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RESEARCH ARTICLE

Angiotensin I-converting enzyme type 2 expression is increased in pancreatic islets of type 2 diabetic donors

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Abstract

Aims: Angiotensin I-converting enzyme type 2 (ACE2), a pivotal SARS-CoV-2 receptor, has been shown to be expressed in multiple cells, including human pancreatic beta-cells. A putative bidirectional relationship between SARS-CoV-2 infection and diabetes has been suggested, confirming the hypothesis that viral infection in beta-cells may lead to new-onset diabetes or worse glycometabolic control in diabetic patients. However, whether ACE2 expression levels are altered in beta-cells of diabetic patients has not yet been investigated. Here, we aimed to elucidate the in situ expression pattern of ACE2 in Type 2 diabetes (T2D) with respect to non-diabetic donors which may account for a higher susceptibility to SARS-CoV-2 infection in beta-cells.

Material and Methods: Angiotensin I-converting enzyme type 2 immunofluorescence analysis using two antibodies alongside insulin staining was performed on formalin-fixed paraffin embedded pancreatic sections obtained from $n = 20$ T2D and $n = 20$ non-diabetic (ND) multiorgan donors. Intensity and colocalisation analyses were performed on a total of 1082 pancreatic islets. Macrophage detection was performed using anti-CD68 immunohistochemistry on serial sections from the same donors.

Results: Using two different antibodies, ACE2 expression was confirmed in beta-cells and in pancreas microvasculature. Angiotensin I-converting enzyme type 2 expression was increased in pancreatic islets of T2D donors in comparison to ND controls alongside with a higher colocalisation rate between ACE2 and insulin using both anti-ACE2 antibodies. $CD68^+$ cells tended to be increased in T2D pancreata, in line with higher ACE2 expression observed in serial sections.

Conclusions: Higher ACE2 expression in T2D islets might increase their susceptibility to SARS-CoV-2 infection during COVID-19 in T2D patients, thus worsening glycometabolic outcomes and disease severity.

Guido Sebastiani and Francesco Dotta shared senior co-authorship.

KEYWORDS
ACE2, beta-cell, COVID-19, pancreatic islets, SARS-CoV-2, type 2 diabetes

1 | INTRODUCTION

Previous studies have shown that patients with COVID-19 and pre-existing diabetes are at increased risk of being admitted to Intensive Care Unit (ICU), are more likely to have multi-organ damage, and have a higher mortality rate than non-diabetic (ND) patients.[1–4] In addition, abnormalities in glycaemic control, insulin resistance and pancreatic islet function have been observed in patients with COVID-19 without a pre-existing history of diabetes, thus increasing the risk of developing Type 1 Diabetes (T1D), Type 2 Diabetes (T2D) or dysglycaemia.[5–16] Therefore, it is conceivable to hypothesise a bidirectional relationship between COVID-19 and diabetes.[17,18]

Angiotensin I-converting enzyme type 2 (ACE2) is the canonical receptor for SARS-CoV-2. The entry of SARS-CoV-2 into cells depends on binding of the spike protein to ACE2. Hence, the presence of ACE2 dictates SARS-CoV-2 infectivity in multiple tissues [i.e., airway respiratory tract[19]] and is indispensable for SARS-CoV-2 infection.[20] Additional cofactors, such as TMPRSS2 and Neuropilin-1 (NRP-1) can facilitate and potentiate SARS-CoV-2 infection even in the presence of low levels of ACE2.[21]

The presence of the SARS-CoV-2 receptor ACE2 in pancreatic beta-cells was initially highly debated, with observed discrepancies among studies.[22–24] At present, ACE2 expression in beta-cells is supported by multiple reports which clearly showed the expression of ACE2 and other co-factors (i.e. TMPRSS2, NRP1) in beta-cells.[24–29] As a consequence, pancreatic islets are susceptible to SARS-CoV-2 infection mainly due to the expression of ACE2 and its co-factors, as demonstrated in several studies showing that beta-cells can be infected in vitro and in vivo in patients who died as a consequence of COVID-19.[25,26,28–30]

Angiotensin I-converting enzyme type 2 expression has been shown to be modulated by pro-inflammatory stress with multiple studies showing increased expression of ACE2 upon exposure to several cytokines and/or pro-inflammatory molecules.[24,31–34] Notably, chronic inflammation is a well-described feature of obesity and T2D.[35–37] Inflammatory processes are also activated in pancreatic islets, as demonstrated by multiple evidence from animal models and humans with obesity and/or T2D (reviewed in[38]). In this context, low-grade inflammation of pancreatic islets has been shown to be associated with progressive beta-cell failure.[39–41]

In T2D, ACE2 expression has been shown to be increased in several organs exposed to a diabetic milieu.[42–44] However, a systematic assessment of ACE2 expression in human pancreatic islets of T2D patients is still missing.

We took advantage of the availability of a cohort of T2D and ND multiorgan donors to measure pancreatic islet ACE2 expression levels employing confocal immunofluorescence analysis and multiple antibodies directed against different ACE2 epitopes.

2 | MATERIAL AND METHODS

2.1 | Ethics statement and multiorgan donors' pancreata

Studies involving human participants were reviewed and approved by the local ethics committee at the University of Pisa (Pisa, Italy). Pancreata not suitable for organ transplantation were obtained with informed written consent from organ donors' next-of-kin and processed following standardized procedures. Human pancreatic tissue sections were obtained from the pancreata of brain-dead adult ND and T2D multiorgan donors, COVID-19 negative. Multiple formalin-fixed paraffin embedded (FFPE) tissue sections were obtained from n = 20 ND controls (CTR) [9F, 11M; age: 70.6 ± 7.0 years (mean ± Standard Deviation (S.D.)); BMI: 26.2 ± 4.1 kg/m^2) and from n = 20 T2D pancreata (6F, 14M; age: 71.7 ± 7.6 years (mean ± S.D.); BMI: 27.1 ± 2.7 kg/m^2 (mean ± S.D.)] whose clinical characteristics are described in ESM Table 1.

2.2 | Immunofluorescence and immunohistochemical analysis of human pancreatic sections

2.2.1 | Triple immunofluorescence for Angiotensin I-converting enzyme type 2 (MAB933)-ACE2 (AB15348)-Insulin

Formalin-fixed paraffin embedded tissue sections (7 µm thick) were obtained from pancreata of ND or T2D multiorgan donors. Formalin-fixed paraffin embedded sections were analysed using quadruple immunofluorescence for insulin (INS) and ACE2 (two different antibodies) and 4′,6-Diamidino-2-phenylindoledihydrochloride (DAPI) for nuclei counterstain.

After deparaffinisation and rehydration (xylene-I and xylene II, 20 min/each; ethanol 100%, 95%, 80%, 75%, all vol./vol., 5 min/each), pancreatic sections were subjected to antigen retrieval through incubation in 10 mM citrate buffer [0.1 M Citric Acid Monohydrate (Cat. C1909 - Sigma Aldrich, St. Louis, MO, USA) and 0.1 M Tribasic Sodium Citrate Dihydrate (cat. S4641 -Sigma Aldrich, St. Louis, MO, USA)] pH 6.0 in microwave (600 W) for 10 min, maintaining boiling conditions. After cooling and 3 washes in Phosphate Buffered Saline 1X (PBS 1X) (Cat. 14040-091 - Gibco, ThermoFisher Scientific, Waltham, MA, USA), sections were incubated with PBS 1X supplemented with 3% Bovine Serum Albumin (BSA) (cat. A1470-25G - Sigma Aldrich, St. Louis, MO, USA) for 40 min at room temperature (RT) to avoid non-specific reactions.

Then, sections were incubated with primary antibody monoclonal mouse anti-human ACE2 (cat. MAB933 - R&D System, Minneapolis, MS, USA) (final concentration: 15 μg/mL) in PBS 1X supplemented with 3% BSA and primary antibody polyclonal rabbit anti-human ACE2 (cat. ab15348 - Abcam, Cambridge, UK) (final concentration: 0.5 μg/mL) in PBS 1X supplemented with 3% BSA, overnight at +4°C. After 3 washes in PBS 1X, the sections were incubated for 1h at RT with ready to use polyclonal guinea pig anti-human insulin (cat. IR002 -Agilent Technologies, Santa Clara, CA, USA) further diluted 1:5 in PBS 1X supplemented with 3% BSA. Then, the sections were incubated for 1 h with related secondary antibodies, all diluted 1:500 (final concentration: 4 μg/mL) in PBS 1X: goat anti-guinea pig Alexa-Fluor 555 conjugate (cat. A21435, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA); goat anti-mouse Alexa-Fluor 488 conjugate (cat. A11029, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA) and goat anti-rabbit 647 conjugate (cat. A21245—Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA). Sections were counterstained with DAPI (cat. D8517, Sigma-Aldrich) diluted 1:3000 in PBS 1X and then mounted with Dako Fluorescence Mounting Medium (cat. S3023—Agilent Technologies, Santa Clara, CA, USA). The sections were stored overnight at +4°C until image analysis.

To confirm the validity of the staining 1 μg of polyclonal rabbit anti-human ACE2 (cat. ab15348 -Abcam, Cambridge, UK) was combined with or without 10 μg of the immunising human ACE2 peptide (cat. Ab15325—Abcam, Cambridge, UK) and the staining was performed as described above.

2.2.2 | Double immunofluorescence for Angiotensin I-converting enzyme type 2 (MAB933)-CD31

After deparaffinisation and rehydration, FFPE pancreatic sections were subjected to antigen retrieval as described above.

After cooling and 3 washes in PBS 1X (Cat. 14040-091 - Gibco, ThermoFisher Scientific, Waltham, MA, USA), sections were incubated with PBS 1X supplemented with 3%

Bovine Serum Albumin (cat. A1470-25G - Sigma Aldrich, St. Louis, MO, USA) for 40 min at RT to avoid non-specific reactions and then incubated overnight at +4°C with primary antibody monoclonal rabbit anti-human CD31 (cat. Ab76533 - Abcam, Cambridge, UK) (final concentration: 3.5 μg/ml). After 3 washes in PBS 1X, the sections were incubated with monoclonal mouse anti-human ACE2 (cat. MAB933 - R&D System, Minneapolis, MS, USA) (final concentration: 15 μg/ml) for 1 h at RT.

After 3 washes in PBS 1X, the sections were incubated for 1 h at RT with ready to use polyclonal guinea pig anti-human insulin (cat. IR002 - Agilent Technologies, Santa Clara, CA, USA) further diluted 1:5 in PBS 1X supplemented with 3% BSA. Then, sections were incubated for 1 h with related secondary antibodies, all diluted 1:500 (final concentration: 4 μg/mL) in PBS 1X: goat anti-guinea pig Alexa-Fluor 594 conjugate (cat. A11037, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA); goat anti-mouse Alexa-Fluor 488 conjugate (cat. A11029, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA) and goat anti-rabbit 647 conjugate (cat. A21245 –Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA). Sections were counterstained with DAPI (cat. D8517, Sigma Aldrich, St. Louis, MO, USA) diluted 1:3000 in PBS 1X and then mounted with Dako Fluorescence Mounting Medium (cat. S3023—Agilent Technologies, Santa Clara, CA, USA). The sections were stored overnight at +4°C until image analysis.

2.2.3 | Immunohistochemical staining CD68-positive cells

Pancreatic CD68-positive cells were detected using enzymatic-colorimetric immunohistochemical staining. After deparaffinisation and rehydration (see above), pancreatic sections were subjected to blocking of peroxidase with 3% hydrogen peroxide in PBS 1X for 20 min. Then, the sections were subjected to heat-induced antigen retrieval using Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA, 0.05% Tween-20, pH 9.0) for 20 min at 100°C. After cooling and incubation in 3% BSA in PBS 1X for 30 min at RT to reduce non-specific reactions, sections were stained in 3% BSA in PBS 1X for 1h at RT with mouse monoclonal anti-human CD68 (cat. M0876 - Agilent Technologies, Santa Clara, CA, USA) (final concentration: 0.4 mg/mL). After three washes in PBS 1X, sections were incubated with secondary antibody polyclonal goat anti-mouse HRP-conjugate (cat.115-036-003 - Jackson ImmunoResearch, Philadelphia, PA, USA) diluted 1:500 in PBS 1X for 1h at RT. Subsequently, the sections were incubated with one drop of 3,3-Diaminobenzidine (DAB) chromogen solution (cat. RE7270-K, Novolink MAX DAB, Leica Microsystems, Wetzlar, Germany) for 5 min, to trigger the colorimetric reaction. After 10 min of incubation in water, the sections were incubated for 1 h at RT with ready to use polyclonal guinea pig anti-human insulin (cat. IR002 - Agilent Technologies, Santa Clara, CA, USA) further diluted 1:5 in PBS 1X supplemented with 3% BSA. After three washes in PBS 1X, sections were incubated with secondary polyclonal antibody goat anti-Guinea Pig conjugated with Alkaline Phosphatase (cat. A18772- ThermoFisher Scientific, Waltham, MA, USA) (final concentration: 0.3 μg/ml). Subsequently, the sections were incubated with one drop of Liquid Fast Red (LFR) (cat. K0640—Agilent Technologies, Santa Clara, CA, USA) (a drop of chromogen in 3 mL of substrate Levamisole (cat. X3021—Agilent Technologies, Santa Clara, CA, USA) one drop per ml of LFR of Levamisole) for 5 min.

Stained sections were then counterstained with haematoxylin (cat. MHS31 - Sigma Aldrich, St. Louis, MO, USA) for 4 min. After 1 h of air drying, the sections were covered with a drop of Faramount, Aqueous Mounting Medium, Ready-to-Use (cat. S302580-2 - Agilent Technologies, Santa Clara, CA, USA).

2.3 | Image analysis

Images were acquired, as a single stack focal plane, employing a Leica TCS SP5 confocal laser scanning microscope system (Leica Microsystems, Wetzlar, Germany).

For the confocal laser scanning microscope system sections were scanned and images acquired at 40X magnification. The same confocal microscope setting parameters (laser power, gain, offset, pinhole aperture) were applied to all stained sections before image acquisition in order to uniformly collect the detected signal related to each channel.

Colocalisation analysis between ACE2 and insulin was performed using LasAF software (Leica Microsystems, Wetzlar, Germany). The region of interest (ROI) was drawn to calculate the colocalisation rate (which indicates the extent of colocalisation between two different channels and reported as a percentage) as a ratio between the colocalisation area and the image foreground. Evaluation of ACE2 expression intensity in human pancreatic islets was performed using LasAF software (www.leica-microsystem.com). This software calculates the ratio between intensity sum ROI (which indicates the sum ROI of the grey-scale value of pixels within a ROI) of the ACE2 channel and Area ROI (μm^2) of human pancreatic islets. In both colocalisation and intensity measurement analysis, a specific threshold was assigned based on the fluorescence background. The same threshold was maintained for all the images in all the cases analysed.

Insulin-positive area was measured using Volocity 6.3 software (Perkin Elmer, Waltham, MA, USA). Relative insulin signal-positive area was calculated as a ratio between Area Intensity sum ROI (μm^2) of insulin channel and Area ROI (μm^2) of each human pancreatic islet.

For CD68-positive cell detection and quantification, images of the entire section were acquired using a NanoZoomer S60 Digital slide scanner (cat. C13210-01 - Hamamatsu Photonics, Hamamatsu City, Japan) and were displayed using the proprietary NDP.view2 software. Manual count of $CD68^+$ cells on the entire section area was performed.

2.4 | Statistical analysis

Results were expressed as mean ± Standard Deviation (S.D.). Comparisons between two groups were carried out using Mann-Whitney *U* test for non-parametric data (normality checked using Kolmogorov-Smirnov test). Differences were considered significant with *p* values less than 0.05. Clinical variable associations with ACE2 expression were checked using multiple least square regression analysis. Statistical analyses were performed using Graph Pad Prism 8 software.

3 | RESULTS

To detect pancreatic ACE2 protein expression and distribution, and to evaluate differences between ND and type 2 diabetic (T2D) donors, we performed a quadruple immunofluorescence analysis on FFPE pancreatic sections obtained from $n = 20$ ND and $n = 20$ T2D multiorgan donors (Table 1 and ESM Table 1). To cross-validate the ACE2 staining results, we used two different anti-ACE2 antibodies: (*i*) a monoclonal mouse IgG2a anti-human ACE2 (R&D, MAB933), whose specificity was confirmed through an isotype primary antibody staining (ESM Figure 1A) and (*ii*) a rabbit polyclonal anti-ACE2 (Abcam, Ab15348), whose specificity was tested through a peptide competition assay and subsequent staining in ND donors pancreatic sections (ESM Figure 1A).

In the ND and T2D donor pancreata, both antibodies showed signals indicating that ACE2 is expressed in pancreatic islets where it is mostly colocalised with insulin signal (ESM Figure 2A). Triple staining on ND FFPE pancreatic sections using ACE2-MAB933, insulin and glucagon antibodies confirmed the prevalent colocalisation of ACE2 with insulin in comparison to ACE2 with glucagon (ESM Figure 2A), in line with our previous data.[24]

The present results confirm that ACE2, in pancreatic islets, is prevalent in beta-cells as expected.

Outside pancreatic islets, ACE2-positive cells showed a vasculature-like morphology and distribution; such results were confirmed by using two different anti-ACE2 antibodies (ESM Figure 3A); a subsequent immunofluorescence co-staining with ACE2 and vascular-endothelial marker CD31 antibodies showed the juxtaposition of the two signals (ESM Figure 3A), thus confirming our previous observations,[24] in line also with other reports,[23] which demonstrated the localization of ACE2 in pancreatic vascular cells.

Next, we focused on ACE2 expression in pancreatic islets. To evaluate putative ACE2 expression differences between ND and T2D, we performed an analysis of the intensity of ACE2 signals including a total of $n = 1082$ islets. Both antibodies revealed a higher intensity of ACE2 in T2D compared with ND pancreatic islets (Figure 1A). Analysis of ACE2 intensity confirmed the significantly increased expression of ACE2 in T2D pancreatic islets compared to ND donors as measured by R&D and Abcam antibodies (greyscale values of ACE2-MAB933 in T2D = 52.5 ± 34.6 and in ND = 37.1 ± 28.1, $p < 0.001$; greyscale values of ACE2-ab15348 in T2D = 53.2 ± 63.5 and in ND = 27.3 ± 22.3, $p < 0.001$) (Figures 1C and D).

TABLE 1 Main clinical characteristics of non-diabetic (ND) and type 2 diabetic (T2D) subjects included in the study.

Clinical parameters	ND ($n = 20$)	T2D ($n = 20$)	*p* value
Age	70.6 ± 7.05	71.6 ± 7.7	ns
Sex (M:F)	11:9	14:6	ns
BMI (kg/m^2)	26.2 ± 4.2	27.1 ± 2.7	ns
Abdominal circumference (cm)	103.0 ± 20.7	100 ± 10.3	ns
ICU stay (days)	3.9 ± 5.0	3.4 ± 1.9	ns
Cold Ischaemia time (hours)	16.0 ± 5.29	15.76 ± 9.29	ns
Mean glycaemia (mmol/L)	8.03 ± 2.0	11.6 ± 3.7	$p < 0.05$
Diabetes duration (years)	-	10.9 ± 8.8	n/a

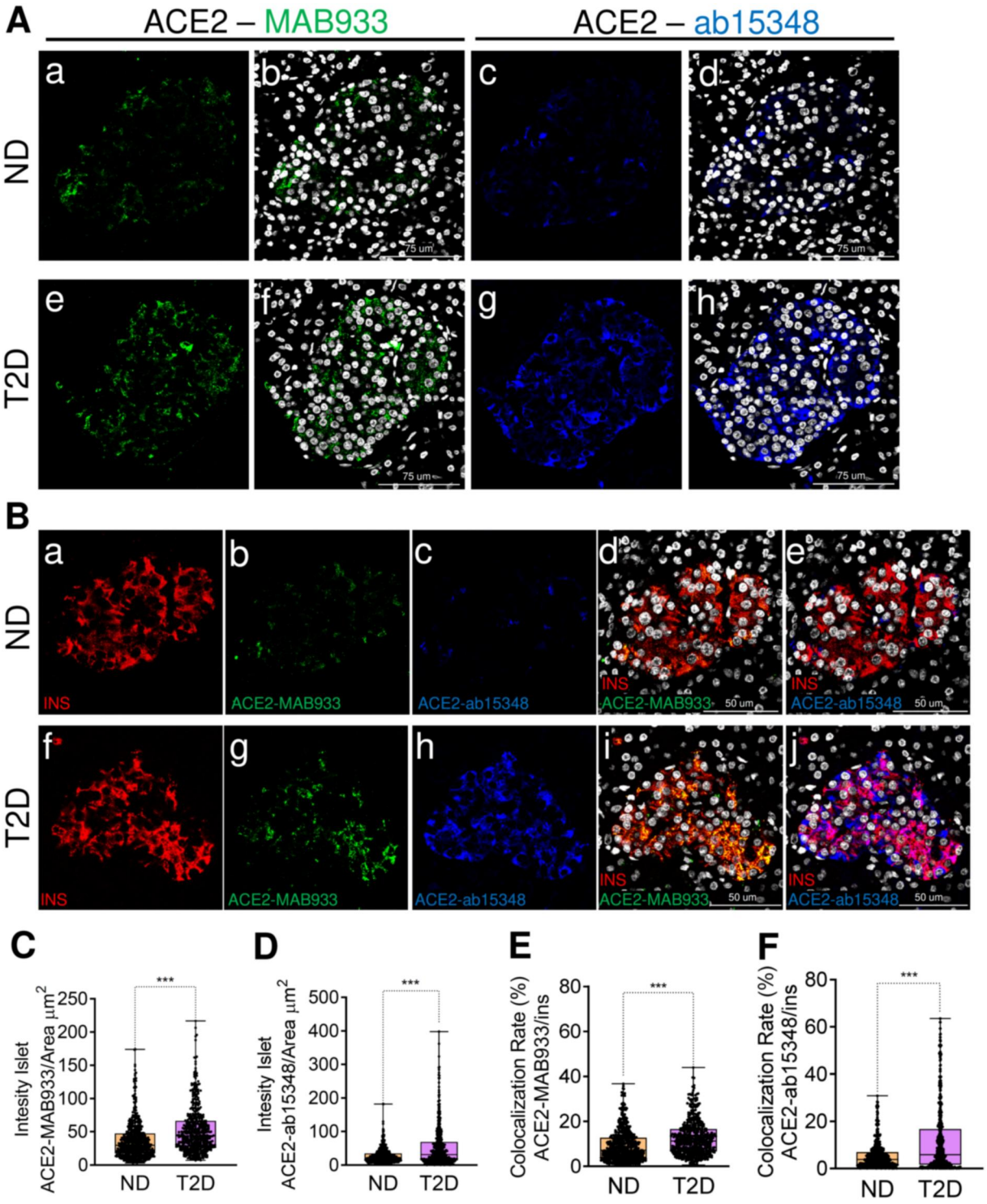

FIGURE 1 Upregulation of Angiotensin I-converting enzyme type 2 (ACE2) in pancreatic islet beta-cells of type 2 diabetic (T2D) donors. (A) Representative confocal images of formalin-fixed paraffin embedded (FFPE) pancreatic section from non-diabetic (ND) (case 13) and T2D donors (case 38). Pancreatic sections were stained with anti-ACE2-MAB933 (green, panels a and e) and the merge with 4′,6-Diamidino-2-phenylindoledihydrochloride (DAPI) (white, nuclei) (panels b and f) showing the expression of ACE2-MAB933 in a pancreatic islet; pancreatic sections were stained with anti-ACE2-ab15348 (blue, panels c and g) and the merge with DAPI (white, nuclei) (panel d and h) showed the expression of ACE2-ab15348 in a pancreatic islet. (B) Representative confocal images of FFPE pancreatic sections derived from a nondiabetic (ND) (case 2) and a T2D donor (case 32). Pancreatic sections were stained for insulin (INS, red, panels a and f), ACE2-MAB933 (green, panels b and g) and ACE2-ab15348 (blue, panels c and h). Colocalisation between ACE2-MAB933 and insulin is shown in yellow (panels d and i), while colocalisation between ACE2-ab15348 and insulin is reported in magenta (panels e and j). Signal intensity analysis measured with anti-ACE2-MAB933 (C) and with anti-ACE2-ab15348 (D) antibody in ND and T2D pancreatic sections; values are shown as fluorescence intensity of each islet detected (ND = 556 islets; T2D = 526 islets) reported as the sum of grey-scale values for each pixel normalised for the islet area (region of interest (ROI), mm^2). (E–F) Colocalisation rate analysis between ACE2-MAB933-Insulin (E) and ACE2-ab15348-insulin (F). Values are reported as the colocalisation rate between ACE2 (MAB933 or Ab15348) and insulin. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, non-parametric Mann-Whitney *U* test, performed after checking normality with the Kolmogorov-Smirnov normality test.

Since ACE2 expression in pancreatic islets is mostly prevalent in beta-cells, we performed a colocalisation rate analysis (reported as the percentage of the overlap of INS and ACE2 signals) of ACE2 MAB933/INS and ACE2 Ab15348/INS in T2D and ND pancreatic islets. We observed a significantly increased colocalisation rate between ACE2 and INS in T2D subjects compared with ND subjects (Figure 1B). The ACE2 colocalisation rate analysis of $n = 1082$ islets across all ND and T2D donors confirmed the increased colocalisation rate between ACE2-MAB933/INS [T2D: 12.5 ± 7.6% (mean ± S.D.) versus ND 9.23 ± 7.0% (mean ± S.D.), $p < 0.001$] and of ACE2-ab15348/INS [T2D: 11.45 ± 13.7% (mean ± S.D.) versus ND 5.3% ± 5.3%; $p < 0.001$] in T2D compared to ND (Figures 1E and F). No differences in insulin-positive area and signals were observed between T2D and ND donors (ESM Figure 4).

In the multiple linear regression analysis (ESM Table 2), ACE2 expression (reported as staining intensity) was not associated with age, BMI, gender, ICU stay, or duration of cold ischaemia time, thus excluding the influence of these putative confounding variables on ACE2 levels. Of note, ACE2 expression was not associated with gender or blood glucose levels (ESM Table 2).

To investigate a possible link between ACE2 expression and inflammation triggered by innate immune cells in the pancreas of T2D donors, we analysed CD68$^+$ macrophages in pancreatic tissue. In the whole pancreatic section (Figure 2A), CD68$^+$ macrophages showed an abundance of about 6.2 cells per mm^2 in T2D and 5.1 cells per mm^2 in ND. Interestingly, CD68$^+$ macrophages in the peri-islets showed an increased abundance trend in T2D compared to ND donors (0.22 vs. 0.15 CD68+ cells/islet) (Figure 2B); although not statistically significant, this result is consistent with the increased expression of ACE2 observed in pancreatic islets of previous serial sections.

Overall, these data show an increased expression of ACE2 in pancreatic beta-cells in T2D compared with ND donors. Although such an increase is independent of available clinical variables related to glycometabolic outcomes, we observed a tendency to increase in peri-islets CD68$^+$-macrophages, thus putatively associating ACE2 expression increase to inflammatory insults.

4 | DISCUSSION

The bidirectional relationship between SARS-CoV-2 infection and diabetes mellitus has been hypothesised at the beginning of the pandemic. As a matter of fact, evidence of hyperglycaemia, inflammation and vascular dysfunction, and abnormalities in glycometabolic control after or during SARS-CoV-2 infection have been demonstrated in several studies.[5–16,45] Notably, a recent meta-analysis showed 59% increase in the risk of developing diabetes in the post-acute phase of COVID-19 infected individuals in comparison to both healthy controls and versus matched non-COVID-19 respiratory infections.[46] Moreover, diabetic patients showed a more severe outcome, a poor prognosis and higher mortality rate after SARS-CoV-2 infection.[1,2,5–8] Notably, it has been demonstrated that SARS-CoV-2 can infect human host cells through the binding of viral spike protein to the extracellular N-terminal domain of the ACE2 receptor.[25,26] As a matter of fact, we and others have previously shown that beta-cells do express ACE2, thus being susceptible to SARS-CoV-2 infection.[24–29]

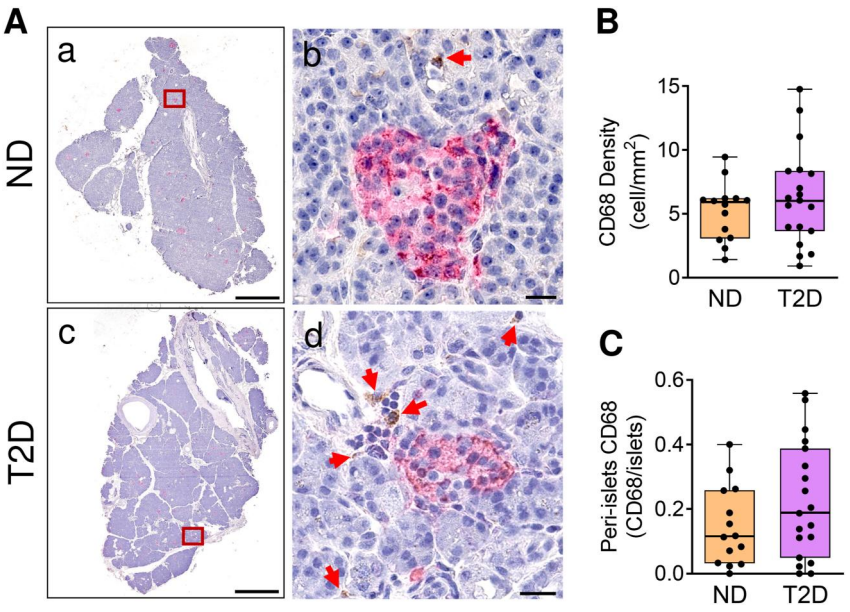

FIGURE 2 Peri-islets CD68$^+$-macrophages in Type 2 diabetes (T2D) pancreas. (A) Representative whole-slide image of formalin-fixed paraffin embedded (FFPE) pancreatic section from non-diabetic (ND) (panel a) and T2D donor pancreatic tissue sections (panel c) stained for insulin (red) and CD68 (brown). Zoom-in insets of the peri-islets CD68+ macrophages are reported in panel b (ND) and in panel d (T2D). (B) CD68 density in the whole pancreas is reported as number of cells/mm^2. Peri-islets CD68+-macrophages (C), considered within 250 μm from islet edge, are reported as the number of positive cells per islet.

In this study, we analysed an extended cohort of T2D patients in comparison to age- and sex-matched ND multiorgan donors to evaluate ACE2 expression and distribution. We demonstrated that ACE2 is increased in pancreatic islets of T2D donors and showed a higher colocalisation rate in beta-cells of T2D versus ND donors. Notably, we considered a total of $n = 1082$ pancreatic islets across all ND and T2D donors, and the results were obtained using two different anti-ACE2 antibodies (monoclonal ACE2-MAB933 from R&D and polyclonal ACE2 Ab1538 from Abcam) adopted in the immunofluorescence analysis in an experimental cross-validation approach. The higher colocalisation rate between ACE2 and insulin in T2D suggests that, in pancreatic islets, ACE2 hyperexpression is mainly occurring in beta-cells; however, at this stage, we cannot decipher whether (*i*) ACE2 expression is increased in beta-cells already expressing the receptor, (*ii*) it is increased due to de novo expression of ACE2 occurring in previously ACE2-negative beta-cells, or (*iii*) a combination of both mechanisms. Additional analyses using imaging machine learning approaches and single cells segmentation are required to further decipher the intra-islet expression pattern of ACE2 in T2D. Overall, our data support an increased expression of the SARS-CoV2 receptor in beta-cells of T2D donors and are in-line with a recent report demonstrating the upregulation of ACE2 in pancreatic islets of T2D donors subjected to microarray and RNA sequencing[47]; indeed, Taneera and colleagues showed that ACE2 is elevated in diabetic islets but no correlation between its expression and HbA1c, age or BMI was detected, similarly to what we have observed in the present study. In contrast to these results, other previous reports did not observe the upregulation of ACE2 in T2D islets[22,23]; this can be due to the high heterogeneity of ACE2 expression or differences among T2D cohorts and/or reagents adopted. It is worth noting that in the present study we adopted two different antibodies after a detailed analysis of their specificity and efficiency testing.

Previous studies showed that other organs exposed to a diabetic milieu such as the lung, kidney and heart, showed the upregulation of ACE2, thus corroborating our findings in a different context.[42–44] Indeed, ACE2 expression was found to be increased in the bronchial epithelium and alveolar tissue of T2D donors and a linear relationship was detected between blood glucose levels and ACE2 expression in alveolar tissue.[42] In the heart tissue, ACE2 expression was significantly increased in cardiomyocytes of T2D patients with poor glycaemic control compared with ND patients and T2D patients with good glycaemic control.[43] In kidney organoids, ACE2 was expressed in tubular-like cells and an oscillatory glucose regimen induced the expression of ACE2.[44] Collectively, we can hypothesise that ACE2 expression is increased upon exposure to inflammation and/or high glucose or other stressors and that such chronic stress stimuli also exert their deleterious effect on beta-cells favouring the upregulation of ACE2. However, unlike other reports, we cannot find a significant correlation between ACE2 expression and blood glucose levels. This can be explained by the high level of glycaemia already observed in ND patients during the ICU stay (Table 1); alternatively, we can hypothesise that, at least in beta-cells, high glucose is not the main factor leading to the hyperexpression of ACE2 and that pro-inflammatory molecules may play a major role in ACE2 modulation. The latter hypothesis is also supported by Van der Heide and colleagues who did not find any association between ACE2 expression and high glucose exposure in beta-cells.[27] In addition, our previous study showed that the in vitro exposure of the beta-cell line EndoC-βH1 or primary pancreatic islets to pro-inflammatory molecules (i.e. IFNγ+IL-1β+TNFα or IFN α), but not metabolic stressors such as palmitate, can significantly increase ACE2 expression,[24] thus supporting the hypothesis of a major inflammatory-mediated mechanism governing ACE2-hyperexpression in beta-cells.[45]

In line with this hypothesis, we explored the potential contribution of inflammation in ACE2 upregulation in T2D pancreatic islets by analysing pancreatic-tissue resident $CD68^+$-macrophages. We observed an increase in peri-islet $CD68^+$ macrophages though not significant. However, such increase is supported by other previous observations, which showed a peri-islet increase in $CD68^+$-macrophages in T2D pancreata in comparison to ND donors.[48,49] Thus, additional analyses should be considered to further explore the inflammatory mechanisms leading to ACE2 upregulation in beta-cells in T2D.

It has been suggested that increased expression of ACE2 may explain the increased infectivity or severity of COVID-19 in patients with diabetes.[50] In this context, we can argue that increased expression of ACE2 in beta-cells may lead to increased susceptibility to SARS-CoV-2 infection, making them more prone to virus tropism in patients already infected with SARS-CoV-2. However, further analyses are needed to explore the expression of ACE2 cofactors (i.e. NRP1, TMPRSS2) in T2D islets and to decipher their contribution in the putative enhancement of the susceptibility of beta-cells to SARS-CoV-2 infection during COVID-19.

In conclusion, we observed the upregulation of ACE2 in pancreatic islet beta-cells of T2D donors, putatively driven by inflammatory-mediated mechanisms. Higher ACE2 expression in T2D islets might increase their susceptibility to SARS-CoV-2 infection during COVID-19 in T2D patients, thus exacerbating glycometabolic outcomes and worsening the severity of the disease.

AUTHOR CONTRIBUTIONS

All authors contributed substantially to the conception and design, data acquisition and analysis, interpretation and drafting the article or critically revising it. All authors approved the final version of the manuscript and its content. Daniela Fignani, Guido Sebastiani and Francesco Dotta are guarantors of this work and as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of data analyses.

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CONFLICT OF INTEREST STATEMENT

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.

DATA AVAILABILITY STATEMENT

The data generated in this study are available from the corresponding authors upon request.

ETHICS STATEMENT

Studies involving human participants were reviewed and approved by the local ethics committee at the University of Pisa (Pisa, Italy). Pancreata not suitable for organ transplantation was obtained with informed written consent by organ donors' next-of-kin and processed following standardized procedures.

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PEER REVIEW

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