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List of Abbreviations

ACE2: angiotensin converting enzyme 2
COVID-19: coronavirus disease 2019
ECs: endothelial cells
EVs: extracellular vesicles
EC-EVs: endothelial extracellular vesicles
miRNAs or miRs: microRNAs
SARS-CoV-2: severe acute respiratory syndrome coronavirus-2
ABSTRACT

Emerging evidence indicates that the relationship between COVID-19 and diabetes is twofold: 1) it is known that the presence of diabetes and other metabolic alterations poses a considerably high risk to develop a severe COVID-19; 2) patients who survived a SARS-CoV-2 infection have an increased risk of developing new-onset diabetes. However the mechanisms underlying this association are mostly unknown and there are no reliable biomarkers to predict the development of new-onset diabetes. In the present study, we demonstrate that a specific microRNA (miR-34a) contained in circulating extracellular vesicles released by endothelial cells reliably predicts the risk of developing new-onset diabetes in COVID-19. This association was independent of age, sex, BMI, hypertension, dyslipidemia, smoking status, and D-dimer.

Key words: Biomarker, COVID-19, endothelium, exosomes, extracellular vesicles, long COVID, microRNA
SIGNIFICANCE STATEMENT

We demonstrate for the first time that a specific microRNA (miR-34a) contained in circulating extracellular vesicles released by endothelial cells is able to reliably predict the risk of developing new-onset diabetes mellitus after having contracted COVID-19. Strikingly, this association was independent of age, sex, BMI, hypertension, dyslipidemia, smoking status, and D-dimer. Our findings are also relevant when considering the emerging importance of post-acute sequelae of COVID-19, with systemic manifestations observed even months after viral negativization (Long-COVID).
INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has led to a global healthcare crisis: >110 million Americans and >700 million worldwide have been diagnosed as infected by SARS-CoV-2, leading to ~1.2-million and ~7-million deaths, respectively.

The relationship between COVID-19 and diabetes is twofold: while it is known that the presence of diabetes and other metabolic alterations poses a considerably high risk to develop a severe COVID-19 (Zhu et al., 2020; Kastora et al., 2022; Kazakou et al., 2022; Kim et al., 2022), emerging evidence indicates that patients who survived a SARS-CoV-2 infection have a significantly increased risk of developing new-onset diabetes (Rubino et al., 2020; Accili, 2021; Kamrath et al., 2021; Khunti et al., 2021; Metwally et al., 2021; Nassar et al., 2021; Sathish et al., 2021a; Sathish et al., 2021b; Lai et al., 2022; Rahmati et al., 2022; Rathmann et al., 2022; Ssentongo et al., 2022; Steenblock et al., 2022; Wander et al., 2022; Xie and Al-Aly, 2022; Zhang et al., 2022; Abumayyaleh et al., 2023; Ali et al., 2023; Bellia et al., 2023; Cefalu, 2023; Chourasia et al., 2023; Gorchane et al., 2023; Izzo et al., 2023; Kim et al., 2023; Li et al., 2023; Naveed et al., 2023; O'Mahoney et al., 2023; Weiss et al., 2023; Zhou et al., 2023; Pantea Stoian et al., 2024). Of note, a retrospective analysis of more than 27-million patients (Cerner Real-World data) revealed that COVID-19 is associated with an increased risk of diabetes, and that Black, Asian/Pacific Islander, and American Indian/Alaskan Native populations are disproportionately at risk (Qeadan et al., 2022); a significant rise in new-onset type-2 diabetes has been observed among children and
adolescents of Alabama during the COVID-19 pandemic (Schmitt et al., 2022); equally important, a meta-analysis has evidenced that the incidence of diabetic ketoacidosis among pediatric patients has significantly increased during COVID-19 pandemic (Alfayez et al., 2022).

Quantifying the expression levels of microRNAs (miRNAs) shuttled by extracellular vesicles (EVs) has been shown to be extremely valuable in clinical practice (Cha et al., 2021; Esmaeili et al., 2021; Gu et al., 2021; Liao et al., 2021; Liu et al., 2021; Papiewska-Pajak et al., 2021; Silvestro et al., 2021; Sonoda et al., 2021; Wang et al., 2021; Yang and Rhee, 2021; Chen et al., 2022; Fullerton et al., 2022; Pavani et al., 2022). We have recently demonstrated that endothelial EVs (EC-EVs) enriched in miR-24 independently predict cerebrovascular events in COVID-19 (Gambardella et al., 2021). In a preliminary assay comparing circulating levels of EC-EVs of COVID-19 patients who developed diabetes to COVID-19 patients who did not develop diabetes, we identified miR-34a as one of the top upregulated miRNAs. Therefore, we hypothesized the existence of a significant association between the onset of diabetes and plasma levels of EC-EV miR-34a in patients that have been hospitalized for COVID-19.

MATERIALS AND METHODS
Study design and participants

In this prospective study, we obtained plasma from consecutive patients hospitalized for COVID-19, enrolled at the “Ospedali dei Colli”, Naples, Italy. The diagnosis of
diabetes was defined according to the guidelines of the American Diabetes Association (Draznin et al., 2022). The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Inclusion criteria: age >18 years old; willingness to participate (informed consent signed by each patient or an authorized representative); diagnosis of COVID-19 confirmed by RT-qPCR on nasal swab, as described (Fiorentino et al., 2021; Gambardella et al., 2021; Trimarco et al., 2022; Trimarco et al., 2023).

Exclusion criteria: we excluded patients for whom admission blood samples were unavailable, patients with cancer, patients with a pre-existent diagnosis of diabetes or prediabetes (Mone et al., 2023a), patients who were not treated with steroids, and patients who received the first diagnosis of diabetes less than 30 days after discontinuation of steroid-based treatments.

**Experimental procedures**

EC-EVs were isolated from plasma via serial centrifugation and magnetic isolation (Miltenyi Biotec, Auburn, CA), as we described and validated (Morelli et al., 2019; Wang et al., 2020; Gambardella et al., 2021) and DNA was extracted as reported earlier (Gambardella et al., 2021; Jankauskas et al., 2023; Mone et al., 2023b); EC-EVs miR-34a levels were quantified via droplet digital polymerase chain reaction (ddPCR) using a QX200 ddPCR System (Bio-Rad, Hercules, CA) following the manufacturer's instructions. A SARS-CoV-2 test (RT-qPCR) was performed in all subjects to confirm or rule out the COVID-19 diagnosis. Blood levels of Interleukin-6 (IL-6), D-dimer, high-sensitivity C Reactive Protein (hs-CRP), and Tumor Necrosis Factor α (TNFα) were
measured in all patients on admission as we described (Fiorentino et al., 2021; Gambardella et al., 2021).

**Statistical Analysis**

All data were analyzed using the SPSS software (version 29.0; SPSS, IBM, Armonk, NY, USA), establishing a significant difference at a p-value < 0.05. Data are expressed as means ± SD or numbers and percentages. The unpaired 2-tailed t-test using (when appropriate) Welch's correction for unequal variances was performed. The chi-squared test was applied to compare categorical variables. A multivariable linear regression analysis was used to assess the association between miR-34a and new-onset diabetes, adjusting for potential confounders. Receiver operating characteristic (ROC) curves were analyzed to identify the optimal cut-off value of miR-34a levels, calculating the Youden’s index to integrate sensitivity and specificity information, as we previously described (Santulli et al., 2019; Gambardella et al., 2022).

**RESULTS**

We obtained plasma from 388 COVID-19 patients. We excluded 73 patients who did not fulfill the aforementioned inclusion/exclusion criteria; consequently, the study was conducted in 315 subjects, with a median follow-up of 12 months.

Table 1 reports the main clinical parameters of our population. New onset diabetes was diagnosed in 28 COVID-19 patients. No significant differences in therapeutic
management and in the presence of comorbidities were observed. As per our exclusion criteria, all subjects received steroids as standard therapy for hospitalized COVID-19 patients.

We found that circulating levels of EC-EV miR-34a were significantly upregulated ($P<0.001$) in patients with vs without new-onset diabetes (Table 1).

Applying ROC curves, we determined that 3300 copies/10 nl was the optimal cut-off value for miR-34a levels in order to predict new-onset diabetes, yielding the following results: sensitivity 71.43% (95% C.I.: 51.33% to 86.78%), specificity 98.61% (95% C.I.: 96.47% to 99.62%), positive predictive value 83.33% (95% C.I.: 64.76% to 93.15%), and negative predictive value 97.25% (95% C.I.: 95.17% to 98.45%).

Using a stepwise multiple regression analysis, adjusting for age, sex, BMI, hypertension, dyslipidemia, smoking status, and D-dimer (Table 2), the association between EC-EV miR-34a and new-onset diabetes in COVID-19 patients was confirmed ($P<0.001$).

**DISCUSSION**

To the best of our knowledge, this is the first study showing a significant association between EC-EV non-coding RNAs and new-onset diabetes in COVID-19 patients.

In the Spring of 2020, we were among the first groups to indicate a link between COVID-19 and endothelial dysfunction (Gambardella et al., 2020; Santulli et al., 2020; Sardu et al., 2020), and our view was later confirmed by other investigators, associating the systemic manifestations of the disease to a direct or indirect involvement of the endothelium (D’Agnillo et al., 2021; Gelzo et al., 2021; Higashikuni et al., 2021;
Kalicinska et al., 2021; Qin et al., 2021; Beltran-Camacho et al., 2022; Casciola-Rosen et al., 2022; Haffke et al., 2022; Kelliher et al., 2022; Ma et al., 2022; Maldonado et al., 2022; Montiel et al., 2022; Otifi and Adiga, 2022; Santoro et al., 2022; Seitz and Ong, 2022; Tarnawski and Ahluwalia, 2022). Indeed, endothelial cells (ECs) express all cofactors necessary for the internalization of SARS-CoV-2 in host cells, including angiotensin converting enzyme 2 (ACE2), transmembrane protease serine 2, cathepsins B and D, neuropilin-1, vimentin, and others, thereby representing a natural target of SARS-CoV-2 (Gambardella and Santulli, 2021; Mone et al., 2021; Kansakar et al., 2022; Gambardella et al., 2023). Furthermore, the systemic inflammatory viral reaction observed in patients affected by COVID-19 has been shown to be linked to endothelial dysfunction (Bernard et al., 2020; Loo et al., 2021; Sabioni et al., 2021). Hence, COVID-19 affects not only the epithelial cells of the lung parenchyma, but also ECs across the whole body, thus leading to a generalized endothelial damage. Such a damage, caused directly by SARS-CoV-2 infection and/or by the ensuing cytokine storm, can shift the vascular equilibrium towards an altered vascular tone, and an increased permeabilization; most of these findings have been substantiated from autopsies of COVID-19 patients since the pandemic outbreak (Otifi and Adiga, 2022). Further supporting our theory of a central role of ECs in COVID-19, clinical trials testing whether interventions that ameliorate endothelial dysfunction can have beneficial effects in COVID-19 patients are ongoing and the available results are very encouraging (Adebayo et al., 2021; Fiorentino et al., 2021; Trimarco et al., 2023).
Consistent with our findings, a significantly augmented risk of newly diagnosed diabetes after SARS-CoV-2 infection, independent of steroid use (which was instead linked to transient hyperglycemia), has been recently demonstrated in a large study (Barrett et al., 2022) performed by the Centers for Disease Control and Prevention (CDC). Current COVID-19 guidelines recommend the use of steroids in hospitalized patients up to 10 days or until discharge and should not be prescribed beyond discharge (Huang et al., 2022). Notably, in our study we ruled out cases of transient hyperglycemia due to steroids by including only patients in which the first diagnosis of new-onset diabetes had been formulated at least 1 month after the discontinuation steroid-based therapies.

A Nature paper (Al-Aly et al., 2021) demonstrated that survivors of COVID-19 had a 39% increased likelihood of receiving a new diabetes diagnosis within six months following infection compared with noninfected patients (Al-Aly et al., 2021); the cohort included 73,435 COVID-19 patients of the Veterans Health Administration, and ~5 million control subjects who did not have COVID-19 (Al-Aly et al., 2021). The same team of researchers lately published another paper evidencing that people with COVID-19 exhibit a markedly augmented risk of new-onset diabetes that increased according to the severity of the infection as proxied by the care setting: non-hospitalized, hospitalized, and admitted to intensive care (Xie and Al-Aly, 2022). Another study conducted in 47,780 hospitalized patients with COVID-19 found out that these patients had a ~50% increased likelihood of developing diabetes ~20 weeks following discharge compared with matched control patients (Ayoubkhani et al., 2021). In full alignment with these reports, we demonstrated in a population of more than 200,000 individuals that
the risk of new-onset diabetes is markedly increased when comparing the years of the pandemic (2020-2022) to the triennium 2019-2019 (Izzo et al., 2023). All these findings provide solid support for a diabetogenic effect of COVID-19, beyond the stress response associated with severe illness.

Our results are particularly relevant if we consider that chronic manifestations of the disease, including metabolic disturbances, have been reported even months after the initial infection occurred (“Long COVID”) (Feldman et al., 2020; Iqbal et al., 2021; Montefusco et al., 2021; Raveendran and Misra, 2021; Sathish et al., 2021a; Narayan and Staimez, 2022; Scherer et al., 2022).

Our study is not exempt from limitations, including the relatively small size of our population and brief follow-up; moreover, our findings refer exclusively to hospitalized COVID-19 patients and therefore cannot be generalized to subjects with a mild disease. We also reckon that the exact molecular mechanisms linking EC-EV miR-34a to the development of diabetes need to be defined in dedicated research projects, whereas our study merely identified miR-34a as a novel and independent biomarker of disease; nonetheless, a biomarker-centric approach has been shown to be crucial in drug development (Fader et al., 2021).

In conclusion, we identified a significant association between EC-EV miR-34a and new onset diabetes, which could be instrumental towards the understanding of the molecular mechanisms underlying the pathophysiology of metabolic complications in COVID-19 as well as long-COVID. Further analyses in larger groups are warranted to
confirm our results, ratify their prognostic value, and explore the potential role of miR-34a in other metabolic events.
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Author Contributions

*Participated in research design:* Jankauskas, Gambardella, and Santulli.

*Conducted experiments and contributed new reagents or analytic tools:* Mone, Jankauskas, Manzi, Gambardella, Coppola, Kansakar, Fiorentino, Lombardi, Varzideh, Sorrentino, Trimarco, and Santulli.

*Performed data analysis:* Izzo, Santulli.

*Wrote or contributed to the writing of the manuscript:* Mone, Jankauskas, Santulli.

All authors contributed to the article and approved the submitted version.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial
or financial relationships that could be construed as a potential conflict of interest.
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<table>
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<tr>
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<th>No new-onset Diabetes (287)</th>
<th>New-onset Diabetes (28)</th>
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<tr>
<td>Age (years)</td>
<td>60.7±14.1</td>
<td>64.28±15.2</td>
<td>0.213</td>
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<tr>
<td>Sex (male, %)</td>
<td>156 (54.3)</td>
<td>14 (50)</td>
<td>0.660</td>
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<tr>
<td>BMI (kg/m^2)</td>
<td>24.8±3.4</td>
<td>26.2±3.5</td>
<td>0.067</td>
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<tr>
<td>Glycemia (mg/dl)</td>
<td>100.2±12.1</td>
<td>103.1±23.6</td>
<td>0.281</td>
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<tr>
<td>SBP (mmHg)</td>
<td>137.76±19.5</td>
<td>142.2±19.3</td>
<td>0.114</td>
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<tr>
<td>DBP (mmHg)</td>
<td>84.46±9.47</td>
<td>86.3±14.9</td>
<td>0.239</td>
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<tr>
<td>Hypertension (%)</td>
<td>103 (35.9)</td>
<td>10 (35.7)</td>
<td>0.690</td>
</tr>
<tr>
<td>Dyslipidemia (%)</td>
<td>86 (29.9)</td>
<td>9 (32.1)</td>
<td>0.811</td>
</tr>
<tr>
<td>Current smoking (%)</td>
<td>126 (43.9)</td>
<td>12 (42.9)</td>
<td>0.916</td>
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<tr>
<td>Duration of steroid treatment (days)</td>
<td>7 (7, 9)</td>
<td>7 (7, 8)</td>
<td>0.843</td>
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<tr>
<td>Interval from steroid discontinuation (days)</td>
<td>166 (137, 193)</td>
<td>171 (140, 178)</td>
<td>0.929</td>
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<tr>
<td>D-dimer (µg/ml)</td>
<td>2.70±1.6</td>
<td>2.94±1.8</td>
<td>0.463</td>
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<td>IL-6 (pg/ml)</td>
<td>7.5±4.0^*</td>
<td>8.4±5.5^*</td>
<td>0.121</td>
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<tr>
<td>TNFα (pg/ml)</td>
<td>6.5±4.2</td>
<td>5.8±4.7</td>
<td>0.271</td>
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<tr>
<td>hs-CRP (µg/ml)</td>
<td>3.6±3.2^#</td>
<td>4.2±2.9^#</td>
<td>0.144</td>
</tr>
<tr>
<td>EC-EV miR-34a (copies/10 nl)</td>
<td>1867.4±1490</td>
<td>3485.8±1998*</td>
<td>0.001</td>
</tr>
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</table>

**Table 1. Main characteristics of our population at hospital admission.**

Data on quantitative parameters are expressed as mean ± standard deviation or as median and interquartile range; data on qualitative characteristics are expressed as absolute numbers and percentage values. BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; IL-6: Interleukin-6; TNFα: Tumor Necrosis Factor α; hs-CRP: high-sensitivity C Reactive Protein; EC-EV miR-34a: level of miR-34a within endothelial extracellular vesicles. *: P<0.05.
Table 2. Multivariable linear regression analysis assessing the association between miR-34a and new-onset diabetes in COVID-19 patients.

BMI: Body mass index; EC-EV miR-34a: level of miR-34a within endothelial extracellular vesicles.