30.7) vs. 19.5 (17.1–22.8) μg/gr, p < 0.001] and diabetic patients [28.4 (23.2–30.7) vs. 17.2 (13.8–19.5) μg/gr, p < 0.0001] (Fig. 8). On the other hand, Zn/Alb ratio in diabetic patients was significantly lower compared to healthy subjects [17.2 (13.8–19.5) vs. 19.5 (17.1–22.8) μg/gr, p < 0.05].

Zn/Alb ratio was further tested with a ROC curve for healthy donors vs. COVID-19 or diabetic patients (Fig. 9). The cut-off value donors vs. COVID-19 was 23.85 μg/gr [AUC (95% confidence interval): 0.775 (0.633–0.916), p = 0.0005], confirming a significant increase of Zn/Alb ratio in COVID-19 patients. On the other hand, the cut-off value donors vs. diabetics was 15.70 μg/gr [AUC (95%

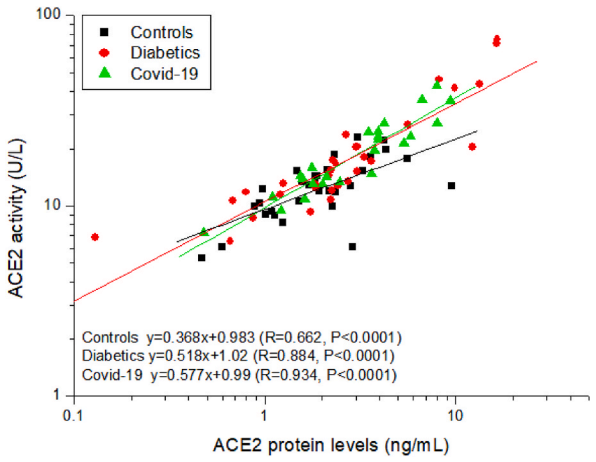

Fig. 5. Linear regression analysis of ACE2 protein levels vs. ACE2 activity for healthy controls (n = 31), diabetics (n = 31), and COVID-19 patients (n = 24). Pearson correlation coefficients and *p* values are reported for each group.

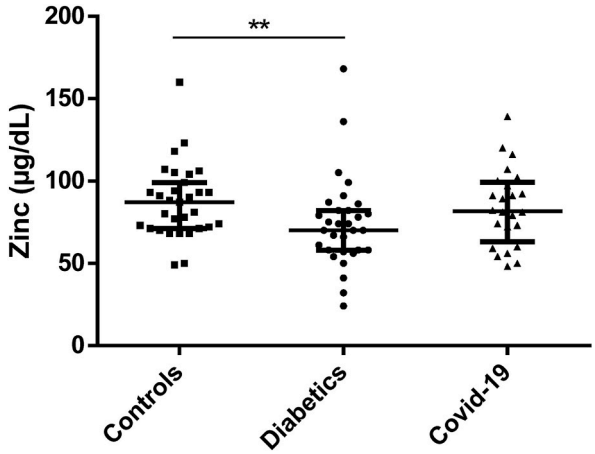

Fig. 6. Circulating total zinc concentration for healthy controls (n = 31), diabetics (n = 31), and COVID-19 patients (n = 24). Bars indicate median and interquartile range. **p < 0.001.

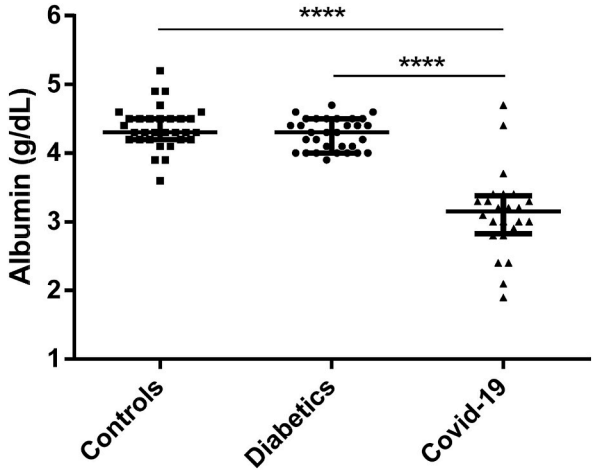

Fig. 7. Albumin levels for healthy controls (n = 31), diabetics (n = 31), and COVID-19 patients (n = 24). Bars indicate median and interquartile range. ****p < 0.0001.

confidence interval): 0.671 (0.535–0.806), p = 0.021], confirming a relative reduction of Zn/Alb ratio in diabetic patients.

Zn/Alb ratio represents a surrogate marker of albumin-unbound zinc species, its increase in circulation of COVID-19 patients suggests a relative shift of zinc species from albumin-bound to albumin-unbound ones, such as that bound to circulating ACE2 zinc-carrying enzyme that has been shown to increase in COVID-19 (**see results above**). On the other hand, diabetes, as well as COVID-19, was characterised by a significant increase of circulating ACE2; therefore, it is possible to hypothesise that the decrease of total zinc concentration in diabetic patients may, at least in part, involve the albumin-bound species of zinc. To verify this hypothesis, we performed linear regression analysis of albumin vs. zinc.

In line with other reports (Craig et al., 1990; Giroux et al., 1976), linear regression analysis of albumin vs. Zn showed a significant positive correlation (r = 0.466; p = 0.008, regression line slope = 33.1, intercept = −57.5) between circulating albumin and zinc in healthy subjects (Fig. 10); however, this was not true in diabetic (r = 0.145; regression line slope = 17.7, intercept = −1.8) and COVID-19 (r = −0.115; regression line slope = −4.4, intercept = 97.5) patients, suggesting that zinc species distribution differ between diseased and healthy groups. In particular, correlation lines in diabetic and COVID-19 patients showed a progressive loss of positive correlation between albumin and zinc, suggesting a swift of zinc from albumin-bound to albumin-unbound species, particularly in COVID-19 patients. Indeed, although COVID-19 subjects had significantly lower albumin levels than the other two groups, zinc levels were similar to healthy subjects and correlation line of albumin vs zinc was even negative.

Since both COVID-19 and diabetes were characterised by increased ACE2 levels but only COVID-19 had a significant increase Zn/Alb ratio, this parameter cannot be used as a surrogate marker of circulating ACE2; nevertheless, this finding may provide a rationale for the use of zinc binding agents in the treatment of COVID-19.

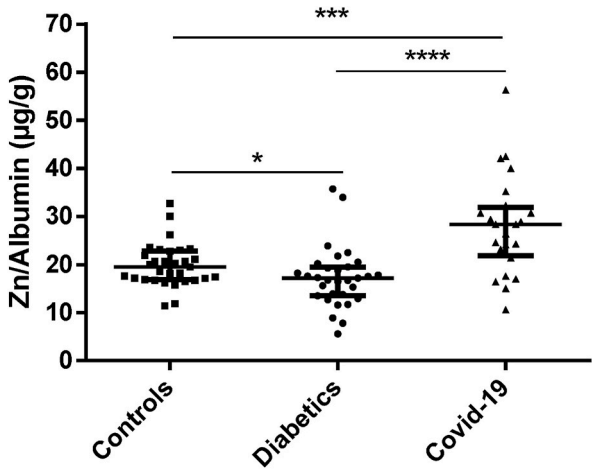

Fig. 8. Zinc/Albumin ratio for healthy controls (n = 31), diabetics (n = 31), and COVID-19 patients (n = 24). Bars indicate median and interquartile range. *p < 0.05, ***p < 0.001, ****p < 0.0001.

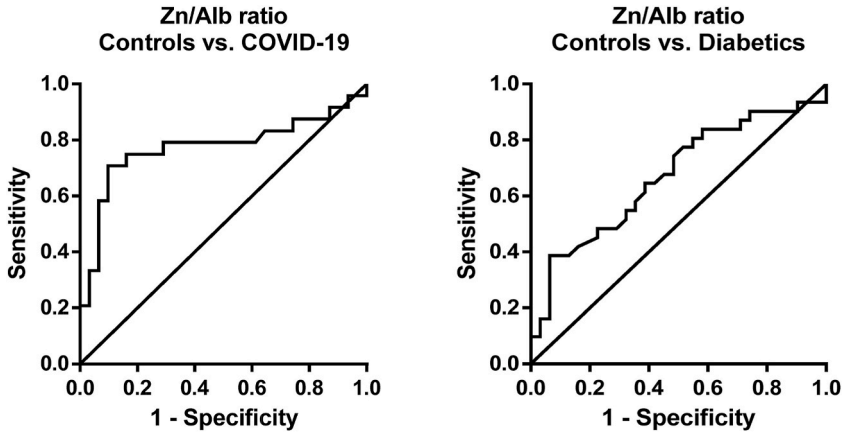

Fig. 9. ROC curve analysis for Zn/Albumin ratio for healthy donors vs. COVID-19 (left panel) or diabetic patients (right panel).

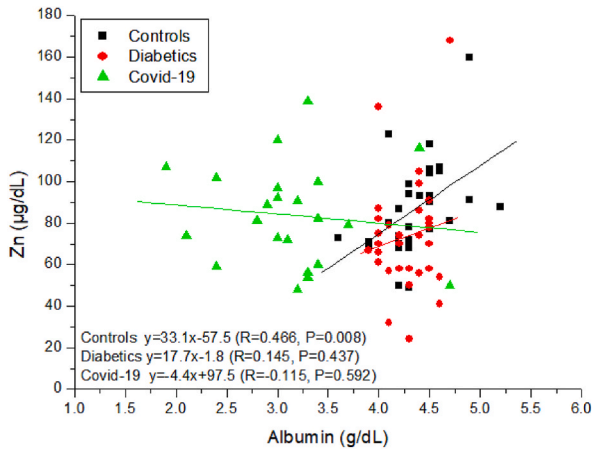

Fig. 10. Linear regression analysis of albumin vs. zinc (Zn) levels for healthy controls (n = 31), diabetics (n = 31), and COVID-19 patients (n = 24). Pearson correlation coefficients and *p* values are reported for each group.

3.3. Multivariate logistic analysis

Multivariate logistic analysis was performed in order to determine the independent variables (among those assessed) associated with the presence of COVID-19 or diabetes (dependent variables). Logistic regression was used to analyse the relationship between sex, zinc, albumin, zinc/albumin ratio, ACE2 activity, ACE2 protein and control/diabetic condition (binary dependent variable). Pseudo R^2 Nagelkeke value was equal to 0.244, showing a fair goodness of fitting. With forward stepwise elimination process, it was found that ACE2 activity (O.R. = 1.109; 95% CI = 1.002–1.227) and zinc (O.R. = 0.978; 95% CI = 0.955–1.001) were significantly associated with the dependent variable (Table 1). ROC curve AUC was 0.737. Using the same model, but control/COVID-19 condition as a dependent variable, pseudo R^2 Nagelkeke value was equal to 0.819, showing a good goodness of fitting. With forward stepwise elimination process, it was found that Zn/Alb (O.R. = 2.575; 95% CI = 1.481–4.478) and Zn (O.R. = 0.793; 95% CI = 0.697–0.902) were significantly associated with the dependent variable (Table 1). ROC curve AUC was 0.951.

Comparison between AUC ROC curve values (Fig. 11) showed that overall accuracy of logistic regression with controls/COVID19s as a dependent variable was significantly higher than the AUC value related to control/diabetic logistic analysis ($p < 0.01$).

4. Discussion

The advent of precision medicine has changed the approach to disease/infection treatment and prevention. Precision medicine uses predictive markers/characteristics in order to select only patients that will benefit from the therapy avoiding ineffective treatments and reducing side effects, which also relieve healthcare costs. Among the predictive markers, those predicting outcomes during disease/infection can drive treatments to reduce hospitalisation and mortality, while those able to predict disease severity before disease/infection (predisposition to susceptibility) allow to identify the most vulnerable individuals and "surgically" direct preventive measures to them, which are the main responsible for pressure on healthcare systems. Indeed, precision-medicine approach maintains both protection of preventive measures on the population and relief of pressure on health services as well as mass medicine approach, while reducing health costs.

Preventive strategies based on individual markers/characteristics (such as age, immunological status and comorbidities) have long been applied with success to influenza, a highly mutable RNA virus. Nevertheless, a mass vaccination/medicine approach has been preferred in preventive strategies against SARS-CoV-2, another highly mutable RNA virus.

Identification of biomarkers that can be easily exploitable for prediction of COVID-19 outcome before infection would be crucial to unveil the most vulnerable people (particularly those rare present among young healthy individuals), the ones that will surely benefit preventive treatments/measures (e.g. vaccination, physical distancing and/or face masks). The possibility of population screening programs using such predictive biomarkers can narrow not only preventive and/or therapeutic interventions (and relative costs) to people for which risk/benefit balance is more favourable, but also their potential adverse effects (treatment-associated morbidity) on healthy people (and relative costs).

Among the individuals challenged by the same etiological SARS-CoV-2 agent, only a minority of susceptible individuals experiences severe COVID-19 and is in need of disease prevention and hospital treatment, suggesting that disease severity mainly depends on host predisposing characteristics/factors rather than on SARS-COV-2 virulence. Therefore, host individual (genetic, epigenetic and/or environmental) factors are expected to play a critical role in driving and consequently predicting abnormal responses to SARS-CoV-2 infection leading to severe COVID-19.

In search for predictive factors/markers of individual risk and specific therapeutic targets for severe COVID-19 that may be used to personalize preventive and/or therapeutic strategies, we have focused our investigation on four circulating markers in three different groups of individuals. Briefly, values and correlations among ACE2 protein and activity, albumin and zinc were evaluated in healthy donors, COVID-19-susceptible diabetics and SARS-CoV-2-negative COVID-19 patients. Among potential predictive markers of COVID-19 outcome, circulating ACE2 zinc-metalloprotease deserves particular attention because it correlates with both COVID-19 severity and comorbidities associated with a higher risk of severe illness and death (Anguiano et al., 2015; Epelman et al., 2009; Fagyas et al., 2022; Nagy et al., 2021; Ortiz-Perez et al., 2013; Patel, S.K. et al., 2021; Pucci et al., 2021; Ramchand et al., 2018; Reindl-Schwaighofer et al., 2021; Soro-Paavonen et al., 2012; Uri et al., 2016; Uri et al., 2014; Valle Martins et al., 2021; van Lier et al., 2021; Walters et al., 2017).

Notably, the initial binding event to ACE2-expressing cells, such as those of blood vessel endothelium, is shared by different

Table 1
Logistic regression analysis for controls vs. diabetics and controls vs. COVID-19s.

Variables	P(χ^2)	O.R. (95% C.I.)
Controls versus diabetics		
Intercept	0.997	
Zn (μg/dL)	0.065	0.978 (0.955–1.001)
ACE2 activity	0.046	1.109 (1.002–1.227)
Controls versus COVID-19s		
Intercept	<0.0001	
Zn (μg/dL)	<0.0001	0.793 (0.697–0.902)
Zn/Alb ($*10^{-6}$)	0.001	2.575 (1.481–4.478)

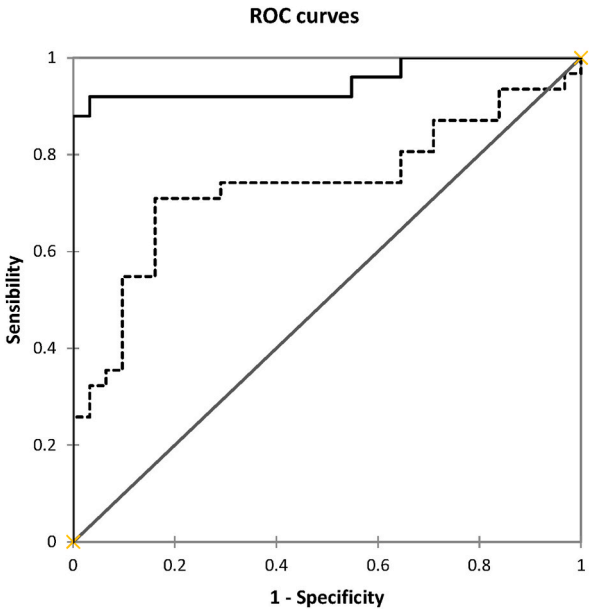

Fig. 11. ROC curves in controls vs. diabetics (dashed line, AUC = 0.737) and controls vs. COVID-19s (continuous line, AUC = 0.951).

coronaviruses, i.e. SARS-CoVs and HCoV-NL63. However, both ADAM17-mediated cell surface shedding of ACE2 zinc-metalloprotease and severe acute respiratory syndrome are exclusive features of SARS-CoVs (Glowacka et al., 2010; Haga et al., 2008; Lambert et al., 2005; Yeung et al., 2021), suggesting that SARS-CoV (but not HCoV-NL63) spikes may drive ADAM17 close to ACE2 membrane-bound protein for its systemic release that may subsequently lead to severe respiratory symptoms (Montanari et al., 2021). In this regard, SARS-CoV spikes has been shown not only to induce ACE2 shedding but also to directly activate ACE2 enzymatic functions (Kiseleva et al., 2021; Lu and Sun, 2020), thus accounting for increased levels of circulating ACE2 enzymatic activities in COVID-19 that may be responsible for severe disease. Indeed, ACE2 is not merely the primary binding site for SARS-CoV-2 infection but also a functional enzyme that can be released from endothelial cell surface in circulation, ultimately producing angiotensin (1–7) and angiotensin (1–9) from angiotensin I and II, respectively. These angiotensin peptides can mediate several systemic effects through the Mas and angiotensin type 2 receptors, respectively. Since every biological parameter has a normal range value between a maximum and a minimum, it is expected that ACE2 pathway hyperactivity could lead to unhealthy consequences. In this regard, some experimental models in the context of excess of angiotensin (1–7) and/or angiotensin (1–9) signalling via the Mas and angiotensin type 2 receptors have also been associated to pathological conditions involving heart, liver, intestine, lung and coagulation and these studies were already extensively described in previous works (for details see Montanari et al., 2021; Zamai, 2020, 2021). Despite ACE2 surface shedding is expected to protect endothelium from virus infection, SARS-CoV spike proteins alone (in the absence of viral infection) has been shown to elicit endothelial both down-modulation of ACE2 surface expression and cell damage in animal models of COVID-19 (Lei et al., 2021; Nuovo et al., 2021). This paradoxical event has suggested that renin-angiotensin system dysregulation induced by circulating SARS-CoV spike proteins may induce COVID-19-like manifestations independently of viral infection (Lei et al., 2021) and enzymatic inhibition of ACE2 has been proposed as a therapeutic approach for COVID-19 treatment (Montanari et al., 2021; Zamai, 2020, 2021, 2023). In this regard, it has been shown that excessive upregulation of pathways downstream ACE2 (i.e. angiotensin (1–7)/Mas receptor and angiotensin (1–9)/angiotensin type 2 receptor) may induce several positive feedback-loops leading to viral independent COVID-19-like manifestations (Montanari et al., 2021; Zamai, 2020, 2021, 2023). Indeed, in our small cohort, the three patients that subsequently died were SARS-CoV-2-negative, indicating that after the initial trigger, SARS-CoV-2 persistence is dispensable to develop severe COVID-19. Among other effects downstream of ACE2 pathway, IL-10, an immuno-suppressive cytokine that may drive COVID-19-associated lymphopaenia and eosinopaenia, is early and strongly upregulated during severe SARS-CoV-2 infection (Montanari et al., 2021; Zamai, 2020, 2021), suggesting that circulating ACE2 may predispose to SARS-COV-2 infection. Moreover, soluble ACE2 protein has been shown to not only bind to integrins (Clarke et al., 2012) but also act as a "bridge" for SARS-CoV-2 endocytosis and infection (independently of ACE2 membrane-bound protein) via angiotensin type 1 and/or vasopressin receptors (Guo et al., 2022; Yeung et al., 2021, 2022), indicating that circulating ACE2 may play a pathophysiological role promoting SARS-CoV-2 infection independently of its systemic enzymatic activity. In line with these findings, a clinical trial with intravenously APN01 (a recombinant ACE2 dimer) that was designed to block SARS-CoV-2 cellular entry, was stopped early because of higher both mortality rates and viral loads than controls (ClinicalTrials.gov, 2021), confirming that systemic ACE2 dimer administration can (directly and/or indirectly) affect SARS-CoV-2 infectivity.

Based on above observations, we have hypothesised that basal levels of circulating ACE2 activity could be not only a mere marker of disease outcome prediction but also a predisposing factor of disease severity. In order to test this hypothesis, we have exploited diabetic patients that are known to both express elevated (basal) levels of ACE2 (Anguiano et al., 2015; Kornilov et al., 2020; Narula

et al., 2020; Sama et al., 2020; Soro-Paavonen et al., 2012; Wallentin et al., 2020) and have an increased risk for severe COVID-19. Indeed, although the prevalence of diabetes in SARS-CoV-2 infected patients was similar to the general population (i.e. similar predisposition to infection), it significantly rose in patients with a severe disease course (Abdelhafiz et al., 2021; Du et al., 2020; Landstra and de Koning, 2021; McGurnaghan et al., 2021; Saha et al., 2021), indicating that diabetics have a higher number of subjects predisposed to develop severe COVID-19 than general population. In line with previous works, we have found that ACE2 levels and activity were significantly higher in the diabetic group than in healthy controls. However, increase of ACE2 levels were due to a subgroup (16–19%) of diabetic patients expressing very high levels of ACE2, while most (81–84%) of them had levels similar to healthy donors. That means that, for some (unknown) individual (genetic, epigenetic, pathological and/or environmental) factors, a few individuals can express abnormal levels of circulating ACE2 and COVID-19-susceptible diabetics have a higher number of these individuals than healthy donors. Interestingly, there was a significant higher percentage of diabetics expressing abnormal high ACE2 levels in the insulin-dependent subgroup (25%) than in the insulin-independent one (9%). In this respect, type 1 diabetics that are typically insulin-dependent have been shown to have both a significant higher activity of circulating ACE2 (Soro-Paavonen et al., 2012) and a higher risk for adverse COVID-19-related outcomes than type 2 diabetics (Landstra and de Koning, 2021; McGurnaghan et al., 2021). In line with this, insulin treatment, as compared with other diabetes drugs, was associated with the highest risk of fatal or critical care unit-treated COVID-19 (McGurnaghan et al., 2021, see appendix p 21), indicating that insulin dependence may predispose to increase both ACE2 levels and COVID-19 severity. This observation supports our hypothesis that high basal levels of ACE2 may both predispose to severe COVID-19 and act as a useful marker to predict individual susceptibility to COVID-19 before infection. In this respect, within our small cohort of patients, only five patients contract symptomatic SARS-CoV-2 infection, all with a mild course of COVID-19. Among them, two expressed high levels of ACE2, but had the booster vaccine about three months before infection accounting for a probable vaccine protection; while among those expressing normal ACE2 levels, two had the booster vaccine about eight months before infection and one contracted SARS-CoV-2 infection before vaccination. Based on these observations, no confirmation or disconfirmation of our hypothesis could be drawn and further studies are needed and welcome to validate (or not) it.

Differently from ACE2 levels, normal albumin concentrations ($\geq$3.9 g/dL) in all diabetic patients indicate that albumin cannot be a predictive marker of COVID-19 severity before infection. However, in line with other reports (Herlekar et al., 2021; Huang et al., 2020), albumin levels in our hospitalized COVID-19 patients were significantly low (usually <3.4 g/dL) and negatively correlated with mortality, confirming that albumin concentration plays a predictive and possibly a protective role during SARS-CoV-2 infection. In this respect, albumin has been shown both to buffer free Zn^{2+} concentration and, curbing its biological effects, to protect cell lines from free zinc cytotoxicity in vitro (Haase et al., 2015). While in vivo, it would act as a zinc chelator that, maintaining a safe concentration of bioactive free Zn^{2+}, protects endothelial cells and modulates endothelial zinc uptake and tissue distribution (Coverdale et al., 2019; Stewart et al., 2009). Among other biological effects, Zn^{2+} ions can stimulate histidine-rich glycoprotein (HRG)-complex formation, a plasma adaptor protein able to neutralize heparins (Kassaar et al., 2015; Stewart et al., 2009). Interestingly, Zn^{2+}-dependent activation of HRG has been clinically linked to thrombotic disorders in individuals characterised by elevated levels of plasma fatty acids, such as diabetics (Kassaar et al., 2015; Stewart et al., 2009). In this respect, elevation of plasma fatty acid levels at physiological relevant ranges has been shown to induce allosteric release of zinc ions from albumin to HRG (Kassaar et al., 2015; Stewart et al., 2009). Indeed, albumin is involved in transport not only of zinc, but also, through lipoprotein formation, of fatty acids, which compete with zinc binding to albumin (Coverdale et al., 2019; Kassaar et al., 2015; Lu et al., 2012; Stewart et al., 2009); therefore, an increase of LCFAs and/or a decrease of plasma albumin can lead not only to decrease of high-density lipoprotein cholesterol (HDL) but also to increase of free Zn^{2+}, an ACE2 activator. However, activation of pathways downstream of ACE2 (i.e. Mas or angiotensin type 2 receptors) has been shown to induce phospholipase A2 activity (PLA-2) and arachidonic acid release (Zamai, 2023), which, in turn, may lead to further increase of circulating LCFAs and HDL decrement, features observed in severe COVID-19 (Nguyen et al., 2021; Thomas et al., 2020). Altogether these observations may provide explanation for the intriguing negative correlations of low HDL levels with circulating ACE2 activity (Rice et al., 2006) or with probability to develop thrombotic complications in diabetics (Sobczak et al., 2019) or severe COVID-19 (Masana et al., 2021). Indeed, both COVID-19 and diabetic patients are characterised by significant increase of LCFAs and/or decrease of HDL levels (Femlak et al., 2017; Krauss, 2004; Liu et al., 2010; Masana et al., 2021; Nguyen et al., 2021; Perez-Torres et al., 2021; Sobczak et al., 2019; Thomas et al., 2020; Wang et al., 2020).

It is known that the albumin-bound fraction of zinc is the major reservoir of plasma zinc (about 75–90%) followed by α2-macroglobulin-bound one (about 10–20%), while only nanomolar concentrations of free Zn^{2+} (less than 2%) are usually present in plasma under normal conditions (Alker et al., 2019; Coverdale et al., 2019; Craig et al., 1990; Giroux et al., 1976). In healthy subjects, variations in total serum zinc concentrations has been shown to mainly depend on albumin-bound zinc and therefore on zinc-binding capacity of albumin, knowing that only about 2% of the albumin is engaged in zinc transport; differently, the concentrations of α2-macroglobulin-bound zinc are substantially constant (Foote and Delves, 1984). However, in diabetes, levels of circulating α2-macroglobulin were found increased compared to matched controls (James et al., 1980). Therefore, the significant decrease of serum zinc concentration, specifically in those treated with insulin (Jansen et al., 2012), may originate from a reduced albumin capacity of zinc storage (possibly consequent to elevated levels of plasma LCFAs) combined with a decrease of renal Zn^{2+} re-absorption (and urinary zinc loss) due to hyperglycaemia-induced diuresis (Coverdale et al., 2019). However, increased Zn^{2+} uptake and/or reduced secretion of inactive insulin-zinc hexameric complex by dysfunctional pancreatic β-cells may also participate in low levels of serum zinc. In this regard, while intracellular Zn^{2+} is necessary to synthesise and stabilise the inactive hexameric form of insulin, free Zn^{2+} buffering by plasma albumin ultimately promotes dissociation of insulin complex into active monomeric form of insulin in circulation (Coverdale et al., 2019). This complex interplay among Zn^{2+}, albumin and insulin may suggest a rational for the diabetogenic effect of COVID-19 (Landstra and de Koning, 2021). Indeed, SARS-COV-2-induced increase of free zinc levels (consequent to hypoalbuminaemia and/or LCFA increase) may take an active role in the inactivation of insulin, thus explaining why severe COVID-19

can have a negative impact on diabetes. Resembling insulin production by pancreatic β-cells, in vitro synthesis of APN01, a dimeric complex of human soluble ACE2, requires high concentrations of Zn^{2+} to stabilizes the structure (Manfred Schuster et al., 2013); however, differently from insulin, the ACE2 complex exhibits improved solubility, bioavailability, and activity compared to the monomeric form, highlighting the crucial role of Zn^{2+} in modulating not only the enzymatic activity but also the synthesis of long-lived soluble forms of ACE2. Indeed, endothelial cells need to uptake free Zn^{2+} from plasma in order to synthesise functional ACE2 enzymes, which are then released from endothelial cell surface in circulation of COVID-19 patients. It is tempting to speculate that low concentrations free Zn^{2+} in plasma, promoting the generation of active insulin monomers, might be functional in insulin-dependent diabetics; however, in some predisposed patients, consequent reduction of ACE2 activity might be compensated by a systemic upregulation of ACE2 levels. Although we did not find correlation between ACE2 increase and total zinc (or Zn/Alb ratio) decrease, multivariate logistic analysis of controls vs. diabetes indicates that decrease of circulating total zinc and increase of ACE2 activity (but not ACE2 protein) were the only independent variables associated with diabetes. It is known that endogenous inhibitors and/or activators, such as chloride ions, may participate in modulating ACE2 enzymatic function in diabetics; however, in our cohort, ACE2 activity strongly correlated with ACE2 protein levels, suggesting that slight differences in variance between ACE2 levels and ACE2 activity may have determined this result. Differently from the "chronic" disease of diabetes, multivariate logistic analysis indicated that "acute" COVID-19 disease was associated with increase of Zn/Alb ratio (a surrogate marker of albumin-unbound fraction of zinc) and decrement of total serum zinc (mainly represented by the albumin-bound fraction), but not with ACE2 activity. Since patients with severe COVID-19 have not only increase of LCFAs (inducing release of free Zn^{2+} from albumin) but also hypoalbuminaemia (Herlekar et al., 2021; Huang et al., 2020; Zamai, 2020; Zamai and Rocchi, 2022), circulating bioactive and toxic Zn^{2+} is expected to temporarily increase in COVID-19. Indeed, the results indicate a shift of circulating zinc species from albumin-bound to albumin-unbound ones. However, differently from other reports (Heller et al., 2021; Maares et al., 2022), we detected only a slight (not significant) decrease of total zinc in COVID-19 patients compared with healthy controls. The reason of this discrepancy may lie in our cohort that was exclusively of SARS-CoV-2-negative patients i.e. in a late phase of COVID-19. Indeed, serum zinc levels of COVID-19 patients have been found significantly low, a feature that characterises COVID-19-susceptible diabetics, at hospital admission (particularly in non-survivors); however, they increased over time during hospitalisation, particularly in sera of non-survivor patients (Heller et al., 2021; Maares et al., 2022), thus possibly accounting for the data obtained from our cohort. Knowing that lowest albumin levels correlated with mortality (Herlekar et al., 2021; Huang et al., 2020), it follows that Zn/Alb ratio should progressively increase during COVID-19 progression to the critical stage. Similarly to severe COVID-19, zinc fume inhalation has been shown to cause both increased levels of serum zinc and acute respiratory distress syndrome with lung radiographic and angiographic pictures that resemble those of COVID-19 (Hjortso et al., 1988; Xie et al., 2017), indicating that excessive circulating zinc independently of SARS-CoV-2 infection may induce activation of systemic zinc-dependent pathways leading to COVID-19-like manifestations (Zamai, 2023). Serum (or plasma) zinc concentration is usually used to assess zinc status of an individual; however, it both represents only a small fraction (about 0.1%) of zinc in body and widely depends on zinc transport proteins and other factors such as LCFAs (Coverdale et al., 2019; Jansen et al., 2012; Kassaar et al., 2015; Lu et al., 2012; Stewart et al., 2009). As an example, many inflammatory diseases are characterised by rapid hypoalbuminaemia that is induced by an increased capillary permeability leading not only to albumin (whose half-life is about 20 days) but also to zinc escape from microvessels to the interstitial space; therefore, serum zinc concentration may not always be a reliable biomarker of cell/body zinc content. Nevertheless, because a (hypothetical) zinc deficiency determined by serum zinc concentration (<70 μg/dL) is frequent in patients with chronic disease comorbidities that predispose to severe forms of COVID-19, several researchers claim that zinc supplementation may work against COVID-19. Despite the hypothesised benefits of zinc in the literature and initial encouraging results from observational studies, randomised controlled trials (RCTs) and/or studies that monitored patients for factors and comorbidities associated with increased risk for severe COVID-19 were inconclusive. Indeed, among papers describing RCTs, most fail to show a treatment effect and/or to reach their target sample size although they started recruitment in April/June 2020, more than two years ago (Abd-Elsalam et al., 2021; Patel, O. et al., 2021; Thomas et al., 2021). The only RCT (ClinicalTrials.gov, 2022) indicating that oral zinc supplementation improves the outcome of COVID-19 was conducted during the Omicron variant wave (from 15 February 2022 to 4 May 2022) in Tunisia (Ben Abdallah et al., 2023). However, it showed a surprisingly high (9.2%) mortality at 30 days in the placebo group (n = 239; age, mean ± SD, 53.7 ± 17.2), knowing that about 40% were non-hospitalized patients and those with severe comorbid conditions were excluded; moreover, percentages of patients with comorbidities associated with severe COVID-19 outcomes were markedly higher in placebo than in zinc group, in particular coronary heart disease (3.8 vs. 2.0) and diabetes (22.6% vs 16%) (Ben Abdallah et al., 2023), raising concerns on the reliability of results. Oral zinc absorption is known to be very low (Patel, O. et al., 2021) and the trial also failed to establish a cause-effect relationship showing that oral zinc supplementation effectively increased serum zinc concentration. In this regard, one RCT based on administration of high-dose intravenous zinc (Australia New Zealand Clinical Trial Register Registration no. ACTRN12620000454976) showed a significant increase of serum zinc levels, but it was unable to prove its benefit for COVID-19 patients. Although the trail started on April 2020, it surprisingly did not reach its target enrolment (Patel, O. et al., 2021), suggesting that zinc supplementation may not actually protect against severe COVID-19.

On the other hand, the negative slope of the regression line of albumin vs. zinc obtained from our cohort of SARS-CoV-2-negative COVID-19 patients further suggests a shift of plasma zinc species from albumin-bound to albumin-unbound ones. Since levels of circulating α2-macroglobulin (with its zinc) have been shown to decrease in COVID-19 patients as compared to matched controls (Medjeral-Thomas et al., 2021), it is likely that in our cohort of COVID-19 patients there was an increased concentration not only of ACE2-bound zinc but also of free (bioactive) Zn^{2+}. In this regard, the concentrations of circulating ACE2 that can carry two zinc ions per monomer (Manfred Schuster et al., 2013) are less than 0.2 nM even when they are elevated, indicating that the increase of ACE2-bound zinc is indeed negligible when compared to albumin-bound one (normal values range between 8.5 and 14 μM). A shift of

zinc species with both a transient increase of free Zn^{2+} (and Zn/Alb ratio) and a transient decrease of albumin (and zinc-binding capacity of plasma proteins) had been described in a rat model of acute haemorrhagic shock and reinfusion (Kelly et al., 2012). This experimental condition causing a brief hypotension and systemic hypoperfusion (hypoxia), may resemble that more stably induced by SARS-CoV-2 infection (Montanari et al., 2021; Zamai, 2020, 2021), suggesting that free zinc concentrations may actually increase in COVID-19. Nevertheless, free zinc concentrations evaluated by a fluorometric assay were reported significantly lower in COVID-19 patients (Maares et al., 2022). The data generated with this assay were obtained by a calculation (dilution independent) based on the assumption that albumin is always sufficient for providing binding sites for zinc and the equilibrium is strongly in favour of complex formation; as a consequence of this, the fraction of free zinc is considered negligible compared to its albumin-bound form, which therefore corresponds the entire total zinc amount (Alker et al., 2019). Consequently, concentration of free zinc in the serum sample dependents only on binding constant of albumin for zinc and on the ratio between total zinc and total serum albumin; an approximation that allows not to take into account dilution of the serum samples when calculating free zinc concentrations (Alker et al., 2019). Notably, in case of low albumin levels and increased LCFAs (that reduce the albumin binding sites for zinc), as occurs in patients with severe COVID-19, the assumptions may no longer be valid because free zinc may not be negligible and/or zinc bound to albumin might be insufficient to maintain free zinc concentration constant after sample (1:50) dilution. There is, therefore, the possibility that free zinc concentrations in samples from severe COVID-19 might be underestimated when evaluated with this assay.

Differently from severe COVID-19, SARS-CoV-2 mRNA vaccination transiently induces both mild symptoms and expression of SARS-CoV-2 spike proteins available not only for immune recognition but also for ACE2 binding; consequently, a transient induction of systemic ACE2 shedding/activity and mild COVID-19-like manifestations are expected (Montanari et al., 2021). In this respect, animal models of COVID-19 using SARS-CoV-2 spike protein in the absence of viral infection has been shown to induce endothelial down-modulation of ACE2 surface expression and cell or lung damage (Lartey et al., 2022; Lei et al., 2021; Nuovo et al., 2021). Moreover, inhibition of ADAM17 responsible for ACE2 shedding has been shown to protect from COVID-19-like lung damage (Lartey et al., 2022), further suggesting that SARS-CoV spike proteins, such as those produced by SARS-CoV-2 vaccination, may induce mild COVID-19-like manifestations independently of viral infection (Montanari et al., 2021). Notably, SARS-CoV-2 mRNA vaccine has been shown to induce a persistent and significant increase of free (but not total) Zn^{2+} (Chillon et al., 2022), indicating that SARS-CoV-2 spike proteins (rather than SARS-CoV infection) may induce plasma free zinc rise, possibly through upregulation of ACE2/PLA-2 pathway (Zamai, 2023). In this regard, it has been recently proposed that most drugs (either NSAIDs or antibiotics) that have been shown to exert some protective effects against COVID-19 possess direct and/or indirect zinc chelating abilities (Zamai, 2023). Indeed, drugs interfering with zinc-dependent functions such as ethylenediaminetetraacetic acid (EDTA) and citrate are expected to protect from COVID-19 at different levels thanks to their both inhibitory activities on ACE2 and/or ADAM17 zinc-metalloproteases and anticoagulant properties (Montanari et al., 2021; Zamai, 2020, 2021, 2023).

Taken all together, the data presented here are consistent with our original counterintuitive hypothesis (Zamai, 2020), suggesting that individuals exhibiting higher levels of ACE2 are not only those with severe COVID-19 (Fagyas et al., 2022; Kragstrup et al., 2021; Lundstrom et al., 2021; Nagy et al., 2021; Patel, S.K. et al., 2021; Reindl-Schwaighofer et al., 2021; Valle Martins et al., 2021; van Lier et al., 2021) but also the most COVID-19-susceptible people before infection. The possibility to identify highly susceptible individuals long before infection and, particularly, to uncover them among young healthy adults having a low probability of developing severe COVID-19, will be crucial to targetedly protect the most productive (susceptible) people with a long life expectancy, while limiting treatment-associated side effects. In this regard, one, a 42-year-old male donor expressing high levels of ACE2 might represent one of the rare cases of healthy "young" adults destined to develop severe COVID-19, for which preventive measures such as vaccination, face masks or physical distancing should be warmly recommended.

However, our study should be interpreted in light of some limitations. First, some potentially important variables such as treatments and disease severity were only partly available (mortality and albumin level were used as indicators of severity); second, the number of subjects were limited. In this regard, it would be interesting to investigate whether a correlation exists between abnormal ACE2 levels such as our outliers and subsequent development of severe COVID-19 in larger cohorts, such as those already analysed for ACE2 protein levels or activity (Anguiano et al., 2015; Kornilov et al., 2020; Kragstrup et al., 2021; Lim et al., 2022; Narula et al., 2020; Ramchand et al., 2018; Sama et al., 2020; Wallentin et al., 2020; Walters et al., 2017).

The strength of this study is the integration and comparison of data coming from three different groups. The new information combined with existing literature lead to converging evidence supporting further studies to validate the determinations not only of circulating ACE2 levels in uninfected subjects as predictive biomarker of COVID-19 outcome before SARS-CoV-2 infection but also of Zn/Alb ratios to individually determine albumin-unbound zinc species in COVID-19 patients for a targeted therapy with zinc chelating agents. Notably, differently from the evaluation of ACE2 activity and free zinc, determination of both ACE2 protein by ELISA and Zn/Alb ratio can be both affordable and routinely performed in diagnostics laboratories; therefore, if validated as biomarkers for COVID-19, they could represent a first step towards a feasible precision medicine approach to COVID-19 that might be used in population-based screening programmes to prioritise high risk individuals for targeted early pharmacological intervention and/or for prevention through vaccination.

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CRediT authorship contribution statement

Serena Benedetti: Data curation, Formal analysis, Investigation, Methodology, Supervision, part of statistical analyses, Writing – review & editing. **Davide Sisti:** Data curation, Formal analysis, statistical analyses, Writing – review & editing. **Daniela Vandini:** Funding acquisition, Methodology, Writing – review & editing. **Simone Barocci:** Methodology, Writing – review & editing. **Maurizio Sudano:** Data curation, Methodology, Writing – review & editing. **Eugenio Carlotti:** Funding acquisition, Writing – review & editing. **Jade Lee Lee Teng:** Funding acquisition, Writing – review & editing. **Loris Zamai:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing, All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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