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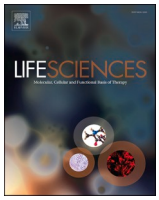

Diabetic individuals with COVID-19 exhibit reduced efficacy of gliptins in inhibiting dipeptidyl peptidase 4 (DPP4). A suggested explanation for increased COVID-19 susceptibility in patients with type 2 diabetes mellitus (T2DM)

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ABSTRACT

Aims: Dipeptidyl peptidase 4 (DPP4) has been proposed as a coreceptor for SARS-CoV-2 cellular entry. Considering that type 2 diabetes mellitus (T2DM) has been identified as the most important risk factor for SARS-CoV-2, and that gliptins (DPP4 inhibitors) are a prescribed diabetic treatment, this study aims to unravel the impact of DPP4 in the intersection of T2DM/COVID-19.

Materials and methods: We analyzed 189 serum human samples, divided into six clinical groups (controls, T2DM, T2DM + gliptins, COVID-19, COVID-19 + T2DM, and COVID-19 + T2DM + gliptins), measuring DPP4 protein concentration and activity by Western blot, ELISA, and commercial activity kits. The obtained results were verified in Huh-7 cellular models.

Key findings: Both DPP4 concentration and activity were decreased in COVID-19 patients, and as in T2DM patients, compared to controls. Despite these lower levels, the ratio of DPP4 activity/concentration in COVID-19 sera was the highest (0.782 ± 0.289 μU/ng *vs.* 0.547 ± 0.050 μU/ng in controls, $p < 0.0001$), suggesting a compensating mechanism in these patients. Supernatants of Huh-7 cells incubated with COVID-19 serum showed a consistent and significantly lower DPP4 concentration and activity. Furthermore, COVID-19 + T2DM + gliptins patients showed a higher serum DPP4 concentration and activity than T2DM + gliptin subjects ($p < 0.05$), indicating that sera from COVID-19 convalescents interfere with gliptins.

Significance: Either SARS-CoV-2 or some metabolites present in the sera of COVID-19-convalescent patients interact with soluble DPP4 or even gliptins themselves since the inhibitory effect of gliptins on DPP4 activity is being prevented. The interactions between DPP4, gliptins, and SARS-CoV-2 should be further elucidated to reveal the mechanism of action for these interesting observations.

1. Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), stopped being considered a public health emergency of international concern by the World Health Organization, on May 5, 2023. However, it is still a

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priority as many aspects of COVID-19 physiopathology remain to be elucidated, and a comprehensive picture that can help in future coronavirus outbreaks or emerging viral infections is lacking. In particular, intriguing questions have been raised about the role of protein dipeptidyl peptidase 4 (DPP4, EC 3.4.14.5) in disease progression. DPP4 was identified as the main functional receptor for the related Middle East respiratory syndrome coronavirus (MERS-CoV) [1], initially named human coronavirus-Erasmus Medical Center (hCoV-EMC). For this purpose, DPP4 was specifically copurified with the spike protein receptor-binding domain (RBD) of MERS-CoV in lysates of susceptible Huh-7 cells. Human DPP4 was also recently described as the cell entry receptor of a bat MERS-like coronavirus circulating in pangolins [2]. Regarding SARS-CoV-2, bioinformatics approaches combining human-virus protein interaction prediction and protein docking based on crystal structures have revealed that the contact residues of DPP4 with the SARS-CoV-2 RBD are consistent with those for binding with the MERS-CoV RBD. Specifically, DPP4 residues K267, R336, R317, Q344, Q286 and T288 interacted with the RBD residues of SARS-CoV-2 Q498, D405, E484, Y489/N487, N501, and Y505, respectively, suggesting a possible role as functional receptors for SARS-CoV-2 [3].

Despite this evidence, the binding of SARS-CoV-2 to DPP4 *in vivo* has not yet been demonstrated, and the canonical receptor for SARS-CoV-2 entry into cells was determined to be human angiotensin converting enzyme II (ACE2) [4], as it was the case for SARS-CoV [5]. However, DPP4 still seems to have an impact on viral entry as coreceptor [6], as well as on COVID-19 physiopathology [7]. For instance, it has been demonstrated that SARS-CoV-2 infects microglial cells that express elevated levels of DPP4 with high effectiveness but do not infect neurons lacking DPP4 [8]. Recently, the importance of DPP4 in susceptibility to SARS-CoV-2 infection and immunomodulation in different types of cancer has also been highlighted [9]. In addition, serum concentrations of DPP4 in COVID-19 patients were found to be significantly lower than those in healthy controls [10], relating low levels of soluble DPP4 (sDPP4) to disease.

Paradoxically or apparently contradictory, gliptins (DPP4 inhibitors used to treat type 2 diabetes) showed mortality improvements and anti-inflammatory effects in diabetic patients with COVID-19, positively impacting in disease progression [7,11–13]. Keys to a comprehensive understanding of this question would be the difference between the soluble and the membrane-bound forms of DPP4, and the difference between protein levels and catalytic activity. Gliptins inhibit DPP4 enzymatic activity, while they have been described to upregulate sDPP4 plasma levels without modifying the extent of the inflammatory effect. Remarkably, mice fed a high-fat, high-fructose, high-cholesterol diet and treated with the selective DPP4 inhibitor MK-0626, a clinical backup candidate for sitagliptin, showed a marked ~4-fold increase in the levels of circulating sDPP4 originating from endothelial or hematopoietic cells [14]. Notably, the authors reproduced this observation in multiple different cohorts of mice, on different diets, and treated for various time periods with MK-0626 (2–14 weeks). Mice treated with either sitagliptin or linagliptin also showed rapid increases in circulating levels of sDPP4. On the other hand, it was hypothesized that sDPP4 could act as a decoy molecule for spike proteins of SARS-CoV-2 and MERS-CoV, blocking the binding of these viral spikes to the membrane-bound DPP4 and thus decreasing virulence by impeding or handicapping cellular entry [15]. In this scenario, gliptins would also help to neutralize viral infection by increasing sDPP4 plasma levels.

Furthermore, altered circulatory DPP4 activity and levels have been found in a wide spectrum of metabolic diseases including diabetes, obesity, cardiovascular and non alcoholic fatty liver diseases (reviewed in [16–19]). Taking into account that type 2 diabetes mellitus (T2DM) has been identified as the most important risk factor for SARS-CoV-2 infection and one of the main comorbidities of COVID-19 [20,21], a systematic measurement of both activity and protein levels of DPP4 and even detailing the tissue of origin important for deciphering the complex interplay among these elements. Despite active research in the field, the interconnection between T2DM and SARS-CoV-2 infection, as well as the relevance of DPP4, establishing causality and determining underlying mechanisms and clinical implications is still lacking. A reason for this lack of clarity is that DPP4 can catalyze the digestion of multiple chemokines, neuropeptides, and regulatory peptides. Given its multifunctional character, this exopeptidase has implications for many physiological and pathological processes, such as glycemic control (and T2DM), cell migration and proliferation (or cancer), as well as the immune system and associated inflammatory responses, adding many confounding factors to patient variability. In addition, the hallmark proinflammatory cytokine storm of COVID-19, driven by an amplified and aberrant immune response to SARS-CoV-2 infection, is also affected by DPP4 regulation, contributing to tissue damage and organ failure [7]. Then, multiple factors affect the outputs of these measurements.

This study aims to unravel the impact of DPP4 on the intersection between T2DM and SARS-CoV-2 infection, to glimpse its role in COVID-19 physiopathology and the inflammatory context of both diseases, and to determine whether the decrease in sDPP4 levels is a cause or a consequence of SARS-CoV-2 infection. For that purpose, we measured the protein levels and activity of sDPP4 in 189 serum human samples from control individuals, as well as patients with and without diabetes and/or COVID-19, distinguishing between diabetic subjects treated with gliptins or other hypoglycemic agents.

2. Materials and methods

2.1. Patients and human serum samples

Samples from patients included in this study were provided by the BioBank Hospital Ramón y Cajal-IRYCIS (National Registry of Biobanks B.0000678), integrated in the Biobanks and Biomodels Platform of the Spanish Institute of Health Carlos III (ISCIII, PT20/00045), and they were processed following standard operating procedures with the approval of the Ethical and Scientific Committees. A total of 189 venous serum samples were purchased from this Biobank, and divided into six groups of patients with and without diabetes and/or COVID-19. Specifically, the clinical groups were as follows: control patients ($n = 40$, without diabetes and without COVID-19), diabetic patients ($n = 38$), diabetic patients treated with gliptins ($n = 19$), COVID-19 patients (n = 40), COVID-19 diabetic patients (n = 38), and COVID-19 diabetic patients treated with gliptins ($n = 14$). Of them, patients classified as COVID-19 were hospitalized from March to May 2020, in Hospital Ramón y Cajal because of COVID-19-related clinical symptoms, varying in severity: mild, moderate, severe, critical, and deceased. In addition to the clinical diagnosis, these patients had a positive result in a reverse-transcription qualitative real-time polymerase chain reaction (rRT-PCR) test when assaying a nasopharyngeal swab. The inclusion and exclusion criteria changed depending on the group but, in general, both sexes were eligible with an age > 18 (adult or older adult). For COVID-19 patients, a clinical diagnosis of COVID-19, and a positive rRT-PCR test were needed, but their lack was considered an exclusion criterion. Demographic data and clinical features, when available, were collected according to the patient record system (for a detailed analysis, see Results). The study was approved by the Ethics Committee of the University of Alcalá (CEI/HU/202/37) and conforms to the principles outlined in the Declaration of Helsinki.

Determination of protein biomarker levels in human serum samples.

The presence of the IgG anti-SARS-CoV-2 spike protein S1 subunit (receptor binding domain -RBD- region) was measured in patient serum samples using an optimized enzyme-linked immunosorbent assay (ELISA)-based serology protocol, previously developed by our laboratory [22]. Meanwhile, DPP4, and IL-6 protein levels were determined using commercially available ELISA kits from Invitrogen, with catalog codes #EHDPP4, and #88-7066-77, respectively (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA). Analyses were performed according to the manufacturer's instructions, assayed in duplicate, and

read at 450 nm on a iMark™ microplate absorbance reader (Bio-Rad, Hercules, CA, USA). Standard curves and concentrations were determined using Microplate Manager® 6 software, Version 6.3 (Bio-Rad, Hercules, CA, USA).

2.2. Measurement of DPP4 activity

DPP4 enzymatic activity was determined in 189 human serum samples, as well as supernatants and cellular extract fractions of Huh-7 cell cultures, using the Dipeptidyl peptidase IV (DPP4) Activity Assay Kit from Abcam (Cambridge, UK, cat. ab204722), according to the manufacturer's indications. For this purpose, Nunc™ F96 MicroWell™ non treated black plates and flat bottom plates were used (Thermo Fisher Scientific, MA, USA, cat. 237,105). The fluorescence per well in relative fluorescence units (RFU) was determined using FLUOstar Omega fluorimeter (BMG Labtech, Germany) at Ex/Em = 360/460 nm, taking measurements every minute for 20 min at 37 °C in the dark. RFU was extrapolated to pmol through a curve pattern and following the manufacturer's instructions, pmol/min/mL (=μU/mL) was determined for each measurement. Then, the mean in the first 10 min (timeline showing optimal activity with linear increases) was calculated.

2.3. Cell cultures

The human hepatocarcinoma cell line Huh-7 was purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (JCRB0403, Tokyo, Japan). Cells were routinely grown in EMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 IU/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B (Invitrogen, Paisley, UK) and 10 % fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). Huh-7 cells were incubated at 37 °C in 5 % CO_2 and routinely tested for *Mycoplasma* infection. For experiments, cells were plated and grown for 24 h. Then, the medium was replaced with EMEM (0.9–1.1 g/L of glucose) or DMEM-high glucose (4.5 g/L glucose) (Sigma-Aldrich, St. Louis, MO, USA). The next day, sitagliptin (PHR1857, Sigma-Aldrich, St. Louis, MO, USA) was added to the corresponding wells. These conditions were maintained for 7 days.

2.4. Western-blot

2.4.1. Reducing conditions

Proteins for Western blotting were isolated by resuspending cells in lysis buffer (50 mM Tris-HCl pH 7.4, 0.8 M NaCl, 5 mM $MgCl_2$, 0.1 % Triton X-100), containing a protease inhibitor and a phosphatase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). They were then incubated on ice for 15 min and cleared by microcentrifugation at 15.870 g for 10 min and 4 °C. For experiments performed with supernatants, they were collected, centrifuged for 5 min at 300*g* to remove cell debris, and transferred to clean tubes. The protein concentration of cell extracts and supernatants was determined by the Bradford method, and samples were maintained at −20 °C.

Then, twenty micrograms of total protein/lane was separated by SDS-polyacrylamide gel electrophoresis (SDS PAGE) and transferred onto a PVDF membrane. Membranes were incubated overnight at 4 °C with primary antibodies againstDPP4 and *β*-actin obtained from Cell Signaling Technology (Danvers, MA, USA), and Sigma-Aldrich (St. Louis, MO, USA), respectively. After washing in T-TBS, the membranes were incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1:5000) for 2 h at room temperature. These immunoglobulins were purchased from Sigma-Aldrich, and Cell Signaling Technology, respectively. The immune complex was visualized with an ECL system (Cell Signaling Technology). Protein expression levels were quantified using ImageJ (National Institutes of Health, Bethesda, MD USA) and were expressed as fold changes relative to the control treatment.

2.4.2. Nonreducing conditions

Samples were prepared using 2× loading buffer without SDS or β-mercaptoethanol (0.5 M Tris-HCl pH 6.8, 50 % glycerol, 1 % bromophenol blue). Electrophoretic separation was performed using polyacrylamide gels and electrophoresis buffer without SDS.

2.5. Statistical analysis

The results are expressed as the mean ± standard deviation (SD), and experiments were performed at least in triplicate to assure reproducibility. Pearson's correlation coefficient was calculated using IBM SPSS statistics version 27 (IBM Corp., Armonk, NY, USA), while one-way ANOVA and correlation tests were used for comparisons between various groups *via* GraphPad Prism 9 (San Diego, CA, USA), assuming a normal distribution and equal SDs in the analysis of the experimental data. For the comparison of clinical and demographic characteristics in patients subdivided into men and women, two-way ANOVA tests were used *via* GraphPad Prism 9. A p value < 0.05 was considered statistically significant, and the legend for differences between groups of patients compared to controls or to other groups are preceded by asterisks (*) as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. For the sake of clarification and Figures interpretation, only the most relevant comparisons with biological sense are included.

3. Results

3.1. Clinical and demographic characteristics of patient samples

In this study, 189 patients were included and subdivided into six clinical groups: control individuals (Control; $n = 40$, without diabetes and without COVID-19), type 2 diabetic patients (T2DM; $n = 38$), type 2 diabetic patients treated with gliptins (DIA-GLI; $n = 19$), COVID-19 patients (n = 40), diabetic patients suffering from COVID-19 (COV-DIA; n = 38), and gliptin-treated diabetic patients suffering from COVID-19 (COV-DIA-GLI; $n = 14$) (Table 1). The proportion of males was higher than that of females, ranging from approximately 75:25 to 60:40, depending on the group. The baseline demographic and clinical characteristics of these patients are summarized in Table 2. The average age of patients was found to be around their fifties in controls, and their sixties in the rest of the groups, except for diabetic men with COVID-19, and gliptin-treated diabetic women with COVID-19, who were in their seventies. All the age differences with respect to controls were statistically significant. The mean weight ranged from 81.3 ± 7.8 kg in men of COV-DIA-GLI patients to 87.6 ± 13.9 kg of gliptin-treated diabetic patients, while women ranged from 65.9 ± 9.8 kg of control individuals to 85.7 ± 20.7 of COV-DIA-GLI subjects, but no significant differences were found in this parameter. Lastly, regarding the body mass index (BMI), unique differences were found in women between any group including 'diabetic' patients and the control, but not COVID-19 alone, indicating that diabetes plays an important role in determining the BMI.

Beyond these clinical and demographic features, other evaluations should be made. Patients classified as COVID-19 (in the last three clinical groups, total $n = 92$) were hospitalized from March to May 2020 (except one patient in December 2020), in Hospital Ramón y Cajal because of COVID-19-related clinical symptoms, varying in severity: mild ($n = 13$), moderate ($n = 48$), severe ($n = 21$), critical ($n = 4$), and deceased (n = 5). All of them were tested for a positive qPCR, to validate and support the clinical diagnosis. The COV-DIA group was composed of 38 patients: 6 without treatment for T2DM, and 32 treated with metformin (one of them supplemented with insulin). Meanwhile, in the COV-DIA-GLI group ($n = 14$), 6 patients received vildagliptin, 6 linagliptin, and 2 sitagliptin, either as unique treatment (1), combined with metformin (8), metformin and insulin (3), metformin and empagliflozin (1), or metformin and dapagliflozin (1). On the other hand, 9 non-COVID-19 diabetic patients were insulin-dependent (24 %), while only 2 (11 %) of the gliptin-treated T2DM subjects needed supplementation

Table 1
Clinical groups used in the study and the abbreviation design.

Group	Control	Diabetes	Diabetes + gliptins	COVID-19	COVID-19 + Diabetes	COVID-19 + Diabetes + gliptins
Clinical condition	Healthy	Diagnosed type 2 Diabetes mellitus	Type 2 Diabetes mellitus treated with DPP4 inhibitors gliptins (Vildagliptin, Sitagliptin, Linagliptin)	Patients with diagnosed COVID-19 by rRT-PCR	Type 2 DM patients with diagnosed COVID-19 by rRT-PCR	Type 2 DM patients with diagnosed COVID-19 by rRT-PCR and treated with DPP4 inhibitors gliptins (Vildagliptin, Sitagliptin, Linagliptin)
n	40	38	19	40	38	14
Abbreviation	Control	T2DM	DIA-GLI	COVID-19	COV-DIA	COV-DIA-GLI

Table 2
Characteristics of the clinical groups considering sex differences for the physical parameters. The six clinical groups employed in this study (and the number of total individuals in each one) are presented, divided by sex with the corresponding percentage. Then, values for age (in years), weight (in kg), size (in cm) and body mass index (BMI) (in kg/m^2) are shown in the following columns. Above, in bold, the mean value + SEM (standard error of the mean) is presented. Below, the median and the maximum and minimum values are in parentheses (MIN - MAX).

		Samples (%)	Age (years)	Weight (Kg)	Size (cm)	BMI
Control [40]	Men	70	**52 ± 5** 52 (42–61)	**83.4 ± 8.6** 82.5 (68–108)	**174 ± 8.2** 173 (150–190)	**27.6 ± 3.1** 26.7 (22.9–36.9)
	Women	30	**54 ± 6** 52 (48–66)	**65.9 ± 9.8** 63.5 (54–90)	**163.7 ± 7** 161.5 (157–180)	**24.6 ± 3.4** 24.4 (21–34.3)
Diabetes [38]	Men	58	**65 ± 10** 68 (46–82)	**82.7 ± 21.8** 79.8 (54–168)	**164.4 ± 18.1**169.5 (84–174)	**27.7 ± 3.9** 27.6 (20.3–36.6)
	Women	42	**67 ± 7** 67 (51–79)	**74.4 ± 16** 71.4 (57–121)	**155.4 ± 8.5** 155.5 (141–170)	**30.6 ± 4.8** 29.1 (22.5–42)
DIA-GLI [19]	Men	68	**65 ± 6** 67 (55–79)	**87.6 ± 13.9** 84.5 (69–111.4)	**166.4 ± 3.4** 166 (160–173)	**31 ± 4** 30.3 (25.5–39)
	Women	32	**69 ± 9** 70 (55–81)	**85.2 ± 8.9** 87 (72.1–95)	**152.4 ± 5.4** 151 (145–160)	**36.9 ± 5.5** 35.3 (30.5–45.2)
COVID-19 [40]	Men	70	**67 ± 11** 65 (46–85)	**82.8 ± 8.3** 82 (68–105)	**170.8 ± 6.4** 170 (158–185)	**28.4 ± 2.7** 28 (21–34.6)
	Women	30	**69 ± 12** 67 (55–98)	**71.1 ± 14.9** 65.8 (51–92)	**155.4 ± 6.5** 155 (148–170)	**28.4 ± 6.1** 27 (23.3–40.9)
COV-DIA [38]	Men	74	**70 ± 10** 71 (50–87)	**85.4 ± 22** 84 (52–150)	**166.2 ± 7.2** 167.5 (147–180)	**31 ± 7.9** 29.8 (17.1–51.7)
	Women	26	**69 ± 10** 68 (58–92)	**73.9 ± 9.8** 75 (60–89)	**155 ± 6.6** 155 (144–165)	**30.9 ± 3.9** 32.4 (22.8–36.2)
COV-DIA-GLI [14]	Men	64	**66 ± 6** 66 (56–77)	**81.3 ± 7.8** 80 (70.4–97.4)	**166.8 ± 6** 169 (157–175)	**29.3 ± 2.5** 28.6 (24.5–33.3)
	Women	36	**73 ± 8** 72 (63–86)	**85.7 ± 20.7** 84 (54.6–119.8)	**158.6 ± 9.1** 155 (148–174)	**33.6 ± 5.2** 35 (24.9–39.6)

with insulin. The DPP4 inhibitors provided in this last group were also vildagliptin ($n = 12$), sitagliptin ($n = 5$), and linagliptin ($n = 2$). Control individuals were not affected by T2DM or COVID-19, although twelve of them presented several mild or acute illnesses (30 %). In these cases, diseases affect the skin (2 individuals), cardiovascular (2) and circulatory (2) systems, bones (3), or ocular alterations (3). Finally, these 189 human samples were used for the following biochemical analysis.

3.2. Antibodies and cytokine concentration

Once the SARS-CoV-2 infection was corroborated by qPCR and the severity of COVID-19 was evaluated by clinical settings, the biochemical characterization of these serum samples was determined by measuring the levels of IgG antibodies against the S1 protein (RBD region) of SARS-CoV-2 and those of the proinflammatory cytokine IL-6 in all patients and controls. As shown in Fig. 1A, control individuals and patients only with diabetes (including those treated with gliptins) did not have antibodies, indicating a lack of infection and vaccination (serum samples were obtained before 2020). As expected, COVID-19 patient groups had a remarkable concentration of IgG antibodies. A large deviation could be observed within these last patients, which could provide an answer to the different immune responses of each individual and to the timing since the infection onset at which the serum was collected, that is the phase or timeline of infection.

Subsequently, we assayed the levels of IL-6, a proinflammatory cytokine that has been involved in COVID-19 physiopathology and is considered a good marker for the cytokine storm, therefore adding an indicator of the clinical presentation and the disease outcome. Therefore, as shown in Fig. 1B, l the concentration of IL-6 was increased in the three groups of COVID-19 patients compared to control individuals, and the differences were statistically significant in all cases ($p < 0.01$ in COVID-19 nondiabetic patients, and $p < 0.0001$ in patients with COV-DIA, treated or not with gliptins). In addition, there was also a statistically significant difference when comparing groups of diabetic patients with or without COVID-19. That is, diabetic *versus* COVID-19 diabetic patients ($p < 0.0001$) and DIA-GLI compared to diabetic plus gliptins individuals also suffering from COVID-19 (COV-DIA-GLI) ($p < 0.001$). Interestingly, when enlarging the graphic for better visualizing values of diabetic sera (Fig. 1B, panel on the right), the IL-6 concentration was slightly higher in diabetic patients relative to controls, indicating a subclinical inflammation that usually accompanies T2DM [23]. Altogether, our results indicated that cytokine and IgG concentrations were according to the clinical classification of patients, and consequently, the serum samples obtained from these patients were suitable for further investigations.

3.3. Alteration of gliptins' inhibitory activity over DPP4 in sera of diabetic COVID-19 patients

To study the role of the exopeptidase DPP4 in the context of COVID-19 and T2DM, we determined the protein concentration in patients' sera by using ELISA. The results showed that the concentration of DPP4 was significantly lower in COVID-19 patients than in controls (987 ± 326 ng/mL *versus* 1654 ± 239 ng/mL, $p < 0.0001$) (Fig. 2A). Furthermore, it

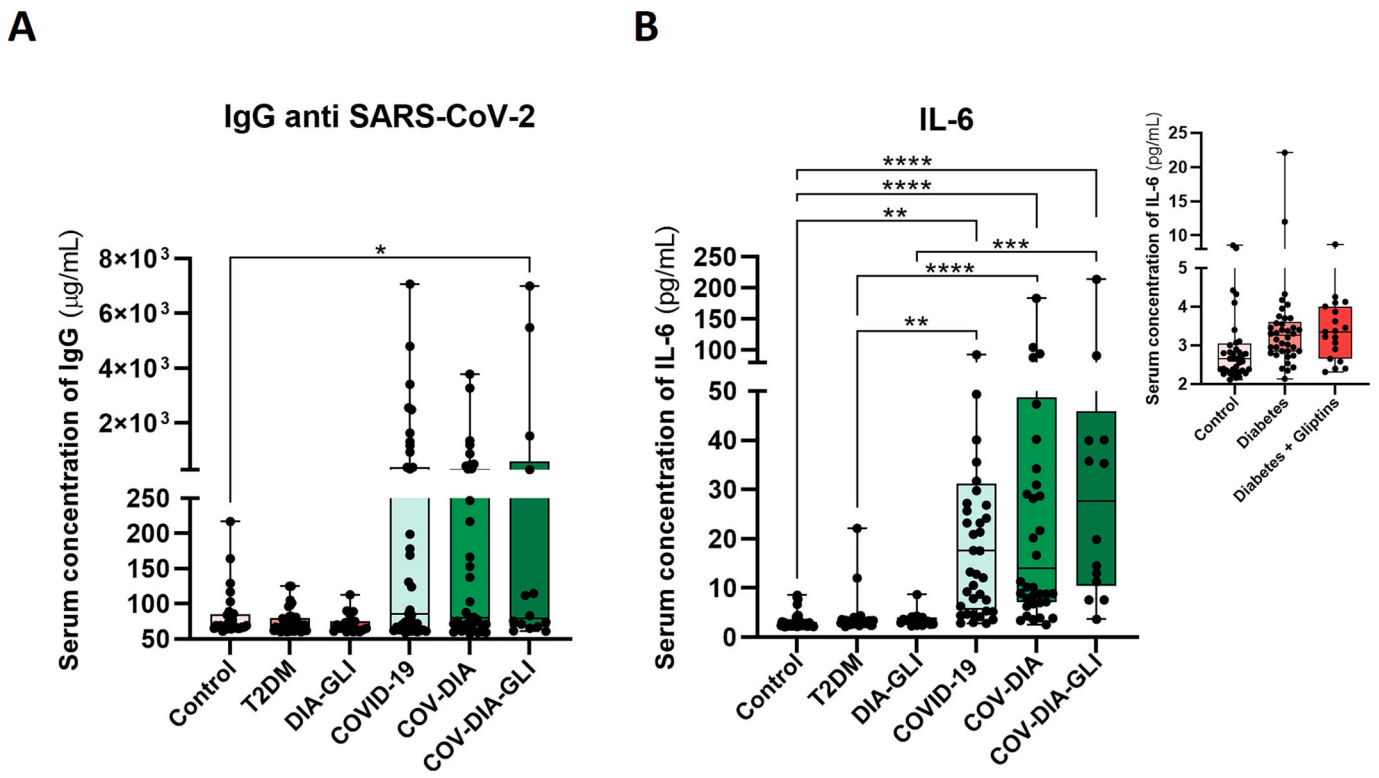


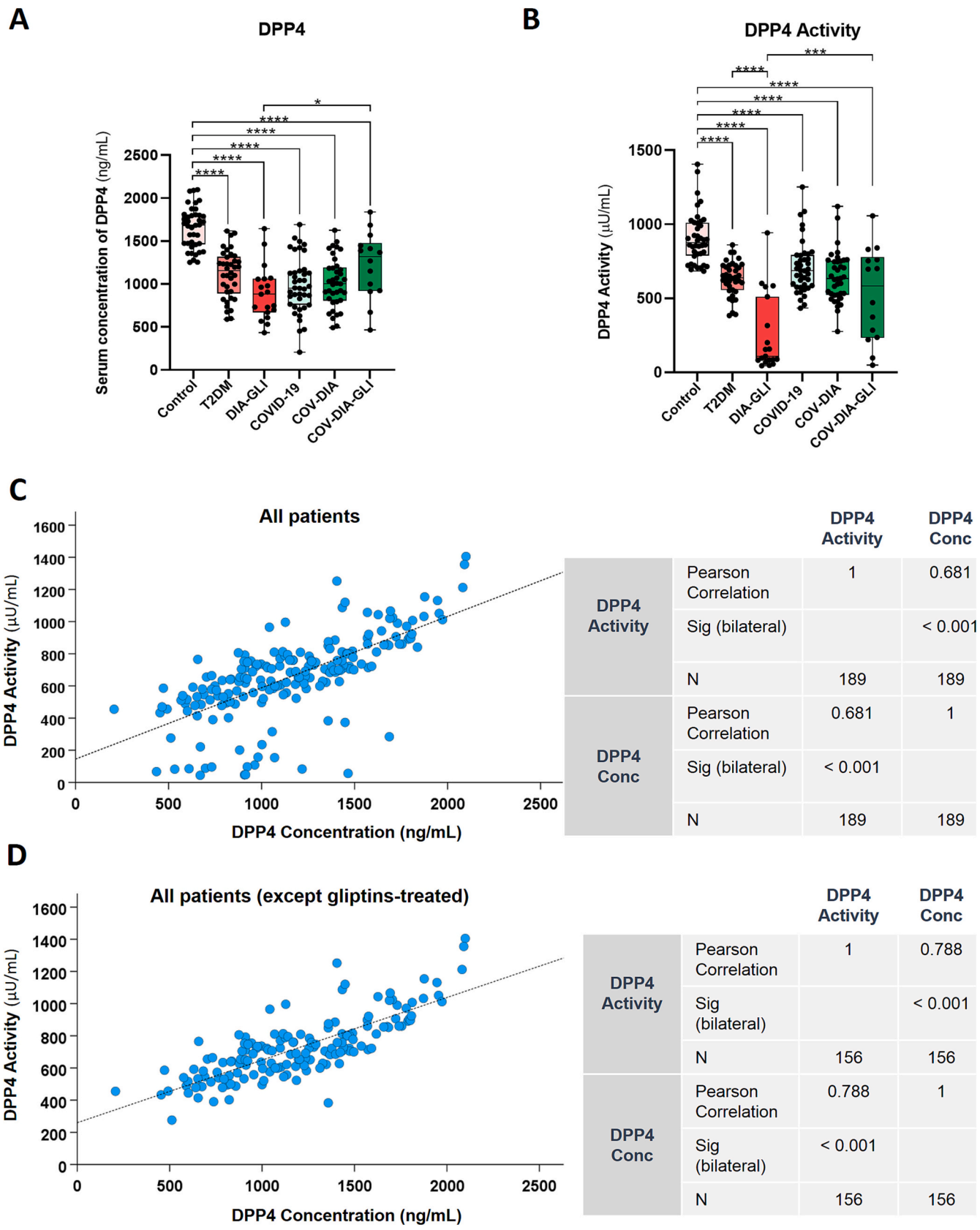
Fig. 1. Concentration of IgG anti-S1 of SARS-CoV-2 and IL-6 in all patients. (A) Concentration of anti-S1 (RBD region) IgG of SARS-CoV-2, measured by an ELISA previously described [22], in the sera of six groups of patients: controls ($n = 40$), type 2 diabetic patients (T2DM, $n = 38$), type 2 diabetic patients treated with gliptins (DIA-GLI, $n = 19$), COVID-19 patients (n = 40), diabetic patients suffering from COVID-19 (COV-DIA, n = 38), and gliptin-treated diabetic patients suffering from COVID-19 (COV-DIA-GLI, $n = 14$). Twelve control samples collected in 2021 were excluded from this particular analysis to avoid misconstructions. (B) Concentration of the pro-inflammatory cytokine IL-6, measured by ELISA in the serum of all patients (on the left), and enlargement of diabetic and control individuals for proper visualization (panel on the right).

is worth noting that diabetic patients, both treated or not treated with DPP4 inhibitors (*i.e.*, gliptins) and suffering or not suffering from COVID-19, also exhibited decreased concentrations of DPP4 relative to controls ($p < 0.0001$). In summary, data showed that control individuals displayed significantly higher concentrations of DPP4 than any other group. However, it should be highlighted that the concentration of DPP4 in COV-DIA-GLI was higher than that in COV-DIA under other therapeutic regimens or even in nondiabetic COVID-19 patients (Fig. 2A). In this group of gliptin-treated individuals, the DPP4 concentration was not as low as expected, although gliptins have been previously described to reduce the activity of DPP4 while increasing their circulating soluble protein levels, so these results are also in line with the literature discussed above [14]. Interestingly, COV-DIA-GLI patients showed increased DPP4 serum concentrations when compared with DPP4 levels of DIA-GLI patients not suffering from COVID-19 (1222 ± 389 ng/mL *versus* 893 ± 315 ng/mL, $p < 0.05$), indicating that sera of COVID-19 convalescents interfere in some way with gliptins.

Afterward, we determined the activity of DPP4 in the serum samples by measuring its ability to cleave a proline-containing substrate and then release a quenched fluorescent group (7-amino-4-methyl coumarin) that was easily detected. Fluorescence was monitored for 20 min in all patients, and activity was calculated during the first 10 min to ensure that the reaction was linear (Supplementary Fig. S1). Thus, the results revealed that DPP4 enzymatic activity was decreased in all groups in comparison with controls, yielding statistically significant differences in all cases ($p < 0.0001$) (Fig. 2B). It is remarkable that gliptins inhibited DPP4 activity in diabetic patients ($p < 0.0001$), but not when there was also an infection by SARS-CoV-2, yielding a new alteration in the biochemical characterization of sera between COVID-19 and non-COVID-19 patients. In line with this difference, both DIA-GLI patients with or without COVID-19 showed significant differences in the DPP4 catalytic activity of their sera, slightly decreasing in the former samples, while absolutely dropping in the latter samples ($p < 0.001$). Altogether, as shown in Fig. 2C, DPP4 activity maintained a good correlation with DPP4 protein levels, given that the Pearson correlation coefficient from all patients was 0.681. However, the correlation improved by not considering patients treated with gliptins, since gliptins present in the serum could interfere with the activity assay. Therefore, after the exclusion of patients treated with gliptins, the Pearson correlation coefficient increased to 0.788 (Fig. 2D), which indicated that most of the DPP4 present in the sera was active.

3.4. Interconnection and correlations between biochemical features

To investigate whether DPP4 concentration or activity were associated with the inflammatory response, we calculated their correlation with IL-6. On the one hand, DPP4 and IL-6 concentrations were negatively correlated, with a Pearson coefficient of −0.334 (Fig. 3A), considering all patients except those treated with gliptins and including the controls. Likewise, DPP4 activity showed a negative correlation with IL-6 concentration in these patients (Fig. 3B), although with a lower Pearson coefficient (−0.221). These findings were surprising and did not agree with the proinflammatory role that has been assigned to DPP4 in the literature, as we will discuss later. Nevertheless, the correlation was not strong, so we decided to analyze all the possible correlations between the parameters measured in this study (*i.e.*, IgG, IL-6, DPP4 levels and DPP4 activity), taking into account four clinical groups (controls, T2DM, COVID-19, and COV-DIA patients, putting aside gliptin-treated individuals) (Supplementary Fig. S2). The strongest correlation was that of DPP4 levels and DPP4 activity, as outlined in Fig. 2D.



		DPP4 Activity	DPP4 Conc
DPP4 Activity	Pearson Correlation	1	0.681
	Sig (bilateral)		< 0.001
	N	189	189
DPP4 Conc	Pearson Correlation	0.681	1
	Sig (bilateral)	< 0.001	
	N	189	189

		DPP4 Activity	DPP4 Conc
DPP4 Activity	Pearson Correlation	1	0.788
	Sig (bilateral)		< 0.001
	N	156	156
DPP4 Conc	Pearson Correlation	0.788	1
	Sig (bilateral)	< 0.001	
	N	156	156

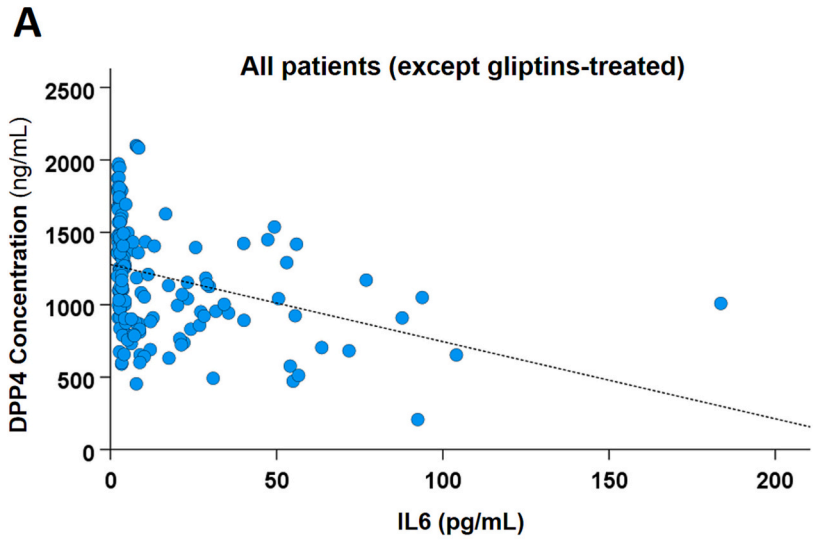
Fig. 2. DPP4 protein levels, enzymatic activity, and correlation between both in human sera samples. (A) Protein levels of DPP4, measured by an ELISA, and (B) DPP4 activity (µU/mL) determined by using a commercially available kit, in the sera of six groups of patients: controls ($n = 40$), type 2 diabetic patients (T2DM, $n = 38$), type 2 diabetic patients treated with gliptins (DIA-GLI, $n = 19$), COVID-19 patients (n = 40), diabetic patients suffering from COVID-19 (COV-DIA, n = 38), and gliptin-treated diabetic patients suffering from COVID-19 (COV-DIA-GLI, $n = 14$). (C) Pearson correlation coefficient of DPP4 activity and DPP4 concentration from all patients. (D) Pearson correlation coefficient of DPP4 activity and DPP4 concentration from all patients (excluding those treated with gliptins).

3.5. COVID-19 patients showed the highest DPP4 activity/concentration ratio

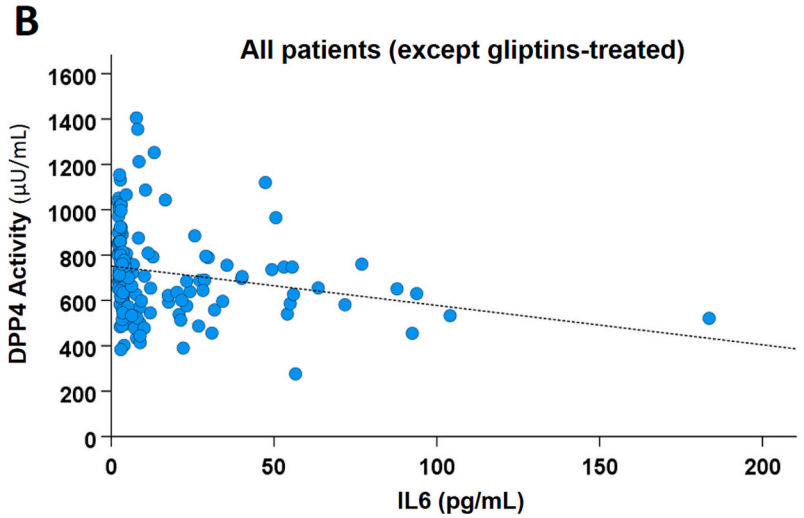
In this analysis, it should be considered that the soluble form of DPP4 (sDPP4) is the circulating extracellular fragment of the transmembrane DPP4 expressed in many different cells, which is active in its homodimer conformation. Therefore, it could be possible that DPP4 concentration does not faithfully reflect its activity, as it has been reported in various pathological conditions [24]. To further investigate this question and examine the different variances in protein concentration and activity in the six clinical groups, we calculated the ratio activity/concentration of DPP4. Surprisingly, although control patients had higher DPP4 concentration and higher DPP4 activity measurements, there was another clinical group that exhibited the highest ratio of DPP4 activity/protein concentration, which was that of COVID-19 patients, with a strong significance (0.782 ± 0.289 μU/ng *vs.* 0.547 ± 0.050 μU/ng, $p < 0.0001$) (Fig. 4A). This fact seems to reflect that in COVID-19 patients, the lower levels of sDPP4 are compensated by an increase in the activity per ng of protein. On the other hand, the levels of COV-DIA and COV-DIA-GLI patients were significantly lower than those of patients with COVID-19. This fact seems to suggest that the decrease in DPP4 concentration and the increase in DPP4 activity may be related to the severity of COVID-19. To address this hypothesis, we presented the activity/concentration ratio of DPP4 *versus* COVID-19 severity. As shown in Fig. S3, there was a positive correlation between the DPP4 activity/concentration ratio and the first three grades of severity. The correlation disappears when patients die from COVID-19 (grade 4) because other factors, such as the decrease in blood volume, may come into play in this fatal scenario. This result suggests that the relationship between DPP4 activity and concentration could play a role in COVID-19 physiopathology. In addition, in gliptin-treated diabetic patients DIA-GLI, the ratio activity/concentration of DPP4 clearly dropped compared to control individuals ($p < 0.0001$) (Fig. 4A). In fact, in this group, there was a poor nonsignificant correlation between DPP4 activity and concentration (Pearson coefficient of 0.331), indicating the ability of gliptins to inhibit DPP4 enzymatic activity (Fig. 4B). Conversely, the inhibitory effect of gliptins on DPP4 activity was not observed in COV-DIA-GLI patients who did have a higher and better correlation between DPP4 levels and activity (Pearson coefficient of 0.547) (Fig. 4C). These results indicate that the ability of gliptins to inhibit DPP4 activity in our assay is lost or hampered in COVID-19 samples, suggesting that there is any factor in the sera of COVID-19 patients that interacts with DPP4.

3.6. DPP4 protein expression and activity in Huh-7 culture cells

Subsequently, we investigated the effect of serum from COVID-19



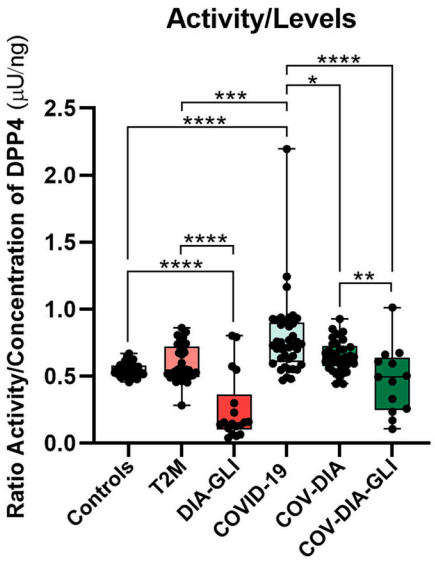
		DPP4 Conc	IL-6
DPP4 Conc	Pearson Correlation	1	-0.334
	Sig (bilateral)		< 0.001
	N	156	156
IL-6	Pearson Correlation	-0.334	1
	Sig (bilateral)	< 0.001	
	N	156	156



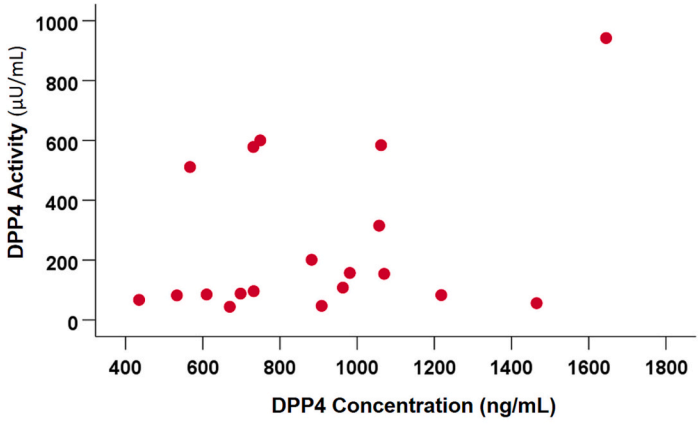
		DPP4 Activity	IL-6
DPP4 Activity	Pearson Correlation	1	-0.221
	Sig (bilateral)		< 0.001
	N	156	156
IL-6	Pearson Correlation	-0.221	1
	Sig (bilateral)	< 0.001	
	N	156	156

Fig. 3. DPP4 correlations with inflammation. Correlation of DPP4 concentration (A) or DPP4 activity (B) with the concentration of the proinflammatory cytokine IL-6 from all patients, excluding those treated with gliptins. Graphical comparison (on the left) and Pearson correlation coefficient (on the right).

A

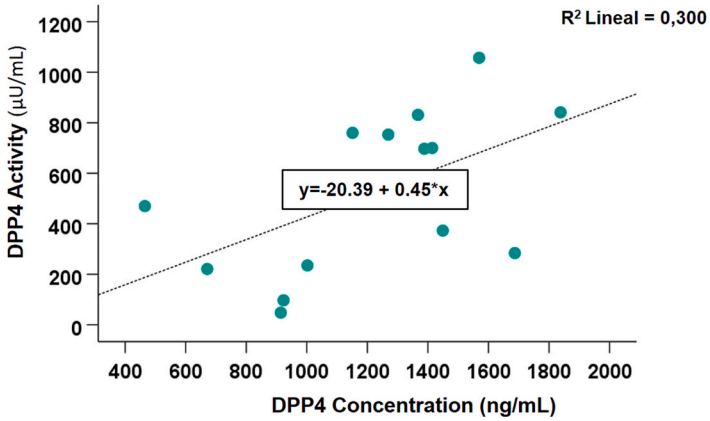


B



		DPP4 Activity	DPP4 Conc
DPP4 Activity	Pearson Correlation	1	0.331
	Sig (bilateral)		0.166
	N	19	19
DPP4 Conc	Pearson Correlation	0.331	1
	Sig (bilateral)	0.166	
	N	19	19

C



		DPP4 Activity	DPP4 Conc
DPP4 Activity	Pearson Correlation	1	0.547
	Sig (bilateral)		0.043
	N	14	14
DPP4 Conc	Pearson Correlation	0.547	1
	Sig (bilateral)	0.043	
	N	14	14

Fig. 4. Ratio of DPP4 activity *versus* DPP4 concentration, and correlations. (A) Boxes and whiskers represent the DPP4 activity/concentration ratio (µU/ng) in the six clinical groups analyzed in this paper (controls, $n = 40$), type 2 diabetic patients (T2DM, $n = 38$), type 2 diabetic patients treated with gliptins (DIA-GLI, $n = 19$), COVID-19 patients (n = 40), diabetic patients suffering from COVID-19 (COV-DIA, n = 38), and gliptin-treated diabetic patients suffering from COVID-19 (COV-DIA-GLI, $n = 14$). (B) Correlation of DPP4 activity and DPP4 concentration in non-COVID-19 gliptin-treated T2DM patients (graphical representation on the left and Pearson correlation coefficient on the right) or in (C) COVID-19 gliptin-treated T2DM patients (COV-DIA-GLI).

patients on the expression of DPP4 at the protein level, along with its enzymatic activity, in Huh-7 cells that have been previously demonstrated to express DPP4 [1]. For that purpose, Huh-7 cells were incubated for 7 days in the presence of pooled serum from control patients, either by themselves or supplemented with glucose or glucose plus sitagliptin, and the same conditions were repeated with pooled serum from COVID-19 patients, to emulate the *in vivo* situation. First, supernatants were collected to determine the presence of DPP4 protein by Western blot, and the obtained bands were analyzed for the densitometric values. As shown in Fig. 5A, levels of DPP4 significantly decreased in the supernatant of cells incubated with the serum of COVID-19 patients (either with low glucose, high glucose, or high glucose + sitagliptin), relative to the control (serum from control patient and low glucose) ($p < 0.05$), in agreement with the results observed in the patient's sera. This decrease was also significant when comparing the same glucose/sitagliptin conditions, in samples cultured with sera from control *versus* COVID-19 patients ($p < 0.05$). In addition, the protein concentration of DPP4 was also determined by ELISA (Fig. 5B), showing a reduction in the supernatants of cells incubated with the serum of COVID-19 patients, although to a lesser extent.

It has been previously described that prolonged DPP4 inhibition increases plasma levels of sDPP4 during inflammation in mice and humans [25]. Therefore, we wondered whether the opposite was possible and whether a decrease in DPP4 levels could induce its activity. Therefore, we determined the enzymatic activity of DPP4 in these samples. Interestingly, supernatants of cells incubated with COVID-19 serum showed a significant decrease in DPP4 activity (Fig. 5C), according to data observed in patient sera. However, in these cellular experiments, supernatants cultured with sitagliptin exhibited a drop in DPP4 activity, not shown in the case of sera from COV-DIA-GLI patients. This could be due to the exogenous addition of this gliptin to the medium (and then the supernatant), which could interfere with the commercial kit for activity measurement. Last, the ratio of DPP4 activity/concentration obtained in Huh-7 supernatants did not display differences, beyond those of sitagliptin-containing samples (Fig. 5D).

Afterward, to investigate whether the decrease in the supernatant concentration of DPP4 was due to lower DPP4 expression in the cells, we determined the DPP4 protein levels and activity in the Huh-7 cellular

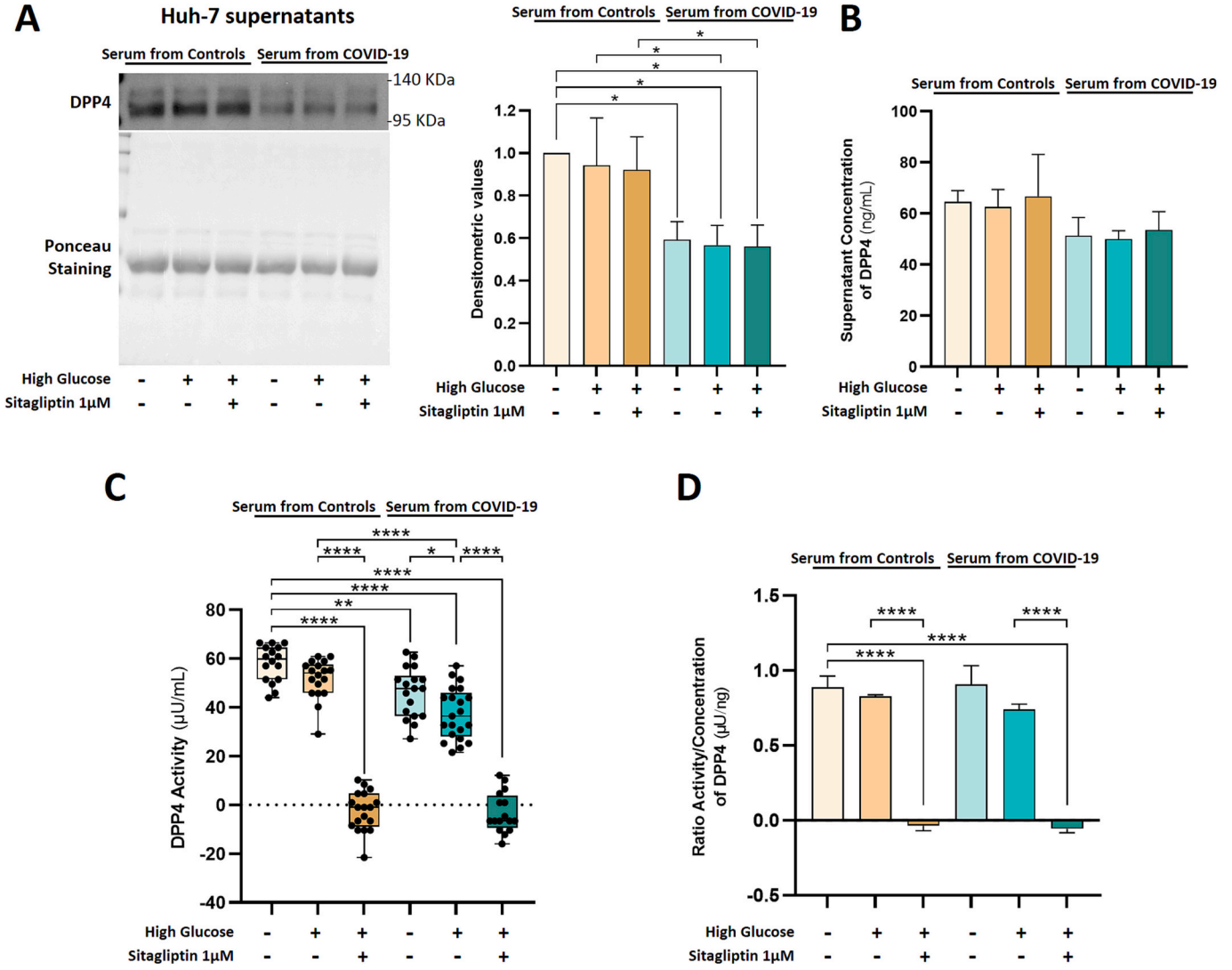


Fig. 5. DPP4 protein concentration, enzymatic activity, and ratio activity/concentration in supernatants of Huh-7 cells. In all panels, Huh-7 cells were cultured for 7 days in the presence of serum from control patients (alone, supplemented with glucose, or glucose plus sitagliptin), and the same conditions were repeated with serum from COVID-19 patients. (A) Protein levels of DPP4 measured by Western blot, showing the gel (on the left) and the densitometric values (on the right). (B) Protein concentration of DPP4 (ng/mL) measured by a commercial ELISA. (C) DPP4 activity (µU/mL) determined by using a commercially available kit. (D) Ratio of DPP4 activity *versus* DPP4 concentration (measured by ELISA) in the six conditions. Experiments were repeated three times with different patient sera, and the results are the mean ± standard deviation (SD) of the replicates.

fraction, under all the conditions indicated above. The sole significant decrease in DPP4 concentration was exhibited by cells cultured with the control serum and supplemented with glucose, while the rest of the conditions (including those incubated with COVID-19 serum patients) were comparable to the control (Fig. 6A). This result is truly fascinating because it reflects that the reduction observed in the supernatants of cells incubated with COVID-19 sera is not due to a lower expression inside the cell but to a lower trafficking to the membrane or even proteolysis of the membrane-bound DPP4. On the other hand, looking at Huh-7 cells cultured with control sera, it should be noted that glucose supplementation to the medium caused a significant decrease in the cellular levels of DPP4, while the addition of sitagliptin to the medium restored the levels of DPP4 (Fig. 6A). Next, we assayed the activity of DPP4 in these cellular fractions, observing similar values in cells treated with control sera as with COVID-19 sera in low glucose (Fig. 6B), while the rest of the conditions showed significant increases relative to these previous ones. Altogether, these results seem to suggest that SARS-CoV-2 infection induces a decrease in the concentration of sDPP4 that is trying to be compensated with elevated expression by the cell and higher activation, although the key trigger and the molecular mechanism for this effect remain to be elucidated.

Aiming to discard the possibility that changes in DPP4 activity are due to the conformational structure of DPP4, we performed electrophoresis under nondenaturing and nonreducing conditions using Huh-7 supernatants under the six cellular conditions tested above. Briefly, DPP4 can be found as a monomer, homodimer, or even as a homotetramer on the cell surface. The active predominant form of DPP4 is a dimer of two identical subunits [26], and any changes in its dimeric structure or stability could impact its catalytic activity. Previous data on DPP4 purification reported that the 290 kDa active dimeric form of DPP4 appears under nondenaturing conditions, while under denaturing and reducing conditions a band of 105 kDa is observed, corresponding to the monomer [27]. Our results showed that the DPP4 secreted by Huh-7 cells in all conditions was in the dimeric 290 kDa active form (Fig. 7); therefore, the observed decrease in DPP4 enzymatic activity reported in this study could not be attributed to altered dimerization of the protein. In this line, the experiment was replicated with patient sera from the different clinical conditions, and a band of 290 kDa was also observed in all the groups (data not shown), indicating that differences in DPP4 activity and the DPP4 activity/concentration ratio were not due to a different protein conformation or dimerization degree.

4. Discussion

Our data showed a significant reduction in serum DPP4 concentration in COVID-19-affected patients compared to control individuals. This observation was in line with previous literature measuring circulating soluble DPP4 (sDPP4) serum concentrations, which were found to be significantly lower in patients suffering from severe COVID-19 infection ($n = 7$) than in healthy individuals ($n = 14$) in a case-control design (242.70 ± 202.12 ng/mL *versus* 497.70 ± 188.13 ng/mL, $p = 0.02$) [28]. Similar results were previously obtained with MERS-CoV [29]. Interestingly, it was shown that sDPP4 was expressed by lymphocytes as a major source [30], and in humans specifically by active T cells [31]. Given that lymphopenia is associated with COVID-19 itself [32], it is unclear, as noted Krejner-Bienias et al., whether sDPP4 reduction was a simple effect of lymphopenia, or should rather be considered as an initial condition and presumable cause of increased susceptibility to MERS-CoV or SARS-CoV-2 infections [15].

Furthermore, as both diabetic and COVID-19 patients had decreased

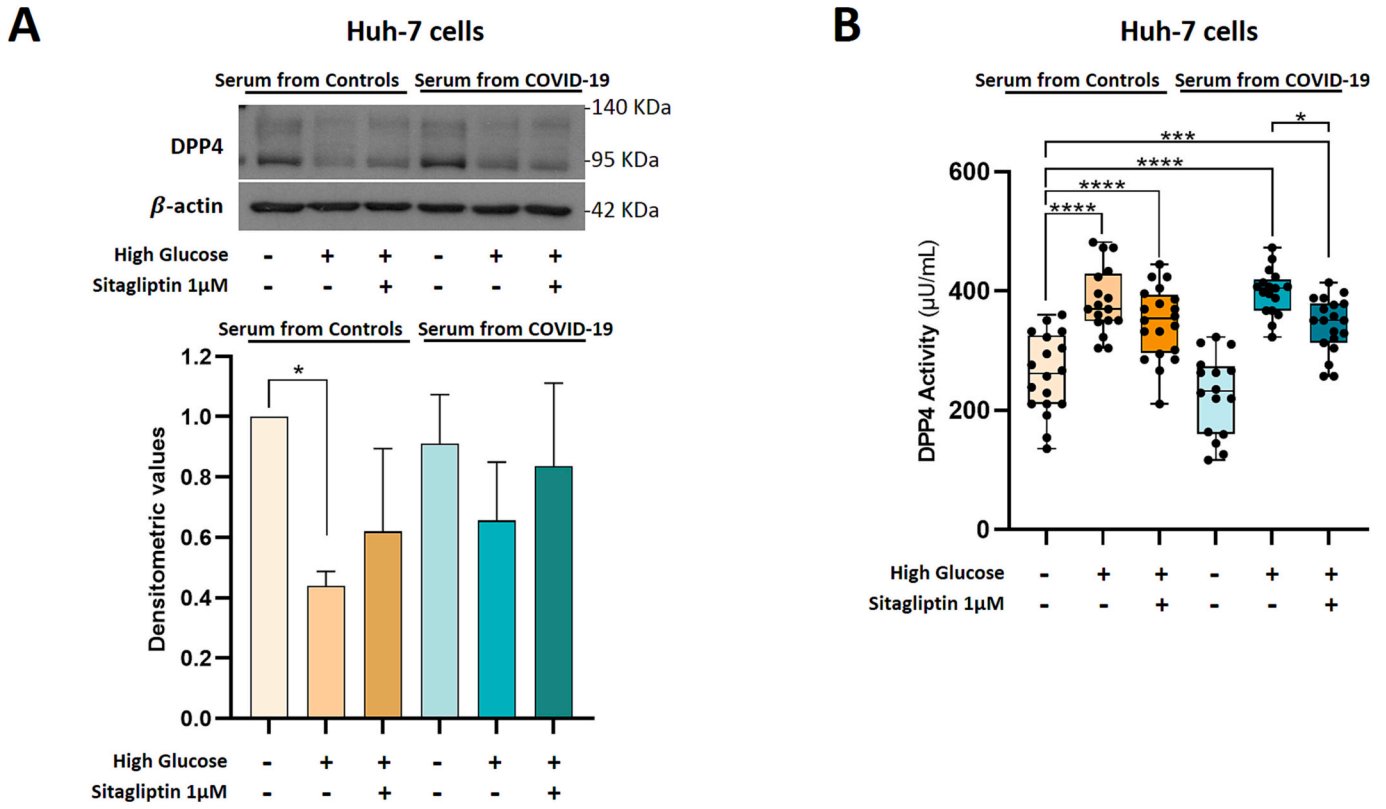


Fig. 6. DPP4 protein levels and enzymatic activity of Huh-7 cellular fractions. In all panels, Huh-7 cells were cultured for 7 days in the presence of serum from control patients (alone, supplemented with glucose, or glucose plus sitagliptin), and the same conditions were repeated with serum from COVID-19 patients. (A) Protein levels of DPP4 measured by Western blot, showing the gel (above) and the densitometric values (below). (B) DPP4 activity (μU/mL) determined by a commercially available kit, and represented by a diagram of boxes and whiskers. Experiments were repeated in triplicate with different patient sera, and the results are the mean ± standard deviation (SD) of the replicates.

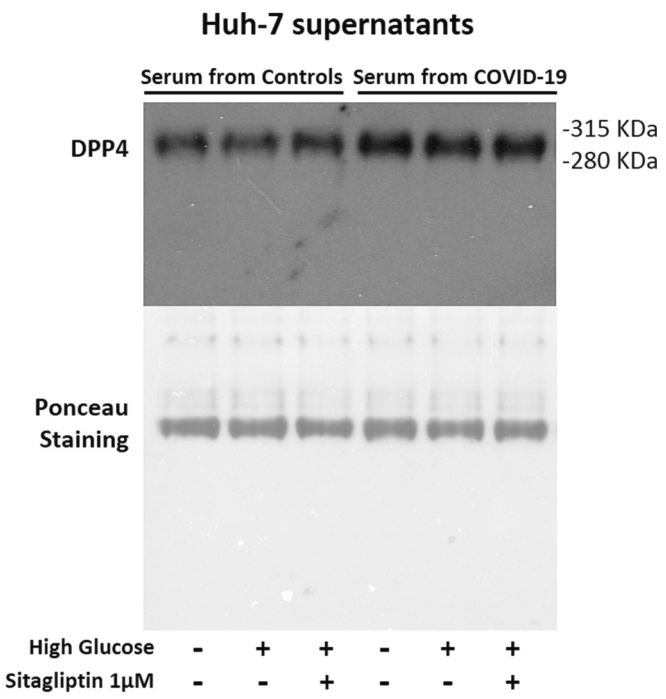


Fig. 7. Structural conformation of DPP4 from Huh-7 supernatants. Supernatants of Huh-7 cells incubated at six different conditions (in the presence of serum from control or COVID-19 patients, either alone, supplemented with glucose, or glucose plus sitagliptin) were analyzed by Western blot for the protein DPP4, after carrying out a polyacrylamide gel electrophoresis (PAGE) under nondenaturing nonreducing conditions.

levels of DPP4, it is interesting to consider the fact that lower levels of sDPP4 in diabetic patients can facilitate SARS-CoV-2 infection. As shown in the introduction, the receptor-binding domain (RBD) of SARS-CoV-2's S1 spike protein has been predicted to bind the glycosylated non-catalytic region of DPP4, in residues consistent with those for binding the MERS-CoV RBD, for which DPP4 is the canonical entry cell receptor. The SARS-CoV-2 residues implicated in the interaction were described to be D405, E484, Y489/N487, Q498, N501, and Y505 of the RBD region in the S1 protein [3]. Considering the current results, the initial hypothesis of DPP4 as a putative primary entry receptor is moving to a complementary coreceptor and, interestingly, to a protective decoy molecule in the serum of patients. In this sense, the binding of sDPP4 to the S1 RBD of SARS-CoV-2 could impede the interaction with membrane-bound receptors disabling infection progression, while the reduction in sDPP4, as occurs in diabetic patients, would be a risk factor.

Furthermore, the SARS-CoV-2 RBD residues involved in the interaction with the canonical receptor ACE2 have been determined to be K417, G446, Y449, Y453, L455, F456, A475, F486, N487, Y489, Q493, G496, Q498, T500, N501, G502, and Y505 [33]. Notably, five of these residues overlap with those blocked by the interaction with sDPP4. In addition, analysis of 144 SARS-CoV-2 genome sequences showed 14 key positions for the binding of SARS-CoV-2 and human ACE, partly conserved with respect to SARS-CoV, and partly semiconservatively substituted. In brief, T402, Y436, N439, Y440, L455, N473, Y475, T486, G488, and Y491, Q493, Q498, and N501 [34]. Therefore, sDPP4 can hinder entry into the cell not only *via* coreceptors such as membrane-bound DPP4 but also by canonical receptors like ACE2 by covering key interactive amino acids in the RBD region. This observation reinforces the idea that diabetic patients with lower levels of sDPP4 have a higher risk of SARS-CoV-2 infection because of its lower shielding. *In vivo* experiments showed that DPP4-overexpressing mice with increased sDPP4 levels were relatively resistant to MERS-CoV infection and developed milder lung inflammation and reduced rates of mortality [35]. To the best of the authors' knowledge, the same model has not been tested for SARS-CoV-2 infection, and it could be important and definitive evidence for the protective mechanism proposed. That is, the infectiveness of SARS-CoV-2 in DPP4-overexpressing mice that exhibit high sDPP4. Anyway, this experiment on MERS-CoV infection indicates that low levels of sDPP4 are a cause of viral susceptibility instead of a consequence, and our low levels of sDPP4 in diabetic and COVID-19 patients are in accordance with this hypothesis. However, it is important to note that further experimental studies are needed to validate and ascertain the effectiveness of sDPP4 as a decoy molecule against different coronaviruses.

On the other hand, our results showed a good correlation between DPP4 levels and activity in COV-DIA-GLI patients (Fig. 4), indicating that the action of DPP4 inhibitors is impeded in these samples. This fact reinforces the idea that either SARS-CoV-2 or some metabolites present in the sera of COVID-19-convalescent patients interact with soluble DPP4 or even gliptins themselves since the inhibitory effect of gliptins on DPP4 activity is being prevented. The binding of SARS-CoV-2 (RBD S1) to DPP4, occurs at positions K267, Q286, T288, R317, Q344, and R336 of DPP4, in the 8-bladed β-propeller domain (N-terminal region) [3], not overlapping with its active site located in the α/β hydrolase domain (C-terminal region). The catalytic domain of DPP4 is found at this α/β hydrolase fold, containing the catalytic triad that is constituted by resides Ser630, Asp708, and His740 [36]. This is in line with our data showing DPP4 activity in samples of patients with COVID-19, indicating that the possible binding of sDPP4 to SARS-CoV-2 S1 does not necessarily interfere with the activity of the enzyme, given that the active site is still in a proper conformation.

However, it is remarkable that in the sera of diabetic patients diagnosed with COVID-19, gliptins did not reduce DPP4 enzymatic activity compared to gliptin-treated T2DM patients not infected with SARS-CoV-2 (Figs. 2B, and 4). We wondered whether there is some structural rationale behind this observation, linked to gliptins binding to DPP4 or even S1. All known gliptins bind to a large pocket in the cavity located at the interface of the α/β hydrolase and β-propeller domains [37]. Going deeper, there are three classes of DPP4 inhibitors categorized on the basis of their binding subsites, and all of them have in common the S_1 and S_2 subsites of the active site [38], with Ser630 of the catalytic triad located at the S_1 subsite [39]. Then, given that the active site is expected to be accessible for allowing DPP4 activity (and specifically for the digestion of the quenched-fluorescent reagent of the commercially available kit), it is unlikely that gliptins enter into the active site. Moreover, the conventional hypothesis for substrates and inhibitors entering or leaving the active site is the 'side opening' between α/β hydrolase and β-propeller domains [38,40,41]. In other words, substrates and inhibitors are proposed to enter by the same pathway, suggesting that its obstruction is not again the reason for gliptin blockage. Altogether, maybe it is not DPP4 but gliptins themselves that are the target and unravel the mechanism of action.

Gliptins seem not to bind to the RBS of SARS-CoV-2, but sitagliptin was in fact proposed as an agent to block and inhibit the activity of two viral proteases, $3CL^{pro}$ and PL^{pro}, related to the processing of SARS-CoV-2 structural proteins [42]. If gliptins were binding viral proteases, it would explain the high DPP4 activity in COVID-19 patient sera compared to non-COVID-19 sera, also acting as a decoy and possibly reducing the viral load and infection by hampering the formation of new viruses. Beyond that, although it is far from the scope of this study, it would be interesting to perform a future metabolomic analysis of sera from SARS-CoV-2-infected patients, to analyze putative metabolites interfering with this system. This approach would help to overcome the main limitation of this study, helping to unravel the mechanism that underlies the present observations.

5. Conclusions

Our study reveals an interesting interference in the action of gliptins over the activity of DPP4 when analyzing sera from diabetic COVID-19 patients. This could be due to interactions with SARS-CoV-2 at different levels or even with metabolites in the sera, whose mechanism of action is still to be resolved. In addition, we unveiled that COVID-19 serum samples showed the highest ratio of DPP4 activity *versus* protein concentration, despite the lower levels of activity and concentration by themselves, pointing to a compensatory mechanism in these patients. These results contribute to disentangling the complex interplay between DPP4, COVID-19, and T2DM.

Abbreviations

ACE2 Angiotensin converting enzyme II
BMI Body mass index
COVID-19 Coronavirus disease 2019
DPP4 Dipeptidyl peptidase 4
ELISA Enzyme-linked immunosorbent assay
hCoV-EMC Human coronavirus-Erasmus Medical Center
MERS-CoV Middle East respiratory syndrome coronavirus
RBD Receptor-binding domain
RFU Relative Fluorescence Units
rRT-PCR Reverse-transcription qualitative real-time polymerase chain reaction
SDS-PAGE SDS-polyacrylamide gel electrophoresis
SARS-CoV-2 Severe acute respiratory syndrome coronavirus 2
sDPP4 Soluble dipeptidyl peptidase 4
SD Standard deviation
SEM Standard error of the mean
T2DM Type 2 diabetes mellitus

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2023.122292.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University of Alcalá (protocol code CEI/HU/202/37). Human samples included in this study were provided by the BioBank Hospital Ramón y Cajal-IRYCIS (National Registry of Biobanks B.0000678), integrated in the Biobanks and Biomodels Platform of the Institute of Health Carlos III (ISCIII, PT20/00045), and they were processed with the appropriate approval of the Ethical and Scientific Committees. Furthermore, for human samples from COVID-19 patients, patient consent was waived due to the extraordinary legal requirements of the Spanish Bioethics Committee as published in the "Report on the ethical legal requirements in research with health data and biological samples in the framework of the Covid-19 pandemic, 2020" (http://assets.comitedebioetica.es/files/documentacion/Informe%20CBE%20investigacion%20COVID-19.pdf), where it states on p. 15–16: "public institutions with powers in public health surveillance may carry out scientific studies without the consent of those affected", and "research groups […] can carry out a secondary use of health data, such as those existing in the medical records of patients treated for SARS-CoV-2 infection in public and private health centres, without requiring a new consent".

Consent for publication

Not applicable.

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CRediT authorship contribution statement

J.M.M.-R. and B.G.S. conducted the experiments, analyzed the data, and prepared the Figures. A.B. analyzed clinical data and prepared the Tables. A.S.-M. and A.D.-Y. analyzed data and reviewed the manuscript. I.D.-L. conceived and designed the experiments and wrote the MS along with A.S.-M. J.M.M.-R. and B.S.G. supervised the study and critically revised the manuscript for intellectual content. R.B.-G. and F.A. provided the samples and participants' data. All authors have approved the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

The datasets used and/or analyses during the current study are available from the corresponding authors on reasonable request.

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