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NEW-ONSET HYPERGLYCEMIA AFTER SARS-COV-2 INFECTION

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Abstract

Patients who were hospitalized for coronavirus disease 2019 (COVID-19) have a higher prevalence of hyperglycemia. By altering glucose homeostasis, cytokine release brought on by The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection may hasten the onset of metabolic changes. Here, they are described different abnormalities, such as glycometabolic regulation, insulin resistance, and loss of beta cell function in COVID-19 patients. Most of those individuals had no prior history of diabetes or diabetes diagnosis. Additionally, it is reported the presence of glycaemic abnormalities in patients who were followed up several months after the onset of the illness. We discovered that 46% of patients in a sample of 551 patients hospitalized for COVID-19 in Milan were hyperglycemic, 27% were normoglycemic, while the remaining 27% were diabetic. Even in individuals with normoglycemia, we were able to identify altered glycometabolic regulation, insulin resistance, and an aberrant cytokine profile using clinical tests and continuous glucose monitoring in a sample of patients. Patients who healed from COVID-19 have been monitored for glycemic irregularities for at least two months.

Moreover, in individuals who developed hyperglycemia following COVID-19, direct and indirect effect of SARS-CoV-2 on human pancreatic islets could possibly occur. The serum of these patients showed toxicity on human pancreatic islets despite the lack of peripheral anti-islet autoimmunity, which could be reversed by the administration of anti-interleukin-1 beta (anti IL-1B), anti-interleukin-6 (anti IL-6), and tumor necrosis factor-alpha (TNF-alpha), cytokines that are known to be significantly upregulated during COVID-19 infection.

It's interesting to note that human pancreatic islets had high levels of expression of the cytokine receptors indicated above. Several COVID-19 patients showed an increase in peripheral unmethylated INS DNA, a sign of cell death. The pancreas of deceased COVID-19-positive hyperglycemic patients showed modest lymphocytic infiltration of the islets and pancreatic lymphonodes. Additionally, postmortem pancreatic tissues contained SARS-CoV-2-specific viral RNA and numerous immature insulin granules or proinsulin, suggesting altered proinsulin processing as well as beta-cell degeneration and hyperstimulation.

Our findings show that COVID-19 is linked to poor glycometabolic regulation, and that these abnormalities can last even after recovery.

In conclusion, SARS-CoV-2 may impair human pancreatic islet survival and function by inducing inflammatory conditions, possibly with a direct tropism, which may then result in metabolic abnormalities observed in COVID-19 patients.

Riassunto

Evidenze cliniche dimostrano che i pazienti con malattia da coronavirus 2019 (COVID-19) abbiano una maggiore prevalenza di iperglicemia. Alterando l'omeostasi del glucosio, il rilascio di citochine causato dall'infezione da parte del coronavirus da sindrome respiratoria acuta grave 2 (SARS-CoV-2) può accelerare l'insorgenza di disordini metabolici. Qui vengono descritte diverse anomalie, come la regolazione glicometabolica, la resistenza all'insulina e la riduzione della funzione beta cellulare negli individui affetti da COVID-19. Una parte di questi soggetti non aveva una storia di diabete antecedente al ricovero o una diagnosi di diabete.

Inoltre è stata dimostrata la presenza di anomalie glicemiche persistenti in pazienti che erano stati seguiti per diversi mesi dall'insorgenza della malattia. Abbiamo scoperto che il 46% dei pazienti su un campione di 551 pazienti ricoverati per COVID-19 a Milano era iperglicemico, il 27% era normoglicemico, mentre il restante 27% era costituito da pazienti diabetici.

Anche negli individui con normoglicemia, siamo stati in grado di identificare un'alterata regolazione glicometabolica, insulino-resistenza e un profilo citochinico aberrante, utilizzando test clinici e il monitoraggio continuo del glucosio in un campione di pazienti. I pazienti guariti da COVID-19 sono stati monitorati per rintracciare eventuali anomalie glicemiche per almeno due mesi.

Inoltre, è stato dimostrato che negli individui che hanno sviluppato iperglicemia in seguito a COVID-19 potrebbe verificarsi un effetto diretto e indiretto di SARS-CoV-2 sulle isole pancreatiche umane. Infatti, il siero di questi pazienti ha mostrato un effetto tossico sulle isole pancreatiche umane nonostante la mancanza di autoimmunità periferica anti-isole, che potrebbe essere invertita dalla somministrazione di anti-interleuchina-1 beta (IL-1B), anti-interleuchina-6 (IL-6) e dal fattore di necrosi tumorale-alpha (TNF-alpha), citochine note per essere significativamente sovraregolate durante l'infezione da COVID-19.

È interessante notare che le isole pancreatiche umane evidenziavano alti livelli di espressione dei recettori delle citochine sopra indicati. Diversi pazienti affetti da COVID-19 hanno mostrato un aumento del DNA INS periferico non metilato, che sta ad indicare la presenza di morte cellulare. Il pancreas di individui iperglicemici deceduti in seguito a COVID-19 ha mostrato una modesta infiltrazione linfocitaria delle isole e dei linfonodi pancreatici. Inoltre, i tessuti pancreatici post-mortem contenevano RNA virale specifico per SARS-CoV-2 e

numerosi granuli immaturi di insulina o proinsulina, a testimonianza di un'elaborazione alterata della proinsulina, nonché di una degenerazione e iperstimolazione delle cellule beta. I nostri risultati mostrano che il COVID-19 è collegato ad una scarsa regolazione glicometabolica e che le anomalie evidenziate possono durare anche dopo il recupero.

In conclusione, è stato dimostrato che SARS-CoV-2 può compromettere la sopravvivenza e la funzione delle isole pancreatiche umane inducendo uno status infiammatorio, possibilmente tramite un tropismo diretto, causando le anomalie metaboliche osservate nei pazienti COVID-19.

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Chapter 1: **INTRODUCTION**

1.1 SARS-CoV-2 infection and diabetes mellitus

1.1.1 Coronavirus: SARS-Cov-2

Several hospitals in Wuhan, Hubei Province, China, reported clusters of patients with pneumonia of unknown origin at the end of December 2019. The Wuhan Municipal Health Commission notified the World Health Organization of a pneumonia outbreak with an unknown etiology on December 31, 2019. (WHO). Despite genetic data indicate a natural origin, it is currently unknown, how The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) made the spillover in human beings. [1] SARS-CoV-2 is a betacoronaviruses, member of the Orthocoronavirinae family [2] The diameter of a SARS-CoV-2 virion ranges between 100 and 200 nm. The virus particles are composed by a protective capsid, which is a lattice of repeating protein molecules known as the coat or capsid proteins, that surrounds the long RNA polymers that are firmly packed into the center of the coronavirus virion. The surface glycoprotein, also known as spike protein (S), the envelope protein (E), and the membrane protein (M), are three structural components of the SARS-CoV-2 virus's envelope, similar to other coronaviruses. The nucleocapsid (N) protein of the viral envelope contains 29,903 bases of positively orientated single-stranded RNA.[3]

1.1.2 Replicative cycle

There are ten open reading frames in the genome of the SARS-CoV-2 (ORFs). The ORF1a and ORF1b genes, which together code for the polyprotein pp1ab, which is further divided into 16 non-structural proteins involved in transcription and genome replication, comprise at least two thirds of the viral genome. Among these proteins, ORF1a encodes the papain-like protease (PLpro) and the 3C-like protease (3CLpro), whereas ORF1b produces the exonuclease (ExoN), helicase (Hel), and RNA-dependent RNA polymerase (RdRp). The remaining ORFs code for auxiliary proteins with uncertain roles, structural glycoproteins S, E, M, and N, as well as a number of other proteins.[4]

Both the non-endosomal route of the cell surface and the endosomal route allow SARS-CoV-2 to enter the host cell. The SARS-CoV-2 virion binds to its target cells via the endosomal pathway by directly attaching to the host receptor angiotensin converting enzyme 2 (ACE2). Glycoprotein S is activated and broken down by transmembrane protease serine 2 (TMPRSS2) upon binding, which causes the viral and cellular membranes to fuse. SARS-CoV-2 may infect cells through non-endosomal entry at the plasma membrane in addition to the typical viral entry through the endosome. The SARS-CoV-2 nucleocapsid and viral

RNA are released into the cytoplasm of the target cells through disassembly, where they can be translated and replicated. After being assembled in the endoplasmic reticulum (ER) with translated proteins to create new virions, SARS-CoV-2 virus particles comes out through exocytosis from the Golgi membrane system and released into the extracellular compartment.[5]

1.2 Prevalence and pathogenesis of COVID-19 in patients with diabetes mellitus

In the first published clinical records approximately 20% of 41 COVID-19 patients hospitalized in China at the time had diabetes. Since then, a number of smaller retrospective Chinese investigations have found that diabetes prevalence ranges from 7% to 21%.[6] Notably, a database of 20,982 individuals with COVID-19 from the Chinese Centers for Disease Control and Prevention (CCDC) revealed a prevalence of diabetes of roughly 5%. The prevalence of diabetes among COVID-19 patients varies greatly over the world. Diabetes is the most prevalent comorbidity, according to the Centers for Disease Control and Prevention (CDC) in the United States, with a prevalence of almost 10% among 122,653 people with COVID-19. [7]

According to Grasselli et al., among 1,043 patients from Lombardy who had COVID-19, diabetes was present in roughly 17% of cases.[8] A prospective observational research by Docherty et al. in the UK revealed that 20.5% of 20,133 people with COVID-19 had simple diabetes. [9] Instead, 121,263 COVID-19 patients in Spain were found to have a 10% prevalence of diabetes, according to Prieto-Alhambra et al. Bello-Chavolla et al. discovered that 15,529 COVID-19 individuals had a prevalence of diabetes of over 18%. [10, 11]

Additionally, a number of meta-analyses have revealed that COVID-19 patients have a varying prevalence of diabetes.

Pooled analysis demonstrated a diabetes prevalence that goes from 8% to 10% among patients hospitalized for COVID-19 [12-15].

Due to data overlap and the inclusion of only studies from China, many of these meta-analyses have limitations, and so, the results may not accurately reflect global prevalence.

Moreover, different meta-analysis of studies conducted in China, United States and Italy, discovered a diabetes prevalence of 11.5% (95% CI 9.7–13.4%) among people hospitalized for COVID-19. In China diabetes incidence in COVID-19 patients was similar to the national prevalence of type 2 diabetes (T2D) (~11%)[16], while in India national prevalence for T2D

(7.3%) was way different from diabetes prevalence among people hospitalized for COVID-19 (47%) [17, 18].

Richardson et al. reported a case series of 5,700 COVID-19 patients admitted to New York City hospitals with a diabetes prevalence of 33.8%. [19]

The relative risk (RR) or odds ratio (OR) of severe COVID-19 in diabetic patients has been significantly increased, according to meta-analyses carried out in various parts of the world, by two to three times.

A considerable (two to three-fold) increase in mortality among those with diabetes and COVID-19 has also been noted in some of these meta-analyses.

Notably, the same meta-analysis that included 18 research (n=14,558) from China, the United States, and Italy discovered that those with diabetes have worse symptoms than those without (RR 2.11, 95% CI 1, 40–3.19).

An extensive national study carried out in England (n=61,414,470) found that individuals with type 1 diabetes (T1D) and T2D had 3.5- and 2-fold increased odds of dying in a hospital setting due to COVID-19, respectively.[20]

Different studies have compared COVID-19 and diabetes patient outcomes with those of patients without diabetes. First, a retrospective observational study with 570 patients in 88 US hospitals found that patients with diabetes [Glycated Hemoglobin (HbA1c) $\geq$ 6.5%] and/or uncontrolled hyperglycemia (defined as more than two independent glycemic values >180 mg/dL within a 24-hour period) had a significantly higher mortality rate (28% vs. 6.2%, p-value< 0.001) than patients with good glycemic control. It's interesting to note that the death rate was considerably greater in COVID-19 patients with uncontrolled hyperglycemia (n = 184) but no history of diabetes (HbA1c < 6.5%) compared to patients with known diabetes (41.7% versus 14.8%, p-value< 0.001, respectively). In this study diabetes was defined as $\geq$ 6.5%. [21]

A similar retrospective study found that patients with diabetes (n = 174) had a statistically significant higher mortality rate than those without (16.7% versus 0%). Despite controlling for confounders, a study of primary care electronic health records in the UK (n = 17,425,445) found a significant link between having moderately controlled (HbA1c < 58 mmol/mol) or uncontrolled (HbA1c $\geq$ 58 mmol/mol) diabetes and an increased mortality rate (hazard ratio of controlled diabetes [(HR) 1.50, 95% CI 1.40-1.60]); uncontrolled diabetes [HR 2.36, 95% CI 2.18-2.56] despite removing potential confounders.[22]

People with diabetes have greater risks of intensive care unit (ICU) admission (42.1% vs. 29.8%, p = 0.007), mechanical breathing (37.1% vs. 23.2%, p = 0.001), and death (15.9% vs. 7.9%, p-value = 0.009) than people without diabetes, according to a retrospective study (n = 178) from Massachusetts General Hospital. In fact, a multivariate logistic regression study found that people with diabetes had higher odds of being referred to intensive care (OR 1.59, 95% CI 1.01-2.52), receiving mechanical breathing (OR 1.97, 95% CI 1.21-3.20), and passing away (OR 2.02, 95% CI 1.01-4.03) at 14 days.

However, there was no correlation between glycemic control and the primary outcome of mechanical ventilation and/or mortality within 7 days of the admission in the prospective observational CORONADO research in patients with diabetes (T2D in 88.5%) and COVID-19 (n = 1,317). [23]

In a retrospective study of 153 patients, it was shown that, compared to non-diabetics, COVID-19 patients with diabetes had significantly higher rates of ICU admission (17.6% vs. 7.8%, p-value = 0.01) and death (20.3% vs. 10.5%, p-value = 0.017).

However, after controlling for confounders, diabetes by itself was not significantly associated with increased mortality rate (HR 1.58, 95% CI 0.84-2.99).[24]

In a similar manner, Agarwal et al. found no correlation between glycemic control (HbA1c levels) and mortality in a retrospective study (n = 1,126) from New York, but they did find that pre-admission insulin treatment in people with diabetes increased the risk of death (adjusted OR 2.30, 95% CI 1.31-4.0). Although T2D was the most common type of diabetes among patients, most studies that were available did not provide outcomes by diabetes type to differentiate between severity and death. [25]

Even after correcting for numerous covariates, the first report of a sizable retrospective analysis (n = 7,337) revealed a significantly higher mortality in COVID-19 patients with T2D (n = 810) compared to those without (n = 6,385) (HR 1.49, 95% CI 1.13-1.96, p = 0.005). In patients with well-managed diabetes (n=282, defined as blood glucose 70-180 mg/dL) compared to patients with poorly controlled diabetes (n=528, defined as blood glucose >180 mg/dL), the adjusted HR for all-cause death was 0.13 (95% CI 0.04-0.44, p-value < 0.001)[26].

Additionally, a poorly controlled versus a well-controlled T2D cohort had a significantly higher complication rate (septic shock: 4.7% vs. 0.0%, p = 0.004; acute respiratory distress syndrome: 21, 4% vs. 7.1%, p< 0.001; acute kidney injury: 3.8% vs. 0.7%, p-value = 0.019; acute cardiac injury: 9.9% vs. 1.4%, p-value < 0.001). According to a large cohort

of T2D patients (n = 2,874,020) from the National Diabetes Audit in England, glycemic control is independently linked to higher mortality in COVID-19 patients. HbA1c >10.0% (>86 mmol/mol), 9.0-9.9% (75-85 mmol/mol), or 7.6-8.9% (59-74 mmol/mol) was linked to a significantly higher risk of death in individuals with T2D and COVID-19 (HR 1.61, 95% CI 1.47-1.77; HR 1.36, 95% CI 1.24-1.50; HR 1.22, 95% CI 1.15-1).[27]

Moreover, when compared to those with a HbA1c of 6.5-7.0% (48-53 mmol/mol), people with T2D and COVID-19 who had HbA1c >10.0% (>86 mmol/mol) had significantly higher odds of dying (HR 2.23, 95% CI 1.50-3.30, p-value < 0.0001).

Even after controlling for numerous confounders, a study of the entire English population found a 3.5-fold increase in hospital-related COVID-19 deaths in people with T1D (n = 263,830) compared to those without diabetes over a 72-day period (OR 3.51, 95% CI 3.16-3.90), and mortality remained significantly high (OR 2.86, 95% CI 2.58-3.18).

The majority of people with poorly controlled diabetes (including T2D and T1D) appear to be more likely than those with well-controlled diabetes or those without diabetes to experience severe COVID-19 and death.[27]

It is unclear what the absolute and relative risks are for COVID-19-related mortality in T1D patients. In a population-based study from Belgium, T1D patients with COVID-19 (n = 2,336) did not have a significantly higher risk of hospitalization compared to non-diabetics (0.21% vs 0.17%), despite having significantly worse glucose control at admission than T1D patients hospitalized for other conditions other than COVID-19. In a US investigation, there was no difference in glycometabolic compensation between T1D patients hospitalized for COVID-19 (n = 7) and those hospitalized for other conditions (n = 28) without COVID-19.

The worst primary outcomes (tracheal intubation and/or in-hospital death up to day 7) were only seen in patients above the age of 75 in a sample of T1D patients (n = 56) from the CORONADO research. [28]

On the other hand, two sizable population-based investigations found that T1D and COVID-19 patients died more frequently. The National Diabetes Audit in England studied on 264,390 T1D patients and discovered an independent link between poor glycemic control and higher mortality rates in those with T1D and COVID-19.

Male gender, older age, and concomitant conditions including cardiovascular disease (CVD), obesity, and/or T1D or T2D are among the traits of patients who are at high risk of developing severe COVID-19 or dying from it. Early research has revealed that patients with COVID-19 admitted to the ICU frequently have underlying CVD and diabetes mellitus.

In particular, glycolysis has been discovered to boost virus replication by activating the hypoxia-inducible factor 1-20, which has been proven to increase SARS-CoV-2 viral replication within human monocytes. Thus, hyperglycemia would seem to encourage the spread of viruses. Thus, morbidity and death in COVID-19 patients may be independently predicted by hyperglycemia or a history of diabetes.[29]

Additionally, T2D as a comorbidity in Middle East respiratory syndrome–related coronavirus (MERS-CoV) infected mice led to a compromised immune response, which resulted in a serious and widespread lung illness. Patients with diabetes mellitus often have a more severe SARS-CoV-2 infection than patients without, and poor glycemic control indicates a greater need for hospitalisation and treatment, as well as a higher mortality rate.[30]

Notably, COVID-19 frequently causes disruption of glycemic control in people with poor glucose control or diabetes mellitus. For instance, SARS-CoV-2 infection was linked to an increased requirement for high insulin dosages in patients who needed it (often close to or greater than 100 IU per day). [31]Increased levels of pro-inflammatory cytokines appear to be linked to changes in insulin needs. While ketoacidosis is often a condition closely related with T1D, it can also happen in people with T2D who have COVID-19. For instance, in one systematic study, T2D was present in 77% of COVID-19 patients who experienced ketoacidosis.[32]

In postmortem examination of the lungs of patients who died of COVID-19, diffuse alveolar injury and inflammatory cell infiltration with significant hyaline membranes were most frequently discovered. Inflammation of the myocardium, lymphocytic infiltration of the liver, macrophage clustering in the brain, axonal damage, microthrombi in the glomeruli, and localized pancreatitis are other postmortem findings.[33]

Additionally, a combined analysis revealed that patients with severe COVID-19 have a severely compromised response to (interferon type 1) INF-1 and low blood IFN activity, which indicates a high blood viral load and a compromised inflammatory response. Additionally, it has been demonstrated that inborn mistakes in B-cell immunity, which is related to Toll-like receptor 3 (TLR3) and Interferon regulatory factor (IRF7), enhance the risk of developing deadly COVID-19 pneumonia in 12.5% of men and 2.6% of women.

From these analyzes therefore emerges a remarkable heterogeneity in the immune phenotypes among patients with COVID-19.

Several patients with severe COVID-19 experience a cytokine storm, which in most cases is fatal for the patient.

Inflammatory responses within 24 hours of hospital admission [interleunkin-6 (IL-6) and lactate dehydrogenase], which were linked with illness severity, were found in a retrospective investigation of 317 individuals with laboratory evidence of COVID-19.

Additionally, the severity of the clinical course of COVID-19 is independently predicted by blood levels of lactate dehydrogenase and IL-6.[34]

Particularly in the setting of innate immunity, IL-6 possesses pro-inflammatory qualities, and studies have shown that levels of IL-6 are related to procoagulant profiles and disease severity. IL-6 can harm DNA, lipids, proteins, and other molecules through an increase in oxidative stress, which eventually compromises our body's ability to function normally.

In patients with diabetes mellitus, this impact may result in a rapid progression of COVID-19 (Figure 1 adapted from Lim S. et al, COVID-19 and diabetes mellitus: from pathophysiology to clinical management, Nature Rev Endocrinol, 2021). Notably, a biological systems assessment of immunity in patients with severe COVID-19 revealed elevated levels of bacterial DNA and lipopolysaccharide in plasma, which were positively correlated with plasma levels of IL-6 and EN-RAGE, a biomarker of lung injury linked to the pathogenesis of sepsis-induced acute respiratory distress syndrome (ARDS).

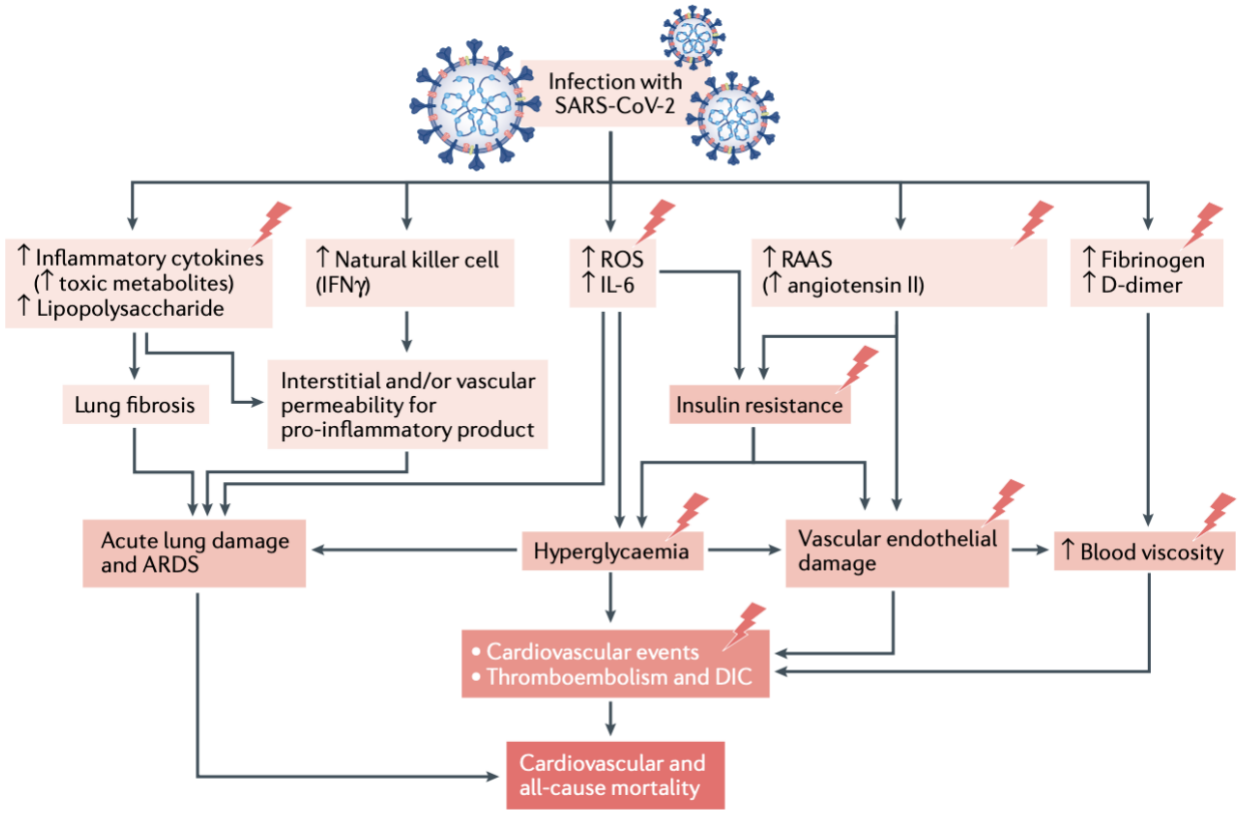

Figure 1 | Potential pathogenic pathways in COVID-19-positive T2DM patients. Adapted from *Lim S. et al, COVID-19 and diabetes mellitus: from pathophysiology to clinical management, Nature Rev Endocrinol, 2021*

These data imply that in severe COVID-19, bacterial products, possibly of lung origin, contribute to an increase in inflammatory cytokine production.

Inflammation brought on by viruses has been linked to an increase in insulin resistance, which has been attributed to a variety of different pathways. For instance, in coronavirus-induced pneumonia, such as SARS and MERS, inflammatory cells infiltrate lung tissue, causing acute injury, ARDS, and/or mortality.[35] This substantial burden of inflammatory cells might affect the functions of skeletal muscle and the liver, which are the two major insulin-responsive organs responsible for the majority of insulin-mediated glucose uptake. People with severe COVID-19 may also have multiorgan failure if they have muscle weakness and an increase in liver enzyme activity, especially during the cytokine storm.[36] In worst conditions, COVID-19 can turn into ARDS, which necessitates the use of positive pressure oxygen and intensive care. ARDS is characterized by severe edema of the lung parenchyma and alveolar walls, as well as a sudden increase in inflammatory indicators including erythrocyte sedimentation rate (VES) and c-reactive protein (CRP) levels. Individuals with COVID-19 also show an increase in other inflammatory markers, including as D-dimer, ferritin, and IL-6, which may increase the risk of microvascular and macrovascular issues as a result of low-grade vascular inflammation brought on by underlying diabetes mellitus.[37]

Microvascular and macrovascular consequences of diabetes were strongly linked to a higher risk of mortality in patients with COVID-19 in a national study conducted in France.

All of these data point to an association between oxidative stress, inflammation, and the molecular pathogenesis of SARS-CoV-2, which may aid in the development of sepsis. [23]

It has already been established that the mechanisms connecting COVID-19 to T1D and T2D share pathways with those controlling immune response. For instance, age is the biggest risk factor for T2D, and aging's impact on immunological function may have a similar impact on the susceptibility to and severity of COVID-19. In addition, immune function can be impacted by hyperglycemia; on the other hand, the macrovascular consequences of diabetes mellitus are associated with a dysregulated immunological condition. As a result, T2D is linked to immune dysregulation, which is possibly equal to accelerated aging. This association may help to explain why people with diabetes mellitus and COVID-19 have a bad prognosis.[38]

Proinflammatory cytokines produced by Th1 cells are known to worsen insulin resistance in obese people, but it is unclear how these cytokines affect COVID-19. Particularly, it is still

unclear whether and how SARS-CoV-2 infections affect people who are at risk of developing diabetes mellitus cause loss of glycemic control. According to a study, acute respiratory virus infection in humans boosts IFN production and results in muscular insulin resistance, which triggers compensatory hyperinsulinemia to maintain euglycemia and intensifies CD8+ T cell antiviral responses. It is possible that such compensation won't work in patients with poor glucose tolerance or diabetes mellitus. [39] Furthermore, by directly stimulating CD8+ effector T-cell activity, hyperinsulinemia may improve antiviral immunity.

Murine CMV infection led to a decline in glycemic control in prediabetic mice with hepatic insulin resistance brought on by diet-induced obesity. As a result, after an infection with SARS-CoV-2, the immunological and inflammatory reactions that follow may alter insulin sensitivity, potentially worsening abnormalities of glucose metabolism.

For instance, respiratory syncytial viruses enhance IFN synthesis, which triggers natural killer (NK) cell activation as a defense strategy. In general, systemic inflammation in muscle and adipose tissue is exacerbated by both increased IFN production and activated NK cells, which has a negative impact on glucose absorption. Furthermore, in patients with poor glucose metabolism, there is a connection between NK cell activity and glucose management. It has been established that people with T2D exhibit decreased NK cell activity than do those with prediabetes or normal glucose tolerance. Additionally, multiple regression analysis demonstrated that in T2D patients, HbA1c level is an independent predictor of NK cell activity.[40]

Patients with diabetes mellitus may be more sensitive to COVID-19 and have a worse prognosis than those without the disease because they have decreased NK cell function as a result of impaired glucose tolerance or diabetes mellitus. To find therapeutic targets, create potent medications, and comprehend the biology of SARS-CoV-2 infections, it is crucial to comprehend how this immunomodulation process occurs during infection.[41]

ACE2 has attracted a lot of attention as a component of the renin-angiotensin-aldosterone system (RAAS) because it can act as an entrance receptor for SARS-CoV and SARS-CoV-2. The respiratory system is where ACE2 is most frequently expressed, according to preliminary investigations. [42] Immunohistochemical analyses carried out in a more recent investigation, however, revealed that ACE2 is also expressed in the colon, kidney, heart, arteries, and pancreas. Thus, evidence points to the expression of ACE2 in a variety of human cells and organs, including pancreatic islets.

Additionally, ACE2 and glucose homeostasis are linked according to the findings of various investigations. For instance, pancreatic beta-cell dysfunction brought on by a high-fat diet was observed to affect Ace2-knockout animals more than wild-type mice.[43]

1.3 Effects of Sitagliptin in T2D patients during hospitalization for COVID-19

Recent researches suggests that SARS-CoV-2 can bind to dipeptidyl peptidase 4 (DPP4 or CD26) in addition to the already well-known ACE-2 when it enters respiratory tract cells. [44] It was also predicted that DPP4, may make it easier for SARS-CoV-2 to enter target cells based on the 3D modeling of the structure of SARS-CoV-2 and its receptors. Inhibiting this binding could improve the COVID-19 clinician's outcome. The interaction between the SARS-CoV-2 spike glycoprotein S1 and human DPP-4 may constitute a potential factor for SARS-CoV-2 entry and virulence. [45]

Sitagliptin, a highly selective oral DPP4 inhibitor increasing glucagon-like peptide 1 bioavailability (GLP-1) exerts hypoglycemic effects and received FDA approval as an anti-diabetic medication in 2006. [46] Moreover, sitagliptin has been demonstrated to decrease hepatitis C virus replication, suppress chemokine release, and lower IL-6 production. This molecule is also well known for its immunoregulatory and anti-inflammatory properties, also on beta cells.[47-49]

These presumptions led to the hypothesis in this study that DPP-4 inhibition would be advantageous for COVID-19 patients, particularly those with T2D.[47, 50]

As previously mentioned, T2D raises the mortality risk in COVID-19 patients, especially in those with more advanced disease. The aim was to determine whether sitagliptin, a DPP4-i with stronger CD26 selectivity, could be effective in treating COVID-19, particularly in patients with T2D who are at a higher risk of comorbidities such cardiorenal or cerebrovascular disease. In a multicenter, case-control, observational, retrospective study setting, the study documented the various clinical and biochemical outcomes of T2D patients hospitalized for COVID-19 who were treated with sitagliptin as add-on medication to standard of care or not.[51] (Full paper in Appendix A)

Characteristic	Standard of care	Sitagliptin	*P* value
Age (years)	69 ± 1.0	69 ± 0.9	0.83
Elderly patients ≥70 years of age, *n* (%)	90 (53)	92 (54)	0.91
Male sex, *n* (%)	115 (68)	123 (73)	0.40
Duration of diabetes (years)	8.7 ± 1.2	9.2 ± 0.8	0.73
Coexisting conditions, *n* (%)			
Cardiovascular disease	53 (38)	65 (40)	0.63
Chronic kidney disease	34 (28)	34 (21)	0.26
Hypertension	80 (67)	118 (74)	0.23
Cancer	17 (14)	27 (17)	0.62
Glucose-lowering medications, *n* (%)			
Metformin	63 (39)	79 (44)	0.16
Insulin	48 (30)	39 (22)	0.15
Other oral antidiabetic agents	50 (31)	61 (34)	0.25
Antihypertensive drugs, *n* (%)			
ACE inhibitors	29 (50)	38 (38)	0.13
β-Blockers	34 (56)	32 (33)	0.007
Diuretics	30 (52)	36 (38)	0.13
Antiplatelet drugs	29 (49)	39 (40)	0.32
Anticoagulant drugs	52 (77)	74 (68)	0.17
Respiratory rate (breaths/min)	25.8 ± 0.7	23.7 ± 0.6	0.04
Clinical score (0–7)	4.4 ± 0.1	4.4 ± 0.08	0.88
BMI (kg/m^2)	30 ± 0.6	29 ± 0.4	0.18
HbA_{1c} (%)	7.5 ± 0.1	7.5 ± 0.1	0.66
HbA_{1c} (mmol/mol)	58.6 ± 1.2	58.6 ± 1.3	0.98
Glycemia (mg/dL)	188 ± 6.8	180 ± 6.7	0.38
Serum creatinine (mg/dL)	1.4 ± 0.08	1.2 ± 0.08	0.10
Lymphocyte count ($\times 10^{-9}$/L)	0.9 ± 0.06	1.1 ± 0.17	0.13
CRP (mg/L)	19 ± 2.3	14 ± 0.7	0.01
D-dimer (μg/mL)	6,377 ± 1,928	5,835 ± 1,391	0.82
Interleukin-6 (ng/L)	95 ± 9.7	89 ± 10.7	0.71
LDH (units/L)	423 ± 43	387 ± 16	0.43
Ferritin (μg/mL)	601 ± 48	688 ± 97	0.43
AST (units/L)	42 ± 3.0	43 ± 2.6	0.79
ALT (units/L)	38 ± 2.4	40 ± 2.9	0.74
Procalcitonin (ng/mL)	12.7 ± 4.4	8.3 ± 3.3	0.42
Oxygen saturation (%)	92 ± 0.7	92 ± 0.5	0.31

Table 1 | Baseline patient demographics and clinical characteristics: In the absence of a clear indication, data are mean ± SEM. Metformin, sulfonylureas, GLP-1-receptor agonists, DPP-4 inhibitors, SGLT2-i, glinides, and thiazolidinediones are additional oral diabetes medications. Lactate dehydrogenase, or LDH.

A total of 169 T2D patients diagnosed with COVID-19 infection were treated with the DPP4-i, sitagliptin, an add-on therapy to standard of care while another 169 T2D patients diagnosed with COVID-19 were treated with standard of care alone. As standard of care, patients were switched from their present T2D treatment, which primarily consisted of metformin but also included insulin, sulfonylureas, DPP4-i, SGLT2-i, GLP1-RA, glinides, and thiazolidinediones, to treatment with insulin (intravenous or subcutaneous). The baseline demographic and clinical characteristics of the two patient groups are presented in Table 1. The mean age was 69 years in both the sitagliptin and control groups.

Additionally, there were no significant variations in patient demographics between the two groups under study.

With fever and respiratory problems as their primary symptoms, all patients were admitted to hospitals. 322 patients (95%) had a known diagnosis of T2D, while 16 patients (5%) had

their T2D discovered by routine metabolic screening while they were hospitalized. At baseline, there were no differences between the two groups in the duration of diabetes, renal function, vital signs, usage of glucose-lowering medications, laboratory testing, or comorbidities like cardiovascular disease (38% vs. 40%), cancer (14% vs. 17%), or hypertension (67% vs. 74%). (Table 1).

The clinical score at the time of hospital admission was evaluated, and neither group's results were different from the other's (Table 1), even after taking into account the various hypoglycemic treatments being used at the time. Between the two groups, blood glucose levels at hospital admission were comparable (Table 1). In general, we may conclude that the two groups' characteristics at the time of hospital admission were equivalent.

There were no differences in the antihypertensive regimens given to the groups, nor were there any differences in the use of other medications including diuretics, anticoagulants, or antiplatelet medications; however, a greater percentage of patients in the standard-of-care group got beta-blockers (Table 1). During hospitalization, 252 patients were administered hydroxychloroquine, while 171 patients were administered antivirals, with a comparable distribution between the two groups.

Characteristic	Standard of care	Sitagliptin	*P* value
Mortality, *n* (%)	63 (37)	31 (18)	0.0001
Clinical score reduction, *n* (%)			
≥2 points	50 (34)	72 (52)	0.0005
<2 points	67 (46)	36 (26)	0.0005
Overall improvement of clinical score, *n* (%)	55 (38)	83 (60)	0.0001
Hospital discharge at day 30, *n*	89	120	0.0008
EIR (IU/day)	31 ± 2.8	30 ± 3.8	0.83
Glycemia (mg/dL)	170 ± 9	139 ± 4	0.002***
Serum creatinine (mg/dL)	1.3 ± 0.1	1.0 ± 0.07	0.008*
Lymphocyte count ($\times 10^{-9}$/L)	1.1 ± 0.07	1.6 ± 0.2	0.03^
CRP (mg/L)	7.1 ± 0.9	3.7 ± 0.5	0.001***^^^
D-dimer (μg/mL)	3,507 ± 1,082	2,693 ± 561	0.50*
Interleukin-6 (ng/L)	81 ± 11	72 ± 10	0.55
LDH (units/L)	302 ± 21	370 ± 18	0.01^
Ferritin (μg/mL)	440 ± 43	411 ± 49	0.66*^
AST (units/L)	42 ± 4.6	28 ± 1.6	0.005***
ALT (units/L)	48 ± 5.5	43 ± 3.4	0.41
Procalcitonin (ng/mL)	8.9 ± 2.9	1.4 ± 0.5	0.01*
Oxygen saturation (%)	92 ± 1.0	96 ± 0.7	0.004***

Table 2 | Patients' clinical results at the follow-up visit (30 days) In the absence of a clear indication, data are mean ± SEM. LDH, Lactate dehydrogenase, EIR, exogenous insulin requirement; IU, international units. Baseline vs Sitagliptin follow-up: *P-value< 0.05; ***P-value<0.001. Baseline vs Standard of care at follow-up: ^P-value<0.05; P-value< 0.001.

	OR (95% CI)	*P* value
Treatment with sitagliptin	0.23 (0.12–0.46)	0.0001
Sex (male)	1.05 (0.51–2.16)	0.88
Age (years)	1.07 (1.04–1.11)	0.0001
Cancer	1.74 (0.78–3.88)	0.17
Cardiovascular disease	2.5 (1.30–4.81)	0.006
Chronic kidney disease	1.12 (0.54–2.35)	0.74
Use of hydroxychloroquine	1.47 (0.55–3.87)	0.43
Use of antiviral agents	0.91 (0.44–1.85)	0.79

Table 3 | Multivariable analysis of the risk factors for mortality in type 2 diabetes and COVID-19 patients receiving sitagliptin or standard of care

Sitagliptin use at admission was linked to lower mortality (Table 2). In the sitagliptin group, a total of 31 out of 169 patients (18%) died, as compared to 63 out of 169 patients (37%) in the control group (P-value = 0.0001). Therefore, sitagliptin treatment was linked to a lower odds ratio (OR) for in-hospital death in T2D and COVID-19 patients (OR 0.37 [CI 0.23-0.62]; P-value= 0.0001), which was sustained even after adjusting for clinically significant variables (age, gender, comorbidities, and current treatments), as shown in Table 3.

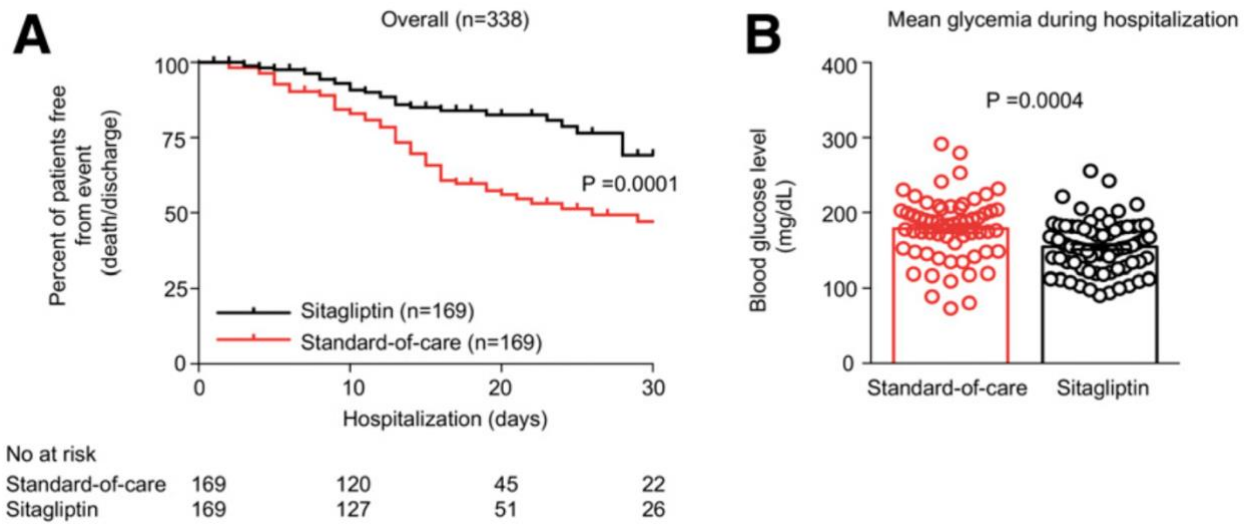

Figure 2 | Mortality in COVID-19 and type 2 diabetes patients getting sitagliptin or conventional treatment. A: Time to reach the clinical end point (death or hospital release) for patients receiving sitagliptin versus those receiving standard therapy. B: A bar graph showing the average blood sugar levels in the two groups during hospitalization. The number of patients is given as No, and the data are shown as mean ± SEM.

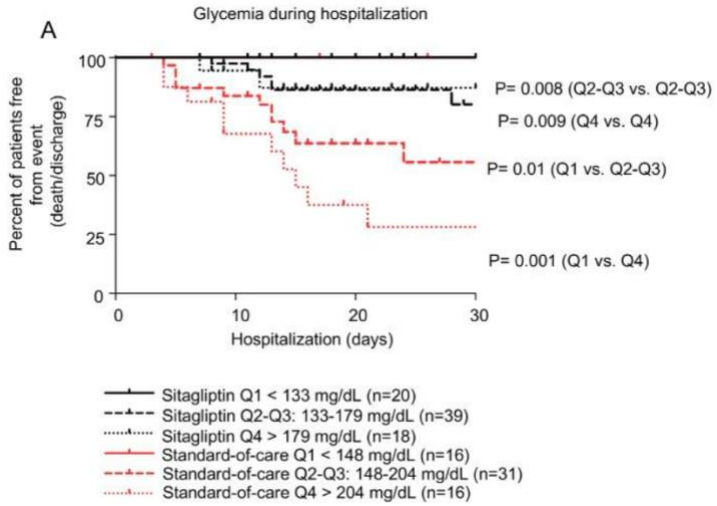

Figure 3 |Clinical outcomes subanalysis in the sitagliptin-treated patients and the control group. Time to clinical endpoint (death/hospital discharge) for sitagliptin-treated patients categorized by mean blood glucose level quartiles derived for each group and for the standard-of-care group.

The sitagliptin-treated group had better outcomes than the control group, according to an analysis of time to clinical endpoint (death/discharge) (hazard ratio [HR] 0.44 [95% CI 0.29-0.66; p-value= 0.0001]) (Figure 2A). We examined the mean blood glucose level recorded during hospitalization and found that it was lower in sitagliptin-treated patients than in the group receiving standard treatment, which was significant given the potential role that glycometabolic management may have in raising the mortality rate (Figure 2B). In an examination of the time to clinical end point (death/discharge), we discovered that improved glycometabolic control during hospitalization was related with better outcomes after classifying patients by quartiles of mean glucose level (Figure 3).

No variations were identified between groups in the necessary units of insulin supplied daily (Table 2).

Clinical outcomes for patients receiving sitagliptin add-on medication upon admission significantly improved (Tables 2 and 4). When compared to standard of care treatment for the relevant clinical indicators, sitagliptin use was linked with a lower probability of needing mechanical breathing (HR 0.27 [CI 0.11-0.62]; p-value = 0.003). (Table 4).

Secondary end points	*N* at risk (*N* with end point), sitagliptin vs. standard of care	HRs (95% CI) for sitagliptin vs. standard of care	*P* value
Intensive care	118 (15) vs. 102 (25)	0.51 (0.27–0.95)	0.03
Mechanical ventilation	118 (6) vs. 102 (17)	0.27 (0.11–0.62)	0.003
ECMO	118 (8) vs. 102 (7)	1.15 (0.41–3.17)	0.77

Table 4 | Measure of the secondary clinical outcomes' HRs (need for intensive care unit, mechanical ventilation, and ECMO).

Sitagliptin users had a slightly lower probability of needing intensive care than those receiving standard treatment (HR 0.51 [CI 0.27-0.95]; p-value = 0.03), but there was no change in the need for extracorporeal membrane oxygenation (ECMO) (HR 1.15 [CI 0.41–3.17]). Clinical improvement, which is measured relative to the 30th day of follow-up, is defined as a decrease of two points or more on the seven-point category scale. These data were available for 145 patients receiving normal care and 139 patients receiving sitagliptin treatment.

Only 50 patients (34%) in the group receiving standard therapy showed clinical improvement, compared to 72 patients (52%) in the sitagliptin group who had a drop of at least two points (Table 2 and Figure 4).

In addition, more patients receiving standard of treatment (67 [46%]) than sitagliptin (36 [26%]) experienced deterioration of clinical outcomes, which is defined as any rise in clinical score from baseline (Table 2 and Figure 4).

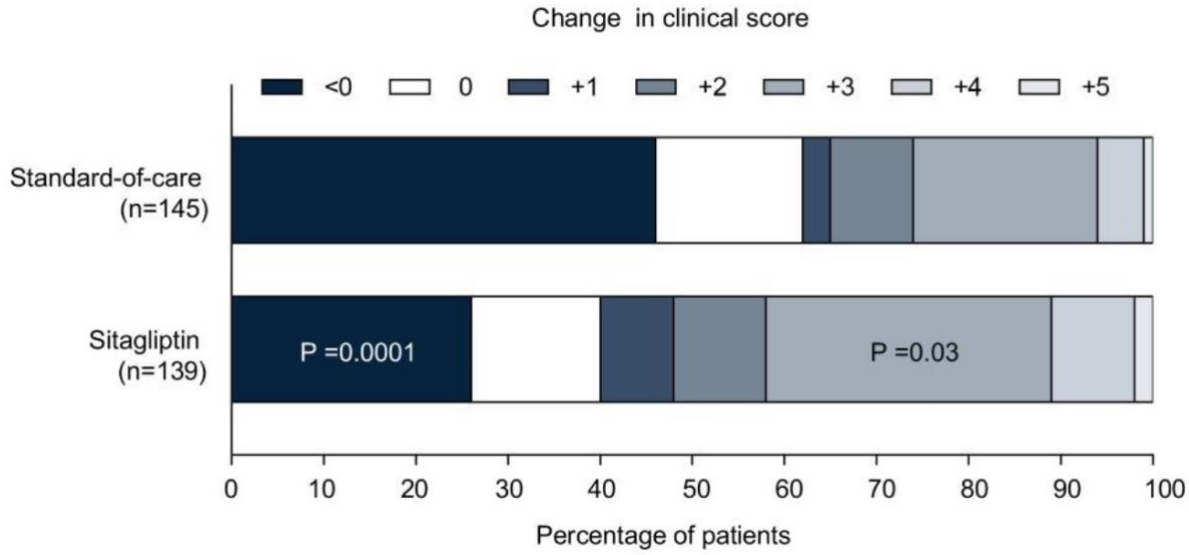

Figure 4 |Change in the clinical score in the sitagliptin-treated and control patient groups after 30 days of follow-up. A bar graph showing the percentages of patients who had evaluated their clinical outcome at a follow-up. These patients were classified according to their clinical outcome improvements. As compared to the clinical outcome at baseline, scores ranged from a worsening outcome to an improvement of 1–5 points.

Comparing patients in the standard-of-care group to those receiving sitagliptin treatment at admission, a more thorough study revealed this association to be true (Table 2). Additionally, more patients receiving sitagliptin were released from the hospital on day 30 than those receiving standard of care (Table 2). In individuals who were recruited in the sitagliptin-treated group, there were no major adverse events connected to the medication. A reduction in inflammatory parameters such as procalcitonin and CRP was also found among the patients included in the study (Table 2). Lymphocyte counts of patients treated with sitagliptin increased from baseline and compared to patients treated with standard of care (Table 2). Finally, an improvement in glycemic control was evident at follow-up in patients treated with sitagliptin compared with patients who had been treated with standard insulin treatment alone (Table 2).

The sitagliptin group outperformed the standard care group in terms of time to clinical endpoint and mortality, according to a sub-analysis in patients under the age of 70 (HR 0.54 [CI 0.34-0.85]; p-value = 0.009) (Figure 5). Males and females were compared in the sitagliptin vs. standard care groups in this investigation that tracked time to the clinical endpoint, but no difference between the sexes was discovered (Figure 5).

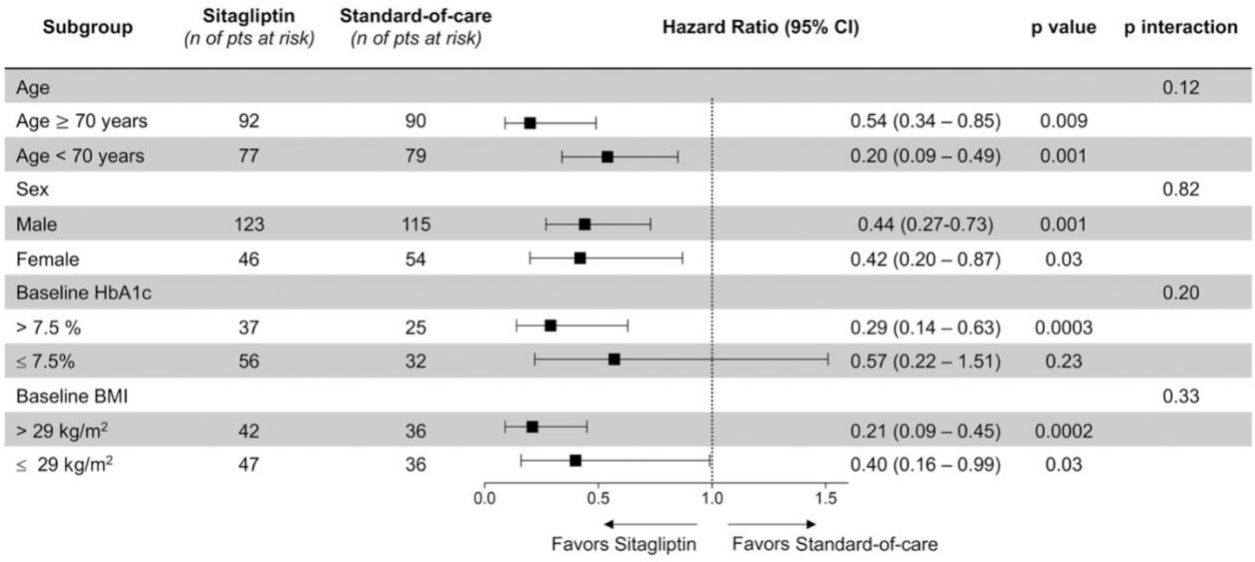

Figure 5 |Analysis of subgroups of COVID-19 patients (pts) receiving sitagliptin or standard of treatment for type 2 diabetes. Forest plots of subgroup analyses examining the impact of standard of care and sitagliptin medication in COVID-19 participants with type 2 diabetes. Age (≥ 70 or < 70 years), sex (males or females), baseline HbA1c (≥ 7.5% or <7.5%), and baseline BMI (≥ 29 kg/m^2 or < 29 kg/m^2) are all subgroups.

It is crucial to remember that both man and females control groups (standard of care) fared worse than the sitagliptin groups in terms of outcomes. Sitagliptin females vs. standard of care females: p-value=0.03, HR 0.42 [CI 0.20-0.87]; Sitagliptin males vs. standard of care men: p-value=0.001, HR 0.44 [CI 0.27- 0.73]. Patients with HbA1c levels below the median (7.5%) shown improvement, although on par with patients in the treated and control groups (Figure 5). There were no apparent interactions between any of the subgroup factors and the therapy. Metformin, insulin, or other hypoglycemic medications pre-hospitalization therapy had no impact on clinical outcomes.

A higher HbA1c value, notably in treated control individuals, was linked to a worse outcome (sitagliptin vs. standard of care, HR 0.29 [CI 0.14-0, 63]; p-value=0.0003). Patients who received sitagliptin and had a BMI below the median of 29 kg/m^2 had a better outcome when stratifying patients based on their BMI, which was also evaluated at baseline (HR 0.40 [CI 0, 16-0.99]; p-value = 0.03) (Figure 5). Sitagliptin vs. standard of treatment, HR 0.21 [CI 0.09-0.45]; p-value = 0.0002; showed that higher body mass index was linked to worse outcomes in the group receiving standard of care.

Chapter 2: **AIMS AND HYPOTHESIS**

The COVID-19 pandemic has affected both apparently healthy subjects and subjects with various pre-existing comorbidities, with a worst outcome in the latter. Several retrospective observational studies have highlighted the fact that among hospitalized patients, a large proportion showed impaired glucose metabolism. In some cases, these glucose alterations were attributable to late diagnoses of diabetes, as evidenced by high HbA1c values in these patients.

However, several other hospitalized cases showed recently diagnosed fasting hyperglycemia, as HbA1c values were normal at the time of admission.

This suggests that the latter were not yet undiagnosed cases of diabetes, but full-fledged new findings, presumably due to SARS-CoV-2 infection.

The hypothesis underlying this work was to determine whether in the cohort of patients belonging to our ASST-FBF-Sacco Polo Universitario structure, there was evidence that could confirm that the acute and post-healing glycometabolic decompensation observed in some of the patients hospitalized for the SARS-CoV-2 infection was caused by the infection and by the cytotoxic effect exerted by the virus itself on the pancreatic islets.

To demonstrate this hypothesis and to characterize the effective role of COVID-19 on the onset of new cases of hyperglycemic patients and on the worsening of the clinical condition of diabetic patients, it was conducted a study where clinical and laboratory outcomes were collected respectively from hospitalized patients and in vitro models of pancreatic islets.

A number of cases of patients affected by COVID-19 showed impaired fasting blood sugar during hospitalization without having a history of diabetes. The main objective was to demonstrate the presence of these glucose alterations both in the acute phase and long COVID-19. Starting from clinical observations the following aims were established:

(i) Study glucose alterations through the use of continuous glucose monitoring (CGM).
(ii) Study insulin resistance and beta cell function through serum hormone sampling under fasting condition and arginine test.
(iii) Characterize secretome (cytokine profile) changes to assess its clinical impact on glycometabolic control.
(iv) Investigate the presence of SARS-CoV-2 virus within pancreatic islets isolated from donors who died for COVID-19 in order to demonstrate its direct effect on beta cell disfunction and glucose metabolism.

Chapter 3: **MATERIALS AND METHODS**

3.1 Study design

From 1 February 2020 to 15 May 2020, informations were collected on patients hospitalized at ASST FBF-Sacco Milano, Presidio Sacco, for acute SARS-CoV-2 infection was gathered. Patient positivity to SARS-CoV-2 was identified by RT-PCR in respiratory sample.

The patient's initial clinical score was determined using a modified ordinal score with the following seven key points: (1) Outpatient with the ability to resume normal activities; (2) Not hospitalized but unable to resume normal activities; (3) Hospitalized but not needing supplemental oxygen; (4) Hospitalized but needing supplemental oxygen; (5) hospitalized, requiring noninvasive mechanical ventilation; (6) hospitalized but needing invasive mechanical ventilation or extracorporeal membrane oxygenation; and (7) death.

For both baseline demographic distributions, clinical data, laboratory data, management, and outcome data, all clinical data were collected from patients' electronic medical reports (Table 5). Each patient's blood glucose levels were checked three times: when they were admitted to the emergency room, while they were in the hospital, and when they were discharged. HbA1c results from the hospital admission were also obtained, when available. When information about a patient's medical history or an antidiabetic treatment regimen was available, patients were categorized as having diabetes, following ADA criteria.

Approximately 551 patients with COVID-19 and those who had recovered from COVID-19 were included in this study based on the clinical assessment of the Infectious and Respiratory Diseases Division of the ASST FBF-Sacco Milano and on the basis of a prior positive COVID-19 test. Within these 551 patients were included normoglycemic with no history of diabetes or IFG (impaired fasting glucose), or IGT (with impaired glucose tolerance). These patients were compared to a group of demographically matched healthy controls. Moreover, this study also included a small group of T2D patients as an extra control. Patients with T2D received metformin treatment and/or adhered to a restricted diet. Acute intravenous arginine stimulation insulin secretion testing (assessment of hormone level) and glucose monitoring with a professional CGM device (assessment of continuous glucose level) were performed on small patients' subgroup). Recruitment of male and female volunteers with normoglycemia, age >18 years and < 80 years, no history of diabetes or IFG/IGT, and no usage of medications known to affect glucose metabolism established the inclusion criteria. Age <18 years, a history of IFG or impaired IGT glucose tolerance, drug

usage with known effects on glucose metabolism, and pregnancy were exclusion criteria. Immunoistochemical and Histopatological analysis were performed on samples gathered from ASST Fatebenefratelli-Sacco Hospital's pathology department. The Milan Area 1 Ethics Committee gave its approval to all investigations and research analyses reported in this retrospective cohort study (n. 2020/ST/167). For all experimental analyses performed and reported in this study, signed informed consent was also obtained.

3.2 Continuos glucose monitoring

Within the original cohort of 551 patients, a subset of participants who were normoglycemic, including healthy controls (n = 12), COVID-19 patients (n = 8), and recovered COVID-19 patients (n = 8), underwent glycemic profiling utilizing a professional retrospective CGM (Medtronic Envision Pro CGM, Medtronic Minimed). The system comprises of a fully calibrated device that includes a CareLink Pro software license, an Envision Pro application, a CE-approved Envision recorder, and an Envision Pro sensor. Throughout the study, CGM results were kept blind from patients and clinical site staff. Each recording on CareLink software for expert CGM systems required informed patient consent. During the registration period, the following variables were assessed: mean blood glucose, estimated HbA1c, peak and nadir blood glucose, time above 140 mg dl, AUC above the limit of 140 mg dl, mean postprandial blood glucose values at 60 and 120 min, standard deviation, and coefficient of variability values.

3.3 Arginine test for the evaluation of hormone levels

Using an intravenous arginine stimulation experiment, the production of insulin and C-peptide was assessed. The patient's arm's antecubital vein was used to place an intravenous catheter. When not in use, a steady infusion of 0.9% saline was used to keep the catheter patent. At 0 minutes, reference samples were collected. Then, over a 45-second period, an intravenous injection of arginine hydrochloride at its highest stimulating dose (5 g) was given. Samples were collected at minutes +2, +5, +10, and +30. In order to calculate AIRmax (arginine-induced maximal insulin release) for fasting glucose, basal insulin was subtracted from the mean of the three highest insulin readings from minutes 2, 5, and 10. As a measure of beta-cell activity, the ratio of fasting insulin to proinsulin was computed.

The HOMA-IR formula (model for the assessment of homeostasis of insulin resistance) was used to determine insulin resistance: fasting insulin (mIU ml-1) fasting glucose (mmol l-1)/22,5. The HOMA-B index (model for assessing beta cell function homeostasis) was calculated using the following formula: 20 × fasting insulin (μIU ml-1)/fasting glucose (mmol ml-1) - 3,5 [52].

3.4 Inflammatory score

To define cut-off points and assign a score ranging from 0, which represents the lowest quintile, to 4, which represents the highest quintile, each plasma cytokine value was stratified into quintiles[53].

3.5 Biochemical analysis

Fasting serum samples were taken from patients and controls at predetermined times and stored at -80°C for biochemical analysis. G-CSF, IFN-, IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, MCP-1, MIP-1, TNF-alpha, and IP-10 baseline levels in patient and control samples were assessed in accordance with manufacturer's instructions using a Bio-Plex Pro Human Cytokine 17-plex magnetic bead-based immunoassay (According to the instructions provided by the manufacturers, baseline serum proinsulin (Mercodia, 10-1118-01) and HbA1c (Aviva Systems Biology, OKEH00660) levels were measured by ELISA using commercial kits. On serum samples collected in tubes containing potassium oxalate/sodium fluoride, a colorimetric assay (Life Technologies, EIAGLUC) was used to assess baseline blood glucose levels. Finally, using a Bio-Plex Pro Human Diabetes 10-Plex Assay kit (171A7001M) and a Bio-Plex 200 reader (Bio-Rad), serum samples from participants who underwent an arginine stimulation test were collected at each arginine test time point (T0-T4) and evaluated for insulin, C-peptide, and glucagon concentrations in accordance with the manufacturer's instructions.

3.6 Statistical and power analysis

Categorical variables are shown as proportions, while continuous variables are presented as means with standard errors. To compare categorical variables, we employed a chi-squared test or Fisher's exact test, and to compare continuous variables, we utilized independent-

samples t-tests. One-way or two-way ANOVA with a Bonferroni's post hoc test between the interest group and all other groups was utilized, as appropriate, for multiple comparisons. To assess connections between mortality and other population parameters, Spearman's correlation analysis was used. The survival curves were compared using a log-rank test. P-values with two tails less than 0.05 were regarded as statistically significant. In order to model the associations between risk factors and clinical outcomes, multivariate logistic regression was used (Stata version 12; StataCorp). The multivariate logistic regression analysis carried out for each clinical outcome took into account age and gender. For between-group clinical endpoint analysis, a log-rank test (Mantel-Cox) was employed throughout time (GraphPad Prism version 8.4.3; GraphPad software). The relationship between peripheral IL-6 levels and blood glucose was investigated using Spearman's correlation. P-values with two tails less than 0.05 were regarded as statistically significant. Graphs relating to Fig. 17b were produced using Microsoft Excel version 16.30. Given that the mean AUC insulin response seen in participants with normal glucose tolerance tested for arginine is 1,083 132 pmol l-1, the sample size was fixed at 15 in the control group and 10 in the other subgroups to give the study 80% power and a significance level of $\alpha = 0.05$ to detect at least a 15% difference in mean insulin AUC response to 5 g arginine intravenously between groups.

3.7 Pancreatic islets specific autoantibodies

A completely established ELISA technique was used to assess the autoantibodies to insulin, GAD, islet antigen 2 (IA-2), and ZnT8. This assessment was performed on the serum of 10 patients who developed new onset hyperglycemia and 10 patients who recovered from COVID-19. When assessed in duplicate in 25 mL of serum, the titres of antibodies to insulin, GAD, IA-2, and ZnT8A were expressed in units determined from internal standard curves. Values for anti-insulin and anti-IA-2 were regarded as negative when 10 < IU/mL and positive when >10 IU/mL, respectively, in accordance with the manufacturer's procedure. For anti-GAD and anti-ZnT8, the cutoff was set at 5 units/mL and 15 units/mL, respectively.

3.8 In vitro studies on human pancreatic islets

Human pancreatic islet apoptosis and function were studied in cultures of human pancreatic islets with and without interleukin-1b (IL-1B), tumor necrosis factor-alpha (TNF-alpha), IL-

13, IL-6, and interferon-g-induced protein 10 (IP-10). To assess insulin release by ELISA, supernatants were gathered. Human sera from patients with acute COVID-19 and from patients who had recovered from COVID-19 were added to human pancreatic islets in order to replicate the effects of COVID-19 on the pancreas; serum from healthy controls or normal culture medium (with 10% FBS) was used as a control. Insulin levels were measured using a microparticle enzyme immunoassay (Iso-Insulin ELISA; cat. nos. 10-1113-01 and 10-1247-01; Mercodia, Uppsala, Sweden) with intra-interassay coefficients of variation (CV) of 3.0% and 5.0% after the culture supernatant had been collected after 24 hours. After 24 hours of culture, islet lysates were obtained, and cell death/apoptosis was evaluated by ELISA (cat. no. 11544675001; Roche Diagnostics GmbH, Mannheim, Germany).

3.9 Recombinant proteins

Human pancreatic islets were cultured for 24 hours per the manufacturer's instructions and exposed to 15 pg/mL recombinant human IL-1B (R&D Systems, Minneapolis, MN), 100 pg/mL recombinant human TNF-alpha (R&D Systems), 20 pg/mL recombinant human IL-13 (R&D Systems), 30 pg/mL recombinant human IL-6 (R&D Systems), and 1 ng/mL recombinant human IP-10 (R&D Systems) or serum from acute or long COVID-19. For 24 hours, 10% FBS was replaced with serum from healthy volunteers in the culture medium as a control. In a 24-hour in vitro culture, immunoneutralization tests were carried out using the inhibitors anti-IL-1B (1 mg/mL) from Thermo Fisher Scientific in Waltham, MA, anti-IL-6 (10 mg/mL) from Roche, and anti-IL-13 (10 mg/mL) from Sigma Aldrich in St. Louis, MO.).

3.10 Pancreatic islets

The manufacturer's instructions were followed for cultivating purified human pancreatic islets of Langerhans received from healthy individuals at a commercial source (cat. #35002-04; Celprogen, Torrance, CA).

3.11 Receptome analysis of human pancreatic islets

400 pure islets from healthy donors (n = 4) with unusable pancreases were used to extract and then purified RNA using the Direct-zol RNA Mini Prep Plus Kit (cat. no. R2070; Zymo Research, Irvine, CA). At the Center for Bioinformatics and Functional Genomics of San

Raffaele Hospital, RNA was sequenced and then with the help of the libraries edgeR_3.26.5, DESeq2_1.24.0, and pheat-map_1.0.12, gene expression analysis was carried out using R software (version 3.6.1). Transcripts were normalized to reads per kilobase per million of expression units of reads mapped in order to estimate the relative abundance of transcripts. Then, for surface receptors expressed at a moderate/high level (cutoff >25) in human islets and cells beta, a rank analysis based on previously discovered genes by transcriptome analysis (Affymetrix, Santa Clara, CA) was carried out.

3.12 Immunoistochemical and histopathological analysis

Pancreatic tissues from deceased COVID-19 patients, deceased control participants, or deceased type 2 diabetes patients were obtained from ASST Fatebenefratelli-Sacco Hospital's pathology department. At the Milan Sacco Hospital, all patients provided their informed consent prior to being admitted to the hospital for sample collection. The obtained samples were preserved in a 70% ethanol solution and treated in 10% neutral buffered formalin (4% w/v formaldehyde and 0.05 M acetate buffer). After that, the samples were prepared for paraffin embedding. After deparaffinization, rehydration, and antigen retrieval, 3 mm thick slices were stained with hematoxylin-eosin (H-E). With the use of an Olympus BX41 microscope, photos were taken (Center Valley, PA). Fixed, paraffin-embedded pancreatic lymph nodes (PLN) from COVID-19 patients were subjected to HE and MECA-79 staining.

3.13 Analyzing unmethylated INS DNA to detect beta-cell death

DNA was isolated from 200 mL of serum using the QIAGEN DNA Blood and Tissue Kit and DNA obtained, which was subsequently treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research). Subsequently, a droplet digital PCR was followed. In the next step, a 25 mL assay volume was prepared, which consisted of Droplet PCR Supermix (Bio-Rad Laboratories, Billerica, MA), 900 nmol/L primer, and 250 nmol/L probe. Two probes targeting two methylation-sensitive sites of the human insulin gene (hg19_knownGene_uc021qcd.1; range chr11:2181009–2182439) at nucleotides 21814010 and 21814012, which are +396 and +399 from the transcription start site, were used together with 5 μL of DNA.

A droplet generator (made by Bio-Rad Laboratories) was loaded with the mixture and droplet generating oil, and the created droplets were then transferred to a 96-well PCR plate and sealed. The following PCR settings were used on a thermal cycler: 10 min of activation at 95 °C, 40 cycles of a two-step amplification technique (30 s of denaturation at 94 °C and 60 s of amplification at 58 °C), and 10 min of inactivation at 98 °C. The QX100 Droplet Reader (Bio-Rad Laboratories) was used to read the PCR plate, and QuantaSoft Analysis Software was used to evaluate the results (Bio-Rad Laboratories). Applying a fluorescence amplitude threshold based on the amplitude read from the negative control used as a template allowed us to distinguish between droplets that contained the target (positive) and those that did not (negative). Each sample's ratio of unmethylated to methylated INS DNA was determined.

3.14 Electronic microscope analysis

Following initial fixation in a solution of 2% paraformaldehyde and 2% glutaraldehyde in sodium cacodylate buffer (pH 7.4), paraffin-embedded pancreatic sections of specimens isolated from COVID-19 patients, healthy control subjects, and patients with type 2 diabetes were post-fixed in 1% OsO4 in 0.1 M sodium cacodylate buffer (pH 7.4). Sections were transferred to Propylene Oxide and then implanted in Epon-Araldite after ethanol through a graded sequence of dehydration. A Morgagni 268D transmission electron microscope (Philips, Eindhoven, The Netherlands) was then used to analyze 60 nm-thick sections that had been cut, mounted on nickel grids with Formvar membranes, stained with uranyl acetate, and lastly evaluated. The pictures were then recorded. Pancreatic sections were progressively treated with citrate buffer (pH 6) for 10 minutes at 98°C and sodium metaperiodate for 30 minutes at 98°C for immunocytochemical examination. Sections were treated in 1% egg albumin solution for 5 min. after that, they were transferred to a solution containing guinea pig insulin antibodies (Dako, Carpinteria, CA), diluted 1:50, and incubated overnight at 4°C. The sections were rinsed several times the following day, and they were then incubated for an hour in a colloidal gold solution containing anti-guinea pig antibodies, (from Jackson ImmunoResearch in Philadelphia, Pennsylvania) which had been diluted 1:20. The sections were washed following incubation and then stained with lead citrate and uranyl acetate.

3.15 SARS-CoV-2 detection through RT-PCR

Thermo Fisher Scientific's mirVana miRNA isolation kit was used to purify the total RNA recovered from postmortem pancreatic tissues acquired from COVID-19-positive hyperglycemic individuals. Following the manufacturer's instructions, the 2019-nCoV Taq-Man RT-PCR Kit from Norgen was used to identify SARS-CoV-2 specific RNA in a real-time RT-PCR based on the usage of TaqMan Technology in order to quantify SARS-CoV-2 expression (Norgen Biotek Corp., Thorold, Canada). The 7900HT Fast Real-Time PCR apparatus was used to conduct RT-PCR reactions in a 96-well configuration (Applied Biosystems, Foster City, CA).

3.16 Immunoistochemical and immunofluorescence analysis of pancreatic sections

A primary polyclonal antibody to SARS-CoV-2 spike protein S1 (cat. no. AHP3013; Bio-Rad Laboratories, Hercules, CA) was tested in the autopsied pancreatic specimens to identify the SARS-CoV-2 virus (1:50 dilution). The following mixture of primary antibodies was tested, correspondingly, to determine which cell types the aforementioned antibody would potentially be positive in:

1. An insulin monoclonal antibody (cat. no. 14-9769-82; Thermo Fisher Scientific) at a dilution of 0.5 mg/mL is used to highlight islet beta cells.
2. An antitrypsin 1 monoclonal antibody (cat. no. ab200997; Abcam, Cambridge, U.K.) is used to identify exocrine acinar cells (dilution 1:2,000)
3. A monoclonal anti-keratin 19 antibody (LS-B108; Lifespan BioSciences, Seattle, WA) at a dilution of 5 mg/mL was used to highlight ductal cells.

Tetramethylrhodamine goat anti-rat IgG, cat. no. AP136R; Merck Millipore, was used as a tetramethylrhodamine probe to detect the anti-SARS-CoV-2 spike protein S1 primary antibody (dilution 1:70), while fluorescent goat anti-rabbit IgG conjugated to fluorescein isothiocyanate, cat. no. AP187F; Merck Millipore, was used (dilution 1:70).
A confocal system (STELLARIS 5 Confocal Microscope; Leica Microsystems, Wetzlar, Germany) with a 63 oil objective was used to observe the three antibody pairs. Multiple

optical routes that were independent and sequential were used to acquire the images in multitrace mode.

Chapter 4: **RESULTS**

4.1 Alteration of glycometabolic control both in acute and post-COVID-19 phase

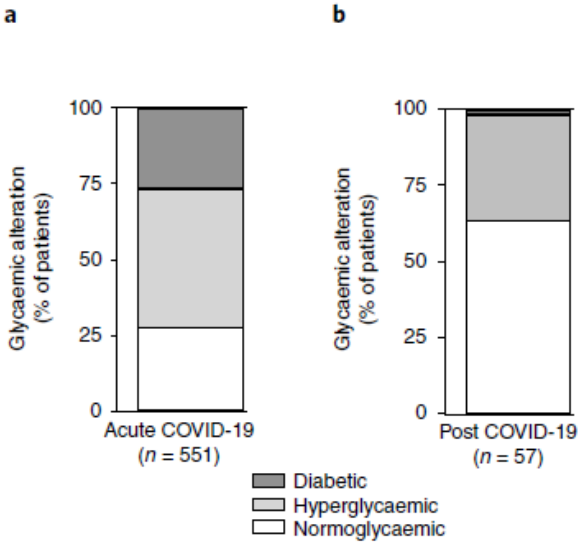

Figure 6 | A: Glycometabolic alterations in a group of 551 individuals who were hospitalized with acute COVID-19. B: Changes in blood glucose levels for the hyperglycemic group at six months after hospital discharge (post COVID-19).

Glycometabolic control was assessed in a cohort of 551 COVID-19 patients who were hospitalized at our academic center as part of the first examination (ASST FBF-Sacco Milano, Presidio Sacco) [54]. The results are fully presented in the published paper in Appendix B. At the time of hospital admission, 151 (27%) out of 551 patients had T2D and clearly abnormal HbA1c results (Figure 6A, 7). Of these 151 patients, 86 had a history of diabetes,

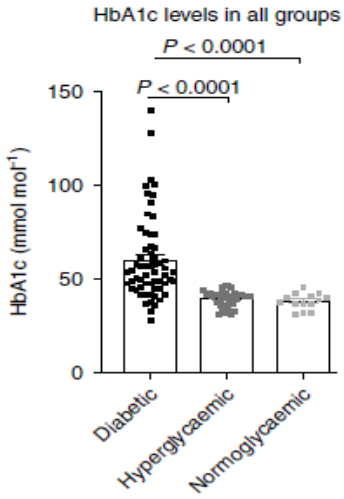

Figure 7 | Patients with diabetes, newly diagnosed hyperglycemia, and normoglycemia had their mean HbA1c values assessed.

while the remaining 65 had their diagnosis of diabetes during their hospitalization using American Diabetes Association (ADA) criteria (Figure 6A) [55]. 253 patients out of 551 (46%) where identified as hyperglycemic during COVID-19 hospitalization. The remaining 147 patients (27%) had normal blood sugar levels (Figure 6A). Among patients who were discovered as new-onset hyperglycaemic during COVID-19 hospital admission and followed up for six months, a significant portion (35%) continued to display this new onset glycemic alteration, while 2% of these patients had acquired and been given a diagnosis of diabetes, and the remaining 63% experienced remissions that resulted in a return to a normoglycemic state (Figure 6B).

	All	Diabetic	Hyperglycaemic	Normoglycaemic	P-value
n	551	151	253	147	
M/F, n (%)	344/207(62/38)	103/48 (67/33)	159/94 (65/35)	82/65 (51/49)	0.28† 0.03§ 0.17 $^{\#}$
Age (years)	61± 0.7	67± 1.1	61± 0.9	55± 1.5	<0.001† <0.001§ <0.001$^{\#}$
BMI (Kg/m^2)	27± 0.5	29± 1.4	27± 0.7	27± 1.1	0.009† 0.009§ 0.009$^{\#}$
Hypertension, n	164	66	68	30	<0.001† <0.001§ 0.18$^{\#}$
ACE/ARB, n	119	47	47	25	0.002† 0.001§ 0.67$^{\#}$
Time to clinical improvement, d	14.9± 0.5	20.2± 1.3	13.6± 0.6	12.8± 0.8	0.001† 0.001§ 0.88$^{\#}$
Death, n (%)	85 (15)	42 (28)	29 (11)	14 (9)	0.001† 0.001§ 0.86$^{\#}$
Time to death, d	11.4± 1.4	10.5± 2.1	11.7± 1.8	12.0± 1.9	0.009† 0.009§ 0.009$^{\#}$
IL-6 (pg ml^{-1})	79.1± 7.4	108.2± 18.7	74.6± 8.0	45.5± 9.5	0.14† 0.007§ 0.38$^{\#}$

Table 5 | M, males; F, females; n, number of patients; ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker. Data are expressed as the mean ± SEM unless otherwise reported. †P Diabetes versus hyperglycaemic; §P Diabetes versus normoglycaemic; #P Hyperglycaemic versus normoglycaemic.

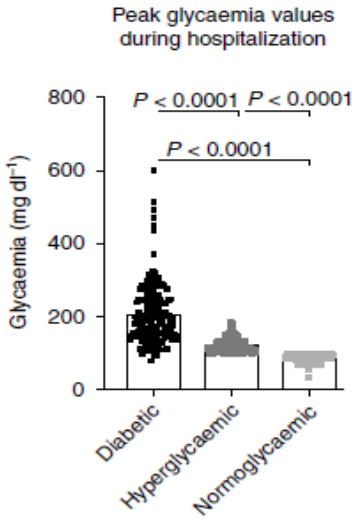

Figure 8 | Patients with diabetes, newly diagnosed hyperglycemia, and normoglycemia had their mean peak blood glucose levels assessed.

In the Table 5, the study's patients demographic and clinical details are listed. According to the data in the table, all patients took an average of 14.9 +/- 0.5 days to see clinical improvement after COVID-19, though diabetic patients took an average of 20.2 +/- 1.3 days. The hypothesis that the hyperglycemia found was of recent onset was confirmed when looking at the mean HbA1c levels, which were found to be significantly higher in patients with established/newly diagnosed diabetes than in patients with new-onset hyperglycemia and normoglycemia and did not differ between normoglycemic patients and patients with new-onset hyperglycemia (Figure 7).

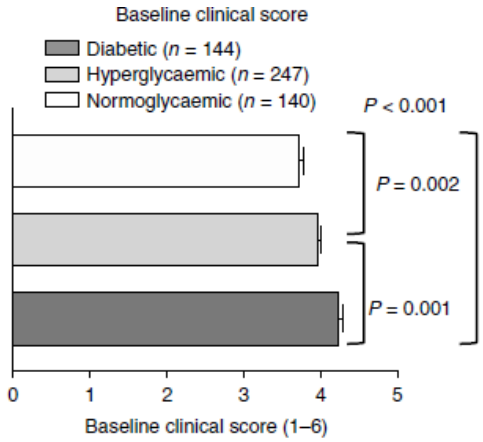

Figure 9 | Clinical score for the three patient groups at hospital admission

The mean glycemia levels of patients with preexisting diabetes were the highest. In contrast, the mean peak glycemic levels assessed during the hospital stay were significantly different between the three groups (Figure 8). According to a time-to-event endpoint analysis, patients with established or newly diagnosed diabetes had a higher risk of dying than those who were normoglycemic (hazard ratio: 2.16; 95% confidence interval: 1.27–3.67) or had newly developed hyperglycemia (hazard ratio: 2.05; 95% confidence interval: 1.28–3.29). Fascinatingly, patients with new-onset hyperglycemia needed to stay in the hospital longer and had higher clinical scores when they arrived (Figure 9). According to this finding, patients with new-onset hyperglycemia require more oxygen and ventilatory support than patients with normoglycemia. Between the groups analyzed, there was no difference in the admission to critical care (Figure 10). After adjusting for age and gender (Figure 11), an elevated odds ratio seen in individuals with hyperglycemia further supported the link between new-onset hyperglycemia and poorer clinical outcomes.

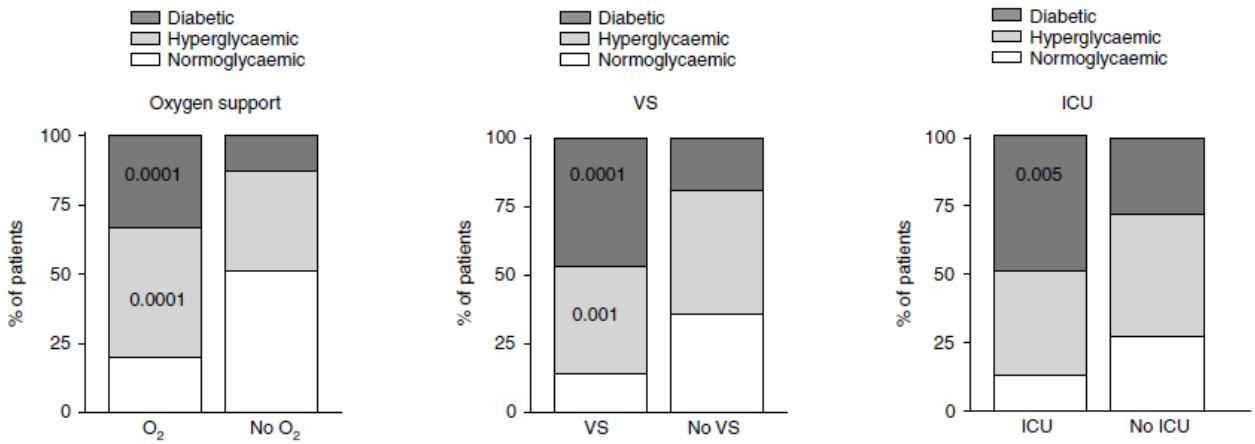

Figure 10 | In the diabetic, hyperglycaemic, and normoglycaemic groups, the rate of oxygen requirement, ventilatory support, and need for intensive care were also reported and compared; individuals with diabetes are represented by dark grey rectangles, individuals with hyperglycaemia by light grey rectangles, and individuals with normoglycaemia by white rectangles.

Furthermore, compared to the other two groups, patients with full-blown T2D had a longer hospital stay, had worse clinical scores, and had worse respiratory metrics (Figure 9/10).

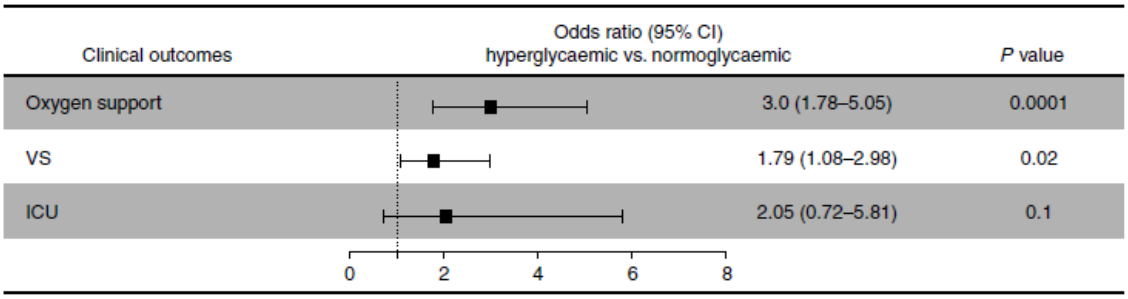

Figure 11 | After correcting for age and sex, forest plots were used to compare the odds ratio of the clinical outcomes (oxygen support, ventilatory support, and need for intensive care) between the hyperglycaemic and the normoglycaemic groups.

These findings and the presented data imply that newly developed hyperglycemia linked with COVID-19 may predispose patients to long-term maintenance of this hyperglycemia status, along with inferior test results and clinical consequences (e.g., longer hospital stays, increased need for oxygen support or positive pressure ventilation).

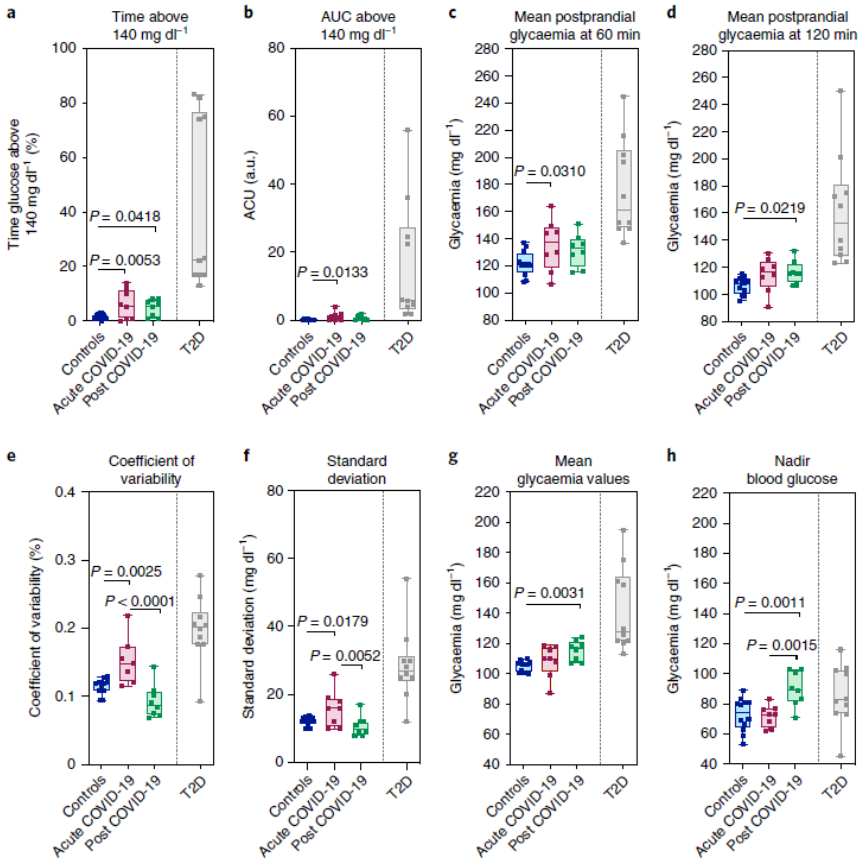

Figure 12 | Patients with COVID-19 had glycaemic abnormalities, according to continuous glucose monitoring. Duration of glycaemia measured above 140 mg dl−1 (a), AUC of glycaemia levels above 140 mg dl−1 (b), mean postprandial glycaemia at 60 min (c), mean postprandial glycaemia at 120 min (d), coefficient of variability (e), standard deviation (f), mean glycaemia values (g) and nadir blood glucose (h) in healthy controls, in patients with COVID-19 (acute COVID-19), in patients who recovered from COVID-19 (post COVID-19) and in patients with T2D.

The CGM was employed to further assess the glycemic anomalies found by the spot measurements taken. Professional CGM monitoring was done on patients with COVID-19 (n = 10) or patients who recovered from COVID-19 (n = 10) as well as on normoglycemic healthy controls (n = 15) and patients with T2D (n = 10; Figure 12). CGM monitoring was done at 62.0 ± 6.5 days following the start of the disease and during the acute phase of COVID-19.

	All	Controls	Acute COVID-19	Post COVID-19	T2D	P-value
n	35	15	10	10	10	
M/F, n	21/14	10/5	4/6	7/3	6/4	0.24† 0.9§ 0.9 # 0.36* 0.65ξ 0.9ψ
Age, years (mean± s.e.m.)	45.9± 2.1	47.2± 3.1	43.0± 4.7	46.9± 3.8	50.7±3.9	0.84† 0.99§ 0.9 # 0.8* 0.53ξ 0.96ψ
Smoking	4	4	0	0	1	0.12† 0.12§ 0.61# 0.9* 0.9ξ 0.9ψ
Familiality T2D, n	13	8	2	3	4	0.21† 0.41§ 0.68 # 0.9* 0.62ξ 0.9ψ
BMI (mean± s.e.m.)	23.4± 0.6	23.3± 0.6	24.8± 2.1	22.4± 1.5	27.2± 0.3	0.72† 0.94§ 0.014 # 0.53* 0.40ξ 0.020ψ
Hypertension (%)	1	1	0	0	5	0.9† 0.9§ 0.022 # 0.9* 0.032ξ 0.032ψ
Time after first symptom, d (mean± s.e.m.)	34.0± 5.7	-	22.9± 4.1	62.0± 3.2	-	<0.001*
Baseline clinical score (mean± s.e.m.)	3.1± 0.3	-	3.7± 0.3	2.0± 0.0	-	<0.001*
Therapies, n						
Hydroxychloroquine	3	0	2	1	0	0.55*
Antiviral	3	0	3	0	0	0.06*
Monoclonal antibody	0	0	0	0	0	0.9*
Steroids	1	0	1	0	0	0.33*
LMWH	6	0	6	0	0	0.001*
Antibiotics	6	2	2	2	0	0.9*
Diabetes-associated therapies, n						
Metformin	0	0	0	0	6	-
Others	0	0	0	0	0	-
No therapies	0	0	0	0	4	-

Table 6 | Demographic and clinical characteristics of subgroups analyzed for the CGM and arginine tests. LMWH, low-molecular-weight heparin. †P Controls versus acute COVID-19; §P Controls versus post COVID-19; #P Controls versus T2D; *Acute COVID-19 versus post COVID-19; ξ Acute COVID-19 versus T2D; ψ Post COVID-19 versus T2D.

Table 6 displays the demographic and clinical traits of these subgroups that received CGM measurements.

Analysis of CGM ratios gathered from different subgroups revealed that COVID-19 was linked to an overall impaired glycemic profile in non-diabetic patients, as shown by a significantly longer time that blood glucose was above 140 mg/dL (Figure 12 a), a significantly higher glycemic area under the curve (AUC) above 140 mg/dL (Figure 12 b), and a higher mean postprandial blood glucose at 60 minutes (Figure 12 c). As shown by a higher coefficient of variability (Figure 12 e) and higher standard deviation (Figure 12 f) in comparison to healthy controls, COVID-19 was also linked to greater glycemic variability. Surprisingly, several of the individuals who recovered from COVID-19 still had these blood glucose abnormalities that were discovered during hospitalization. In fact, patients who recovered from COVID-19 demonstrated longer durations of blood glucose above 140 mg/dL (Figure 12 a), higher mean postprandial blood sugar at 120 min (Figure 12 d), higher mean blood sugar (Figure 12 g), and greatest blood glucose nadir when compared to healthy controls (Figure 12 h). Other variables including variability coefficient and standard deviation for recovered COVID-19 patients were similar to healthy controls but differed from those for acute COVID-19 patients (Figure 12 e/f). Further evidence of a permanent glycometabolic control aberration following recovery from COVID-19 was revealed by the CGM data in the subset of patients who took part in the monitoring, though to a smaller extent than the glycemic changes seen in patients with T2D. The degree of insulin resistance and beta cell function were evaluated by sampling serum hormones under fasting conditions and after arginine stimulation in the patient subgroup receiving CGM, in order to better understand the influence that COVID-19 may have had on the observed glycemic abnormalities.[56]

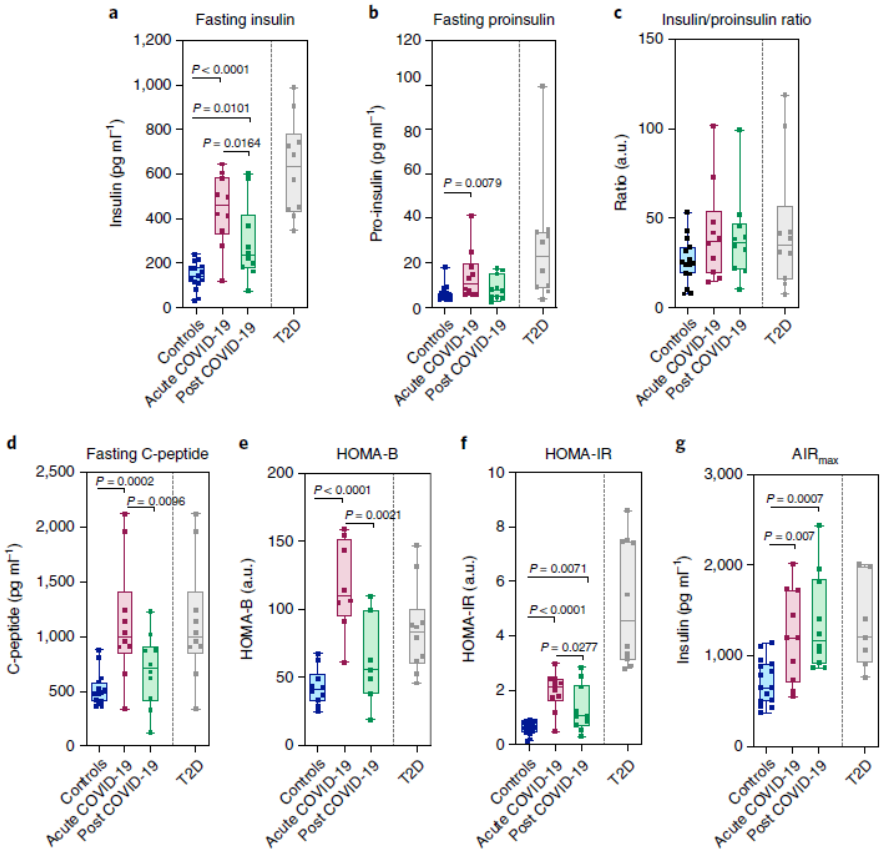

Figure 13 | Patients with COVID-19 have persistent insulin resistance and beta cell dysfunction. Mean fasting insulin (a), mean fasting proinsulin (b), fasting insulin-to-proinsulin ratio (c), fasting C-peptide levels (d), HOMA-B (e) and HOMA-IR (f) are shown for healthy controls, for patients with COVID-19 (acute COVID-19), for patients who recovered from COVID-19 (post COVID-19) and for patients with T2D, AIRmax (g)

Even though the arginine stimulation test is not regarded as the standard approach for evaluating beta cell activity [like the mixed meal test (MMTT) or the oral glucose challenge test (OGTT)], multiple clinical trials have shown that it is more reliable and reproducible than other tests. Patients with COVID-19 had significantly higher mean fasting insulin, proinsulin, c-peptide, HOMA-B, and HOMA-IR readings than healthy controls (Figure 13 a-f). Intriguingly, compared to healthy controls, COVID-19 patients displayed significantly larger maximal acute insulin responses to arginine (AIRmax; Figure 13 g) and higher AUCs for insulin and c-peptide. (Figure 14). Furthermore, compared to healthy controls, recovered COVID-19 patients had significantly higher fasting levels of c-peptide, HOMA-B, and HOMA-IR. (Figure 13 a-f). Similar to this, recovered COVID-19 patients' AIRmax values were considerably higher than those of healthy controls (Figure 13 g).

In addition, recovered COVID-19 patients had significantly greater AUC values for insulin relative to healthy controls, but not for c-peptide (Figure 14).

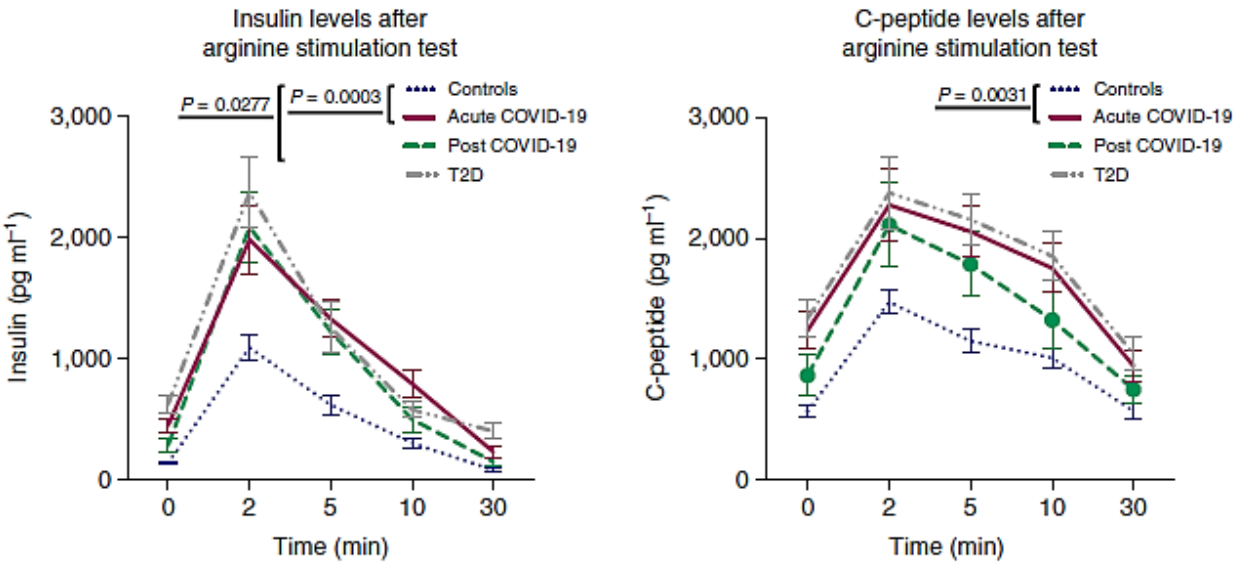

Figure 14 | mean AUC of insulin and C-peptide after arginine test (left and right) are shown for healthy controls, for the acute COVID-19 group or for the post-COVID-19 group, and for individuals with T2D.

According to data from CGM monitoring, both the hormonal profile under fasting settings and the hormonal profile following an arginine stimulation test are altered in patients with COVID-19 and in individuals who have recovered from COVID-19 (post-covid). Particularly from the latter test, persistent insulin resistance is emphasized, and patients with COVID-19 display a hormonal profile resembling those of T2D patients (Figure 14). [57, 58]

The cytokine profile was then analyzed to see if there were any modifications and/or differences between the COVID-19-affected patients, the recovered patients, T2D patients and the healthy controls [59]. It was evaluated the cytokine profile (i.e., the secretome) in the serum of patients with COVID-19 or in those who had recovered from COVID-19 and had also undergone CGM monitoring and arginine stimulation test.

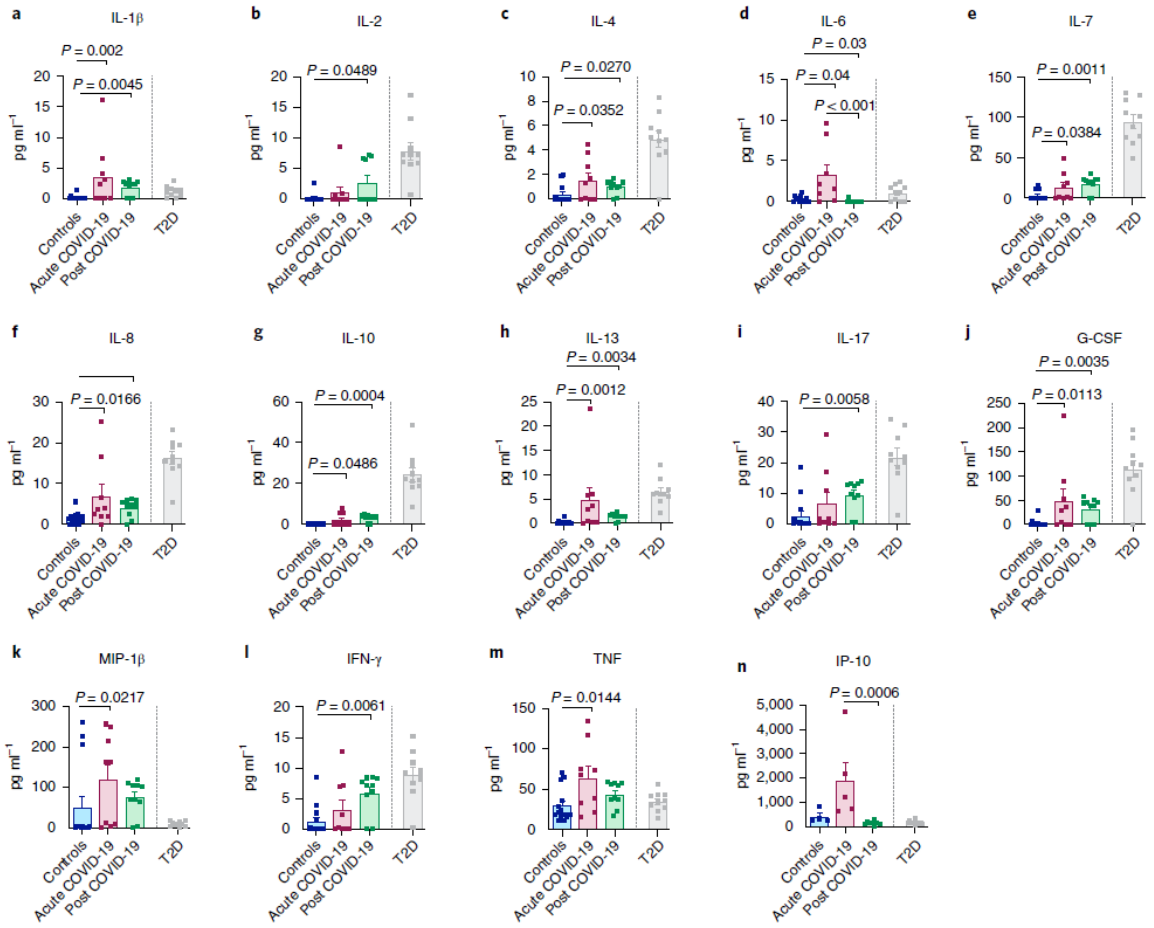

Figure 15 | Changes in the secretome are detected long after recovery from CoVID-19. A Luminex assay was used to measure the peripheral levels of 14 circulating cytokines (IL-1B, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-17, G-CSF, MIP-1beta, IFN-gamma, TNF, and IP-10) using sera from healthy controls, COVID-19 patients (acute COVID-19), COVID-19 patients who recovered from COVID-19, and patients with T2D. Data are visualized as scatterplots that display the mean ± SEM. Each dot (controls (blue), COVID-19 (maroon), and post COVID-19 (moss) represents a distinct sample. The unpaired Kruskal-Wallis test was used to establish statistical significance.

Those evaluation were performed based on the evidence reported in the literature[38, 60, 61], which shows that higher levels of some cytokines were detected in patients with SARS-CoV-2 infection. To do this, it was used a Luminex reader to perform a multiplex immunoassay that evaluates 17 different analytes, including cytokines and other released proteins. Ten cytokines were found to be significantly upregulated in serum of COVID-19 patients compared with healthy controls out of the 17 analytes examined (Figure 15): IL-1B, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, granulocyte colony stimulating factor (G-CSF), macrophage inflammatory protein-1 beta (MIP-1beta), and TNF-alpha. In particular, post-COVID-19 patient serum levels of 10 of the 17 analytes tested (IL-1B, IL-2, IL-4, IL-7, IL-8, IL-10, IL-13, IL-17, G-CSF, and IFN-gamma) were greater (Figure 15). Several analytes were discovered to be elevated in T2D patient sera, mirroring the pattern seen in COVID-

19 patients (Figure 15). In patients who had recovered from COVID-19, IL-6 and interferon-gamma-induced protein 10 (IP-10) levels were lower compared to both healthy controls (for IP-10) and to those who had COVID-19 infection in the acute phase (for IL-6 and IP-10; Figure 15). Some of the cytokines described above (IL-2, IL-17, and IFN-gamma) proved to be exclusively elevated in COVID-19 patients as compared to healthy controls (Figure 15). Additionally, based on these findings, an inflammatory score (IS) was computed, and it was higher in patients with COVID-19 and in patients who had recovered from COVID-19 (Figure 16 a).

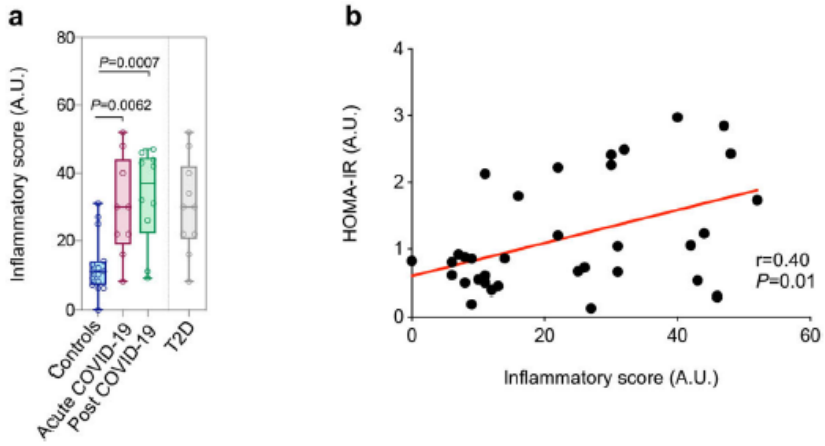

Figure 16 | Patients with COVID-19 exhibit elevated inflammatory scores and insulin resistance. (a) Inflammatory score assessed in patients with COVID-19 (Acute COVID-19), in patients who recovered from COVID-19 (Post COVID-19), in patients with T2D and in healthy controls. (b) Correlation between HOMA-IR and inflammatory score (IS) in patients with COVID-19.

This finding further supported that the insulin resistance linked to COVID-19 has an inflammatory origin by showing a positive correlation between the inflammatory score and the HOMA-IR (Figure 16 b).

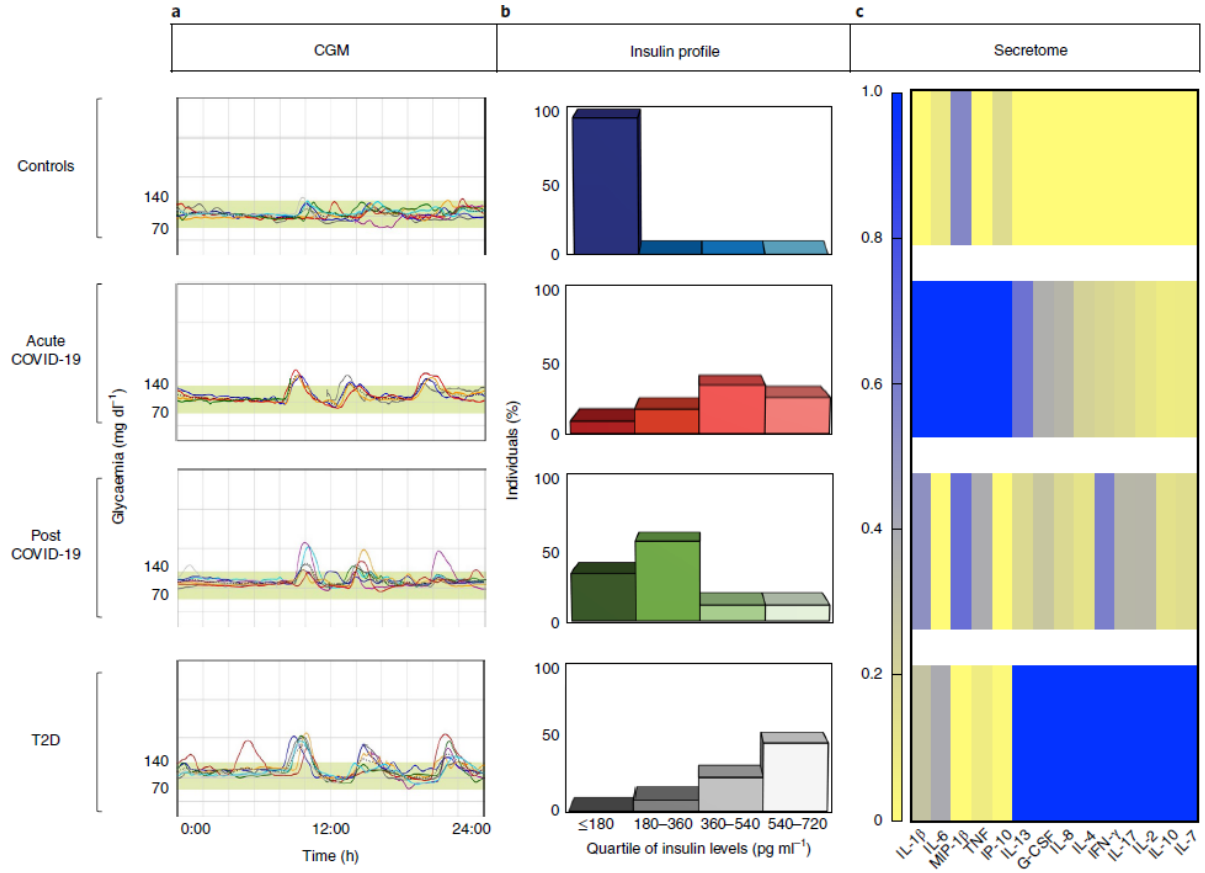

Figure 17 | Evidence of glycometabolic, hormonal and secretome abnormalities in patients with COVID-19. Schematic/analysis of comparisons between patients with COVID-19 (acute COVID-19), those who recovered from COVID-19 (post COVID-19), and patients with T2D, demonstrating abnormalities in CGM (a), insulin levels (b), and secretome profile (c), showing similarities with those found in patients with T2D.

The data revealed a change in the secretome in COVID-19 and post-COVID-19 patients, with an overall rise in numerous cytokine levels in the serum but a distinct profile from that seen in T2D patients.

Overall, COVID-19 patients, COVID-19 who already recovered, and T2D patients differed significantly from controls in terms of their CGM, hormonal profile, and secretome (Figure 17). The information gathered from our patient database was evaluated in order to make the following observations in order to better understand at a mechanistic level the potential impact that high peripheral levels of cytokines may have on the preservation of glycometabolic control: as a first finding, COVID-19 patients who had pre-existing diabetes had greater IL-6 levels determined by peripheral blood sampling at the time of hospital admission than normoglycemic patients, but not as high as newly diagnosed hyperglycemic patients (Table 5).

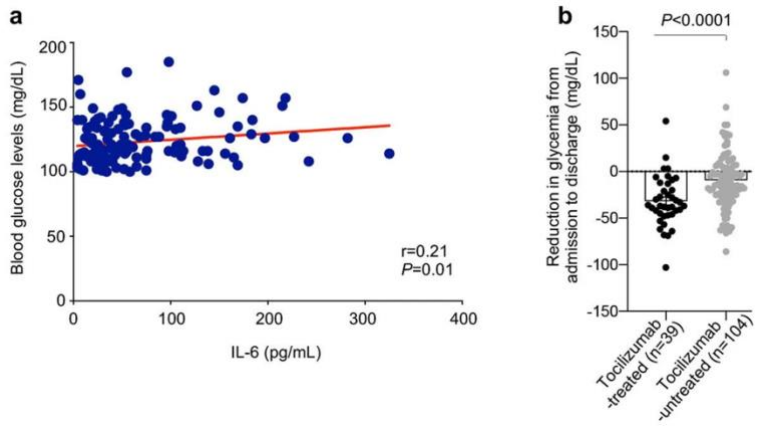

Figure 18 |Correlation between IL-6 and glucose and effect of tocilizumab on the latter in newly hyperglycemic patients with COVID-19. (a) Correlation between peripheral IL-6 levels and blood sugar in COVID-19 patients with new-onset hyperglycemia, and (b) reduction in glycemic levels represented as a delta between admission and discharge in COVID-19 patients with new-onset hyperglycemia who received tocilizumab or not as a treatment.

Indeed, peripheral IL-6 levels and fasting glucose levels were favorably associated in newly diagnosed COVID-19 hyperglycemic individuals (Figure 18 a). Importantly, among the 253 patients with new-onset hyperglycemia, 39 exhibited abnormally high peripheral IL-6 levels and were treated with tocilizumab as an off-label measure to lessen COVID-19-related inflammation. At hospital discharge, tocilizumab-treated COVID-19 patients with concurrent new-onset hyperglycemia showed better blood glucose decreases than tocilizumab-untreated patients (Figure 18 b).

	Controls	Acute COVID-19	Post COVID-19	*P*
N of participants	15	10	10	NS
Age, years ± SEM	45.9 ± 2.1	47.2 ± 3.1	43.0 ± 4.7	NS
Sex, M/F	10/5	4/6	7/3	NS
BMI, kg/m^2 ± SEM	23.4 ± 0.6	23.3 ± 0.6	24.8 ± 2.1	NS
Estimated HbA_{1c}				
mmol/mol ± SEM	34.1 ± 0.4	35.6 ± 1.4	38.0 ± 0.9	NS,* <0.05,† NS‡
%	5.5	5.4	5.6	
Autoantibodies, units/mL ± SEM				
Anti-insulin	2.1 ± 0.2	1.0 ± 0.4	2.5 ± 0.2	NS
Anti-GAD	1.0 ± 0.0	1.3 ± 0.3	1.0 ± 0.0	NS
Anti–IA-2	1.6 ± 0.6	1.0 ± 0.0	1.0 ± 0.0	NS
Anti-ZnT8	8.9 ± 0.0	9.0 ± 0.1	9.0 ± 0.0	NS
COVID-19 therapy, %				
Hydroxychloroquine	0	20	10	NS
Dexamethasone	0	10	0	NS
Antiviral	0	30	0	NS
Tocilizumab	0	0	0	NS
Antibiotics	20	20	20	NS
Heparin	0	60	0	NS

Table 7 | Baseline demographic and clinical characteristics of participants included in this study. NS, not significant. *Controls vs. acute COVID-19. †Controls vs. post COVID-19. ‡Acute COVID-19 vs. post COVID-19.

It was deemed appropriate to assess whether the cohort of patients with acute or post-COVID-19 had developed symptoms related to pancreatic islet autoimmunity after it was shown that COVID-19 causes a fasting blood sugar alteration that lasts for a long time after the resolution of the disease. [62] The results are fully presented in the published paper in Appendix A.

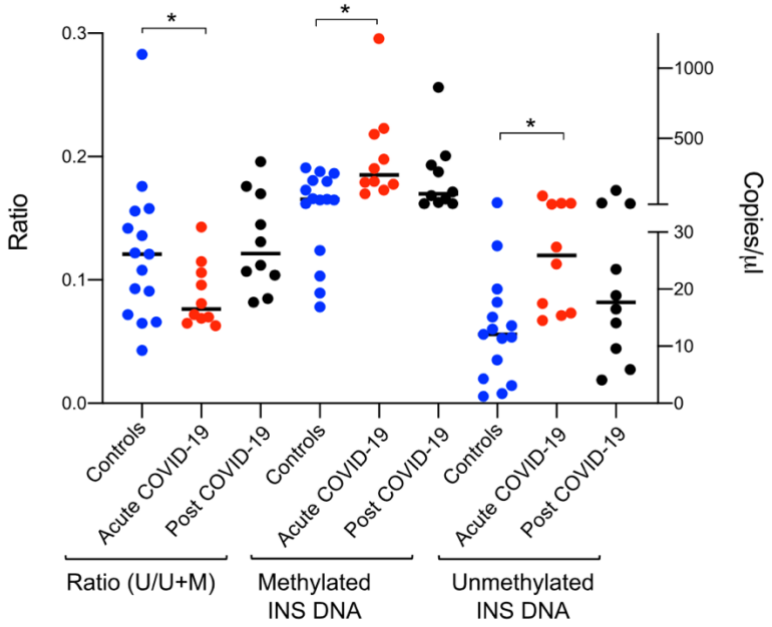

Figure 19 | Evidence of cell death associated with COVID-19 was found in vivo and in vitro. Comparison of the ratio of unmethylated (U) INS DNA/U plus methylated (M) INS DNA was performed in patients with COVID-19 (acute COVID-19), in patients who had recovered from COVID-19 (post COVID-19), and in healthy control subjects.

Despite having a severe disruption of glycometabolic control, the data gathered showed that patients in both subgroups tested negative for the autoantibodies most frequently used to identify autoimmune diabetes (Table 7). When comparing the mean ratios of unmethylated to methylated INS DNA, with a ratio cutoff of 0.196 and values >0.196 indicating beta cell death, a significant decrease in the ratio was discovered in patients with acute COVID-19 but not in post COVID-19, when compared to control subjects (Figure 19). This result indicates that a widespread tissue destruction may occur in patients with COVID-19. In addition, both acute and post COVID-19 patients, showed an increase in unmethylated and methylated INS DNA copies as compared to control subjects (Figure 19). Taken together these informations can be viewed as the first proof of tissue injury in COVID-19 patients.

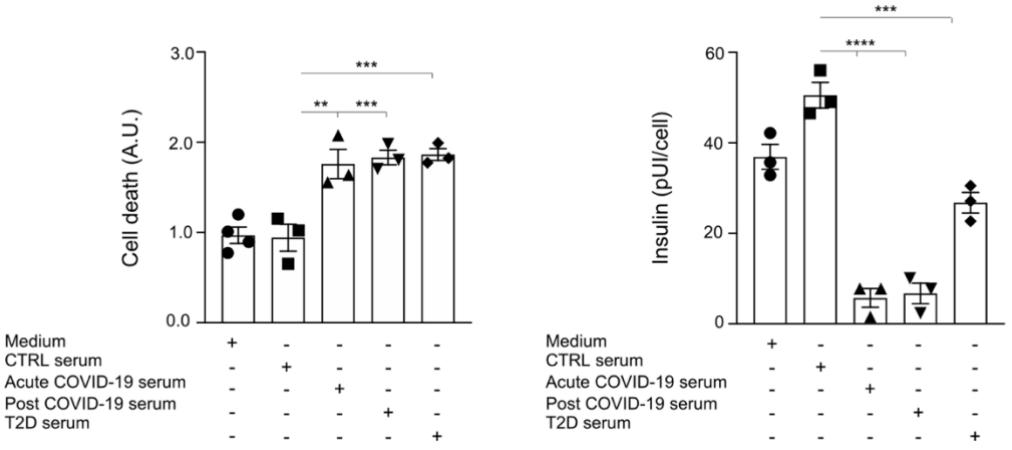

Figure 20 | Rates of cell death (left) and insulin secretion (right) were analyzed for purified human islets upon in vitro challenge with serum from healthy control subjects, from patients with COVID-19 (acute COVID-19), from patients who had recovered from COVID-19 (post COVID-19), or from patients with type 2 diabetes (T2D). Data are representative of n = 15 samples from the subgroup of healthy control subjects and n = 10 from acute COVID-19 and from post COVID-19. Data are representative of a pool of n = 3–4 commercial islet preparations in three separate experiments (n = 3–5 pooled sera tested). Data are represented as mean ± SEM. Ordinary one-way ANOVA test with Bonferroni correction was used for calculating statistical significance between all groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. AU, arbitrary unit.

The pancreatic islets were cultivated in vitro with serum derived from patients with COVID-19 or post-COVID-19 to better understand if the differences found in the secretome related to COVID-19 could explain the functional changes and the lower survival of human pancreatic islets.

When human pancreatic islets were grown with serum from COVID-19 or post COVID-19 patients, increased human pancreatic islet apoptosis and a significant decrease in insulin secretion were discovered (Figure 20).

However, insulin secretion was only slightly reduced when human pancreatic islets were cultured with T2D patient sera, although human pancreatic islet death was similarly increased in this situation (Figure 20).

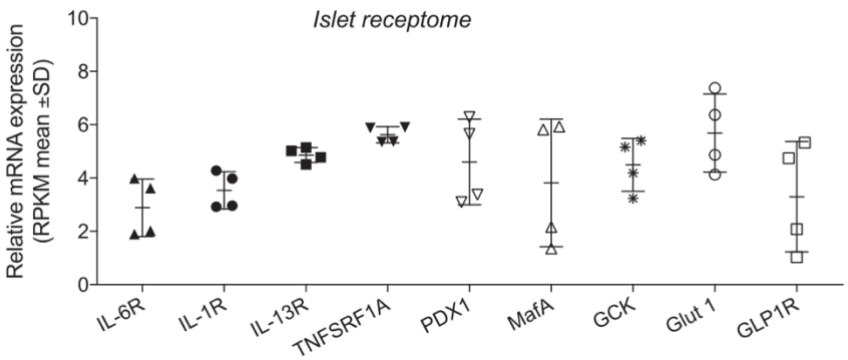

Figure 21 |Receptome analysis of human pancreatic islets depicting the expression of proinflammatory cytokine receptors.

Then it was conducted a receptome study on human pancreatic islets derived from healthy donors to provide proof of the mechanism by which secretome changes might result in pancreatic islet damage. This stage aimed to establish whether and how many of the cytokine receptors that we discovered to be elevated in COVID-19 patients were expressed on pancreatic islets.

Human pancreatic islets were revealed to express TNF-alpha, IL-13, IL-1B, and IL-6 receptors in particular (Figure 21). Starting from secretome and receptome expression profile that were found in COVID-19 patients, islets were cultured with elevated cytokines alone or in combination.

The results of these studies further supported the idea that the increased effect of apoptosis is mediated by both individual cytokines and combinations of them.

In fact, adding IL-1B to the pancreatic islet culture medium along with IL-6, IL-13, IP-10, and TNF-alpha caused a noticeable rise in the apoptosis of human pancreatic islets (Figure 22). Along with the development of apoptosis, the injection of the various cytokines (IL-1B, IL-6, IL-13, IP-10, and TNF-alpha) alone and in combination resulted in a decrease in insulin secretion (Figure 22).

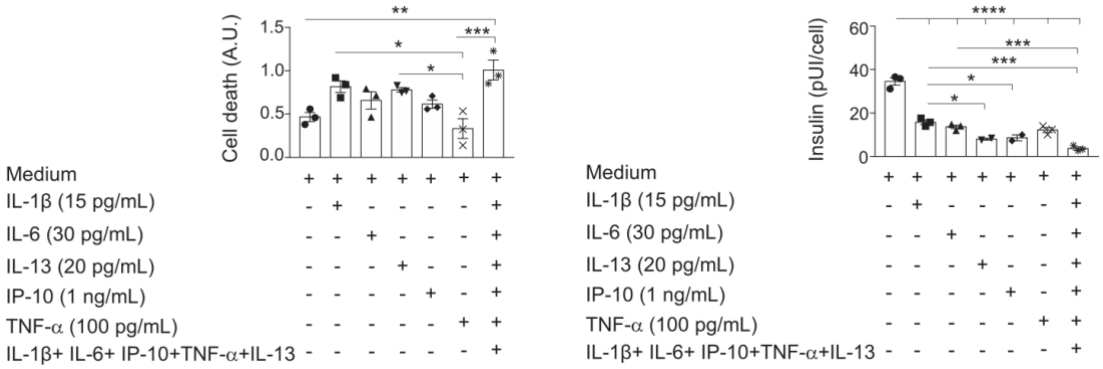

Figure 22|Purified human pancreatic islets were examined for rates of cell death (left) and insulin secretion (right) following in vitro stimulation with a chosen panel of cytokines (IL-1B, IL-6, IL-13, IP-10, and TNF-alpha) that were shown to be elevated in the serum of COVID-19 patients.

An immunoneutralization test was performed to determine if the peripheral secretome was the primary mediator of the insult to the pancreatic islets and, consequently, to determine which was the principal source of the damage to the islets.

Using particular blocking/neutralizing antibodies for each individual cytokine, we looked at the elevated cytokines in the serum of COVID-19 patients.

The results specifically shown that anti-IL-1B and anti-IL-6 blocking/neutralizing antibodies in serum reduced human pancreatic islet apoptosis. Three cytokines (IL-1B, IL-6, and IL-13) were immunoneutralized by antibodies in combination in pancreatic islet culture serum to prevent islet apoptosis to a larger extent, bringing the proportion of apoptotic human pancreatic islets back to baseline levels (Figure 23). Anti-IL-1B, anti-IL-6, and anti-IL-13 were also added, and this restored insulin secretion by human pancreatic islets (Figure 23).

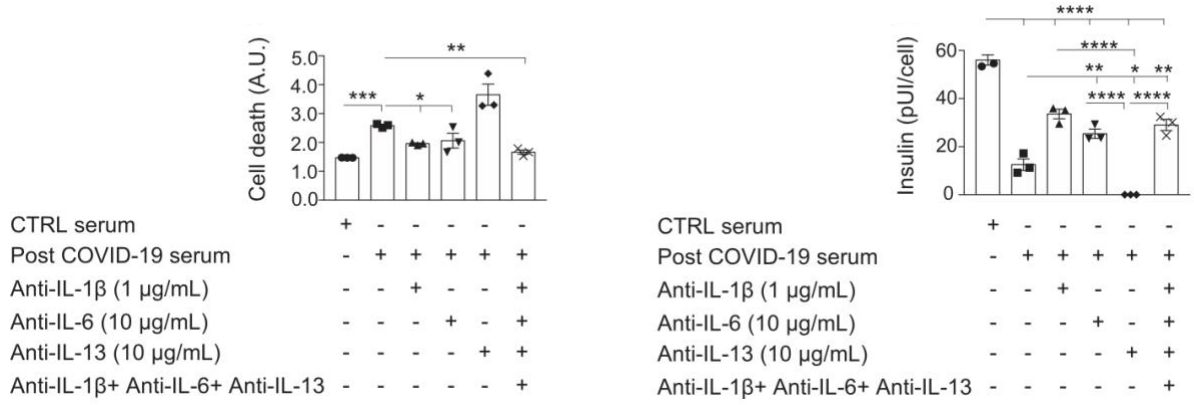

Figure 23 |Effects of the cytokines added alone or in combination on cell death were evaluated. Rates of cell death (left) and insulin secretion (right) for purified human pancreatic islets were examined after in vitro challenge with serum from a patient who had recovered from COVID-19 (post COVID-19) in the presence of neutralizing antibodies (anti-IL-1B, anti-IL-6, or anti-IL-13) alone or in combination, as compared to serum from healthy control subjects.

Then, to check for any specific defects, pathological pancreatic sections isolated from individuals dying from COVID-19 with a diagnosis of recently diagnosed hyperglycemic condition were examined.

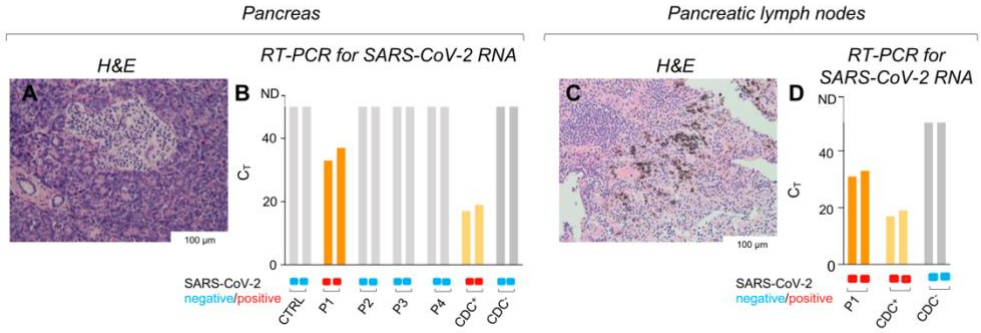

Figure 24 A-D|Histologic examination of pancreatic section retrieved from a patient with COVID-19 stained with H-E showing mild islet lymphocytic infiltration. B: Bar graph showing the results of a SARS-CoV-2 RT-PCR assay using the 2019-nCoV_N1 and 2019-nCoV_N2 primer probe sets performed on RNA samples extracted from pancreatic sections from patients with COVID-19 (P1, P2, P3, and P4) and from healthy control subjects, showing detectable viral RNA in patient P1. C: H-E staining of PLNs from a patient with COVID-19. D: Bar graph depicting the results of a SARS-CoV-2 RT-PCR assay using the 2019-nCoV_N1 and 2019-nCoV_N2 primer probe sets performed on RNA samples extracted from PLNs from a patient with COVID-19 (P1), showing detectable viral RNA in patient P1.

Few lymphocytes were found in the exocrine pancreas, according to histological analysis of the HE-stained pancreatic sections, which showed a minor lymphocytic infiltration of the human pancreatic islets (Figure 24 A).

By analyzing post-mortem samples taken from patients with COVID-19 and recently discovered hyperglycemia, RT-PCR was able to further corroborate lymphocytic infiltration. Particularly, the pancreatic tissues of these patients contained SARS-CoV-2 virus RNA (Figure 24 B).

The PLNs taken from patients who died with COVID-19 had the same finding, i.e., significant lymphocyte activation (Figure 24 C).

Furthermore, SARS-CoV-2 viral RNA was found in the PLNs of numerous samples once more by RT-PCR (Figure 24 D). On pancreatic tissues acquired from the same patients who had died from COVID-19, from participants who had died without COVID-19 (controls), and from patients who had died from T2D, we then conducted an ultrastructural study using transmission electron microscopy.

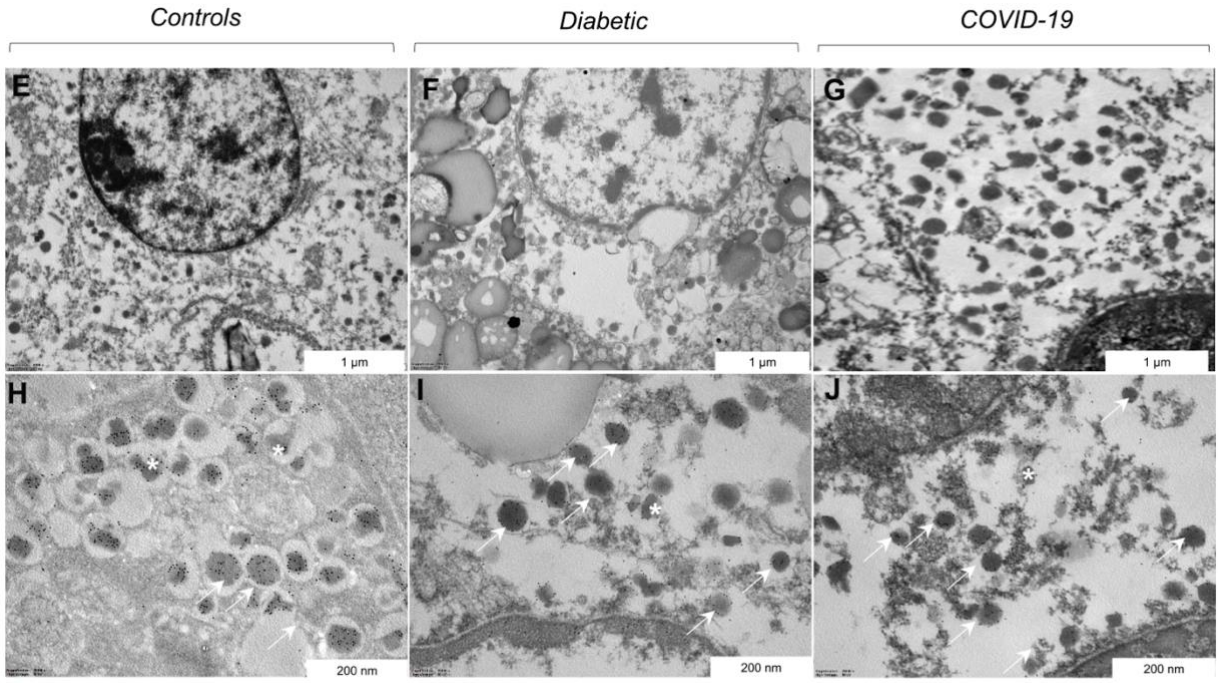

Figure 24 E-J| The presence of insulin secretion granules is indicated by the presence of arrows, which also delineate insulin granules with varying degrees of granulation in healthy control subjects and diabetic samples, respectively. Transmission electron microscopic analysis of pancreatic tissue from patients with COVID-19 as compared with healthy control subjects and patients with type 2 diabetes demonstrates similar islet alterations in pancreatic tissues from patients with COVID-19 and patients with type 2 diabetes. Asterisks in panels H, I, and J show mature insulin granules.

In the pancreatic tissues of COVID-19 patients with newly diagnosed hyperglycemia, changes in islet architecture have been noted. The changes observed in these patients were almost identical to those observed in T2D patients (Figure 24 E-G). The number of mature

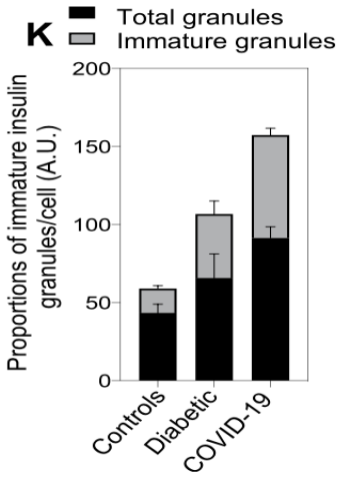

Figure 24 K | Quantification of the proportions of immature insulin granules per total mature insulin secretion granules; n = 3 cases per section were analyzed.

insulin granules was lower in patients with COVID-19 and T2D compared to controls, highlighting abnormalities in beta-cell structure and shape in all patient samples (both COVID-19 and T2D). This finding is connected to more severe beta cell damage (Figure 24 H-J). Notably, a large percentage of beta cells from COVID-19 patients with newly diagnosed hyperglycemia were found to possess immature granules that appeared to contain proinsulin (Figure 24 H–K). A common sign of degeneration and hyperstimulation, beta cells from COVID-19 patients with recent signs of hyperglycemia have a high number of circular granules and some granules with crystalline form (Figure 24 H–K).

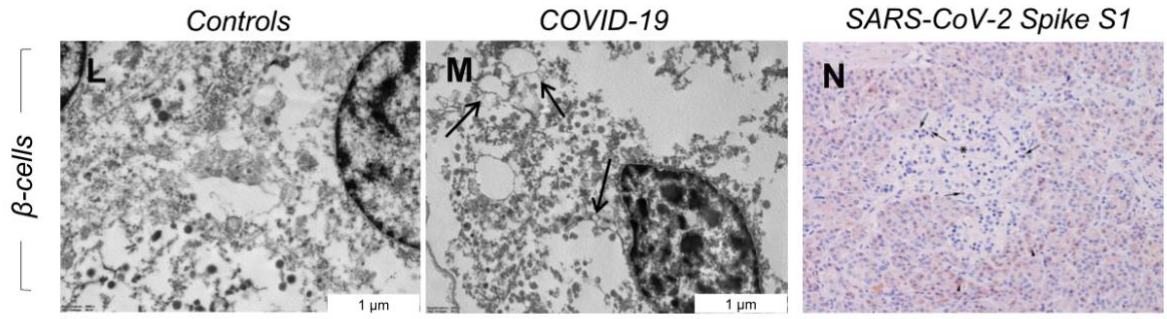

Figure 24 L-N | L and M: Transmission electron microscopic analysis of pancreatic tissue from a patient with COVID-19 as compared with that from healthy control subjects depicting the presence of several vacuoles (shown by black arrows) in the vicinity of b-cells from a patient with COVID-19. **N:** SARS-CoV-2 spike S1 staining depicted within endocrine cells; arrows indicate the positive staining within endocrine cells of the pancreas; asterisk refers to an islet, and arrowheads indicate pancreatic acinar cells. AU, arbitrary unit.

In addition, beta cells (Figure 24 L and M) as well as alpha cells, delta cells, endothelial cells, pericytes, and histiocytes (Figure 24 A-E) were found to have a notable number of vacuoles, which may indicate the presence of the virus, but the viral origin of these vacuoles has not been established. A thorough ultrastructural study of pancreatic sections from COVID-19 patients was also carried out (Figure 24 N). Here, it was clear that the exocrine pancreas as well as endocrine cells contained the SARS-CoV-2 spike protein S1 (Figure 24 N). As shown by confocal analysis showing double immunostaining for S1 spike protein of SARS-CoV-2 and cells insulin-positive autopsied pancreatic islets (Figure 25 A-C), where SARS-CoV-2 RNA was already confirmed by RT-PCR, it was discovered that S1 staining of SARS-CoV-2 spike protein distinguishes beta cells in particular.

As shown by combined immunostaining for S1 spike protein of SARS-CoV-2 and trypsin-positive acinar cells (Figure 25 D-F), as well as from CK19 positive ductal cells, the same S1 spike protein labeling of SARS-CoV-2 was also found within pancreatic exocrine cells (Figure

25 G-I). On pancreatic samples, immunohistochemical evaluation, HE staining and islet size estimation, as well as quantification of insulin expression, were carried out. When compared to controls, pancreatic sections from COVID-19 patients had significantly smaller islets and slightly less insulin expression, according to pathological examination.

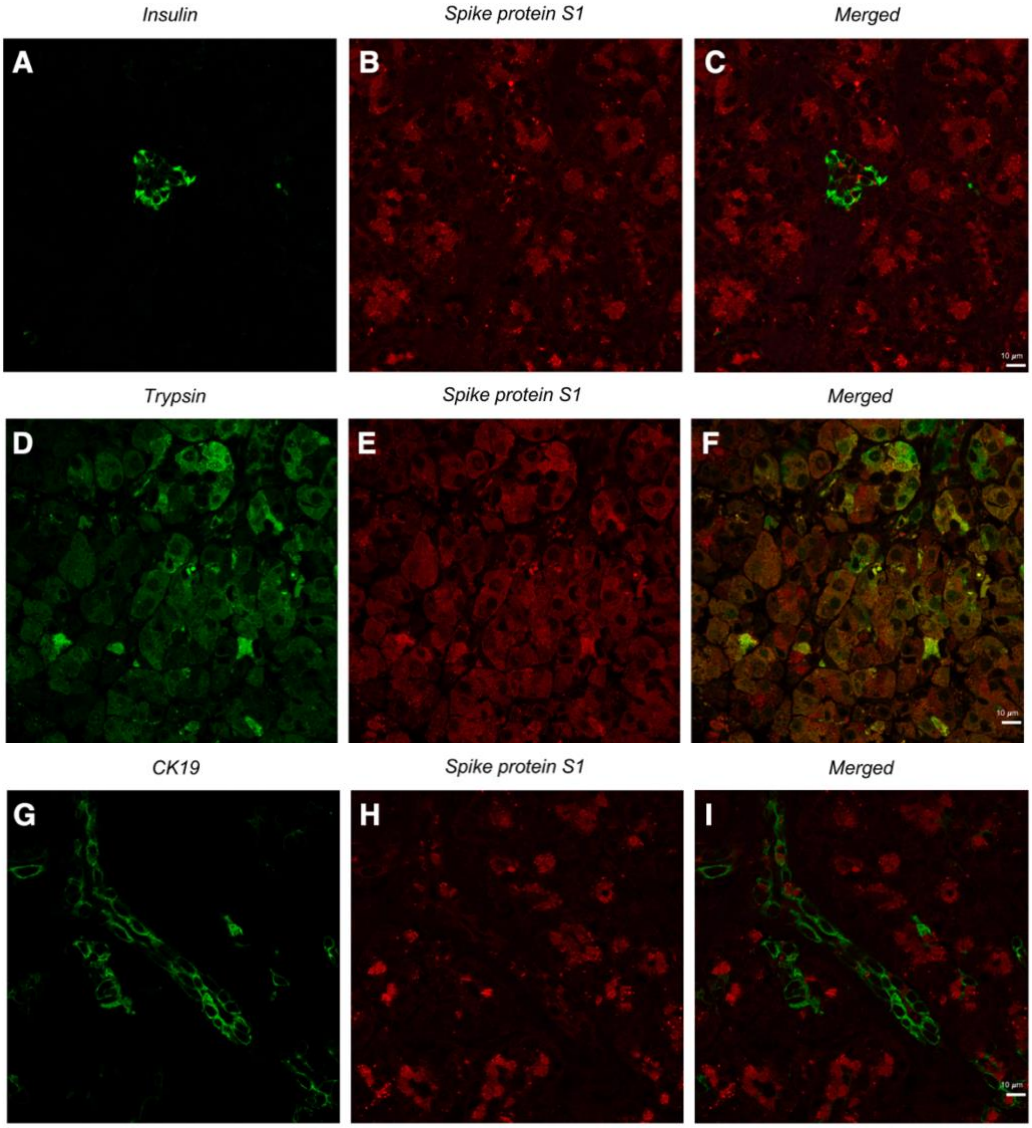

Figure 25 A-I | SARS-CoV-2 spike protein S1 localizes within endocrine pancreatic beta-cells and exocrine pancreatic cells. Confocal microscopic analysis of pancreatic tissue from a patient with COVID-19 depicting the localization of SARS-CoV-2 spike protein S1 within beta-cells (insulin-positive cells) (shown in panel C) and within exocrine cells, trypsin-positive cells (shown in panel F), and CK19-positive cells (as shown in I). Scale bars in panels A–I, 10 mm.

The existence of the viral infection was established after performing caspase 3 immunostaining to assess beta-cell death in pancreatic sections of patients with COVID-19 (Figure 24 M and N). In the aforementioned pancreatic slices, no endocrine cells exhibited

positive caspase 3 staining. It is known that caspase 3 is only one component of the typical apoptotic cascade.
Caspase 3 might not be necessary for beta-cell death, though, or the process might not be as obvious (Figure 24 F).

Chapter 5: **DISCUSSION**

New cases of diabetes are reported to develop as a result of SARS-CoV-2 infection, according to recent investigations. The infection appears to cause a glycometabolic decompensation in people who are not yet diabetic, which eventually manifests as hyperglycemia. On the other side, COVID-19 can cause more severe symptoms in diabetic subjects, which might occasionally result in death or a reduced life expectancy.

In this investigation, we showed that COVID-19 patients without diabetes prior to hospitalization may develop new-onset hyperglycemia, insulin resistance, and beta-cell hyperstimulation. The destruction of glycometabolic compensation observed in the aforementioned cases appears to be caused by a change in the secretome, which persists long after the disease has been recovered. COVID-19 can cause an inflammatory condition that is very similar to, if not even more serious than, that seen in T2D [63], despite the fact that changes in metabolism, including in particular glucose metabolism, have also been identified following other infections [64, 65]. Over time, this changed inflammatory state may result in beta-cell exhaustion due to insulin overproduction and glucose-mediated toxicity. [66]

The subset of new-onset hyperglycemic patients who got off-label tocilizumab experienced a significant drop in blood glucose compared to control individuals because it targets inflammation. Tocilizumab therapeutic effect, that we found in our study appear to benefit glucose metabolism and the endocrine system rather than the resolution of the infection itself, as a recent randomized, double-blind, placebo-controlled study of tocilizumab showed poor efficacy regarding recovery from COVID-19 in treated patients. [67]

Several facts stand out as being interesting.

First and foremost, the high proportion of COVID-19 hospitalized patients who had hyperglycemia, followed by the presence of significant glycometabolic decompensation and altered beta cell function in patients without a history or diagnosis of diabetes (newly detected hyperglycaemic), as shown by normal glycated hemoglobin levels in these patients. Also, intriguing is the significant mortality rate among COVID-19 hospitalized patients who have hyperglycemia.

The study found that the change in blood glucose levels was not just present during the acute stage of the infection but was also present months after COVID-19 recovery.

We are very aware that our study has limitations even though it shows some compelling evidence: first of all, we're conscious that the sample size may in some way constrain the conclusions that may be taken from this data's study. The sample size of 551 patients

ensures a sufficient number of statistical data for the study, however because of this, the significance of the findings from the subgroup analysis may be less strong.
Furthermore, we are aware that the participants in the subgroup analysis have lower ages and BMIs than the patients who were enrolled in the entire study.
We enrolled the patients in the subgroup analysis whenever a new hospital admission took place, preventing any bias in the participant selection process. This is further supported by the observation that there is no statistically significant difference in the demographic traits of patients who belong to the various subgroups.
Because COVID-19 had greater morbidity and mortality in older patients, the mean age of the study's patients was lower than that of the general population.
Additionally, because patients were only recruited from one facility, namely the ASST FBF-Sacco Milan university hospital, Presidio Sacco, the study's conclusions may have been tainted by hospital selection bias.
Despite these drawbacks, which we are aware of, our study has allowed for a number of intriguing discoveries, one of which is the use of CGM to spot glycemic changes that would not have been obvious from self-measurement of capillary glycemia alone in fasting conditions [68].
We also emphasized changes in the hormonal profile in our patients, both at baseline and after stimulation by arginine test, always in line with this significant observation.
In contrast to healthy controls, we discovered greater levels of insulin, proinsulin, and c-peptide in both acute and post-recovery (post-COVID-19) COVID-19 patients.
In addition, the analysis of our data reveals that COVID-19 causes long-term harm to the neurological, renal, and cardiovascular systems in addition to the short-term harm already noted in prior studies on beta cell function and insulin signaling [69]. Again, based on the analysis of our data, it is clear that the emergence of insulin resistance, as is the case with T2D, is caused by the proinflammatory environment that develops after COVID-19 and results from the so-called cytokine storm, in which IL-6 plays a major role [53].
Since only a small portion of patients got steroids and hydroxychloroquine as inpatient medication therapy throughout the course of their sickness, any bias resulting from drug therapy can also be disregarded.
Finally, in our study, we showed that the serum cytokine profiles of COVID-19 and post-COVID-19 patients differed significantly from those of healthy controls and that insulin

resistance, beta cell dysfunction, and hyperglycemia may be caused by the proinflammatory environment brought on by cytokine storm.

After that, SARS-CoV-2 causes insulin resistance and modifies beta cell function, which results in a fresh development of hyperglycemia that lasts even months after the infection has been treated [54]. For a complete overview please see the full published paper in Appendix B.

In this study, we also emphasized a change in the function and survival of human pancreatic islets when those islets were exposed to serum from COVID-19 patients as well as serum from T2D patients. We postulate that circulating proinflammatory factors, which have already been mentioned in the literature, may be the cause of the adverse effect on islets. Proinflammatory substances seen in the serum of T2D patients have long been documented in the literature as having some role in the development of the inflammatory process that affects the pancreatic islets and ultimately results in the exhaustion of beta cells in T2D.[70] This may be a confirmation of our observations regarding the significant decline in insulin production by pancreatic islets exposed to the serum of T2D patients.

We have also discovered that IL-1, a proinflammatory cytokine, has a crucial role in maintaining the survival and homeostasis of pancreatic islets, making it the most significant cytokine identified in the serum of T2D patients. In fact, a proinflammatory cascade that later plays a crucial part in the pathophysiology of T2D and the development of the disease can be brought on by prolonged exposure to or high levels of IL-1. However, the significance of IL-1 in this role seems to be diminished after statistical correction using the Bonferroni test applied to the One-Way ANOVA.

Another crucial investigation carried out in our study was the assessment of autoantibodies in samples taken from patients with COVID-19 or retrieved from COVID-19. The follow-up time of the study may also have contributed to the lack of autoimmunity that was observed in the performed analysis.

Because of this, we are aware that the time of the observations may have been a limiting factor, preventing us from detecting the distinctive features of autoimmunity, such as the exact production of autoantibodies against pancreatic islets. In addition, the presence of a slight lymphocytic infiltration was noted in the pancreatic sections of COVID-19 patients with newly discovered hyperglycemia. This, along with the discovery of SARS-CoV-2 viral RNA in the islets, suggests that SARS-CoV-2 has a preference for beta cells and may directly cause the functional changes found in the pancreatic beta cells themselves. [71]

Different proinflammatory cytokine receptors are expressed by pancreatic islets, which clearly shows that the islets are vulnerable to the mortality brought on by these cytokines. The pancreatic tissue's inflammation and the pancreatic lymphonodes activation, as a result of the histopathological changes, signal the longer-term impact that the COVID-19 infection will have on these patients.

By spotting numerous cytoplasmic vacuoles in certain beta cells, as well as in endothelial cells, pericytes, and histiocytes, we were able to show that SARS-CoV-2 was present at the extrapulmonary level thanks to the analysis of some pancreatic samples from hyperglycemic individuals with COVID-19. According to this information, the cytotoxic effect caused by SARS-CoV-2 may not only be indirect and hence caused by the general proinflammatory status, but also directly by the virus invading the islets and beta cells. [72]

Other investigations have documented the presence of SARS-CoV-2 virus particles in pancreatic tissue as well as the expression of ACE2 in the microvasculature and pancreatic ductal epithelium.

Additionally, in other studies, the presence of the well-known SARS-CoV-2 entry mechanisms, including ACE2, TMPRSS2 and DPP4, as well as a direct detection of SARS-CoV-2 inside the ducts of pancreatic cells, acinar cells, endothelial cells, and in the vicinity of the islets of Langerhans, within the islets, and within insulin-producing beta cells, has been demonstrated [73].

Instead, we did not see a rise in the amounts of beta cell-derived unmethylated DNA. This could, however, be explained by the fact that these beta cells were likely destroyed before the samples were collected. If so, there might have been a sharp rise in INS DNA generated from sources other than beta cells, hiding any signs of beta cell apoptosis [74].

Although the literature is in favor of the theory that beta cell death is precisely reflected by an increase in the frequency of unmethylated INS CpG sites in beta cells and the ratio of methylated to unmethylated INS DNA released into circulation after beta cell death, there are a number of significant problems that would restrict its application. For instance, there isn't any interlaboratory validation for the use of unmethylated INS DNA as a marker of beta cell death in vivo [75].

Second, beta cells may not even produce demethylated insulin when they die because under stressed circumstances, they can methylate the INS DNA.

The published evidence from other research revealing that different cell types can produce even small levels of circulating unmethylated INS DNA and hence the release of these

molecules raises even another issue with the use of unmethylated INS DNA as a marker of beta cell demise. The latter is not only brought about by pancreatic beta cell degeneration [62, 76, 77]. For a complete overview please see the full published paper in Appendix A. This study is one of the first to identify an essential systemic inflammatory process as well as a direct cytotoxic effect brought on by the SARS-CoV-2 virus, which when combined can cause malfunction and ultimately the death of pancreatic beta cells.

Chapter 6: **CONCLUSIONS**

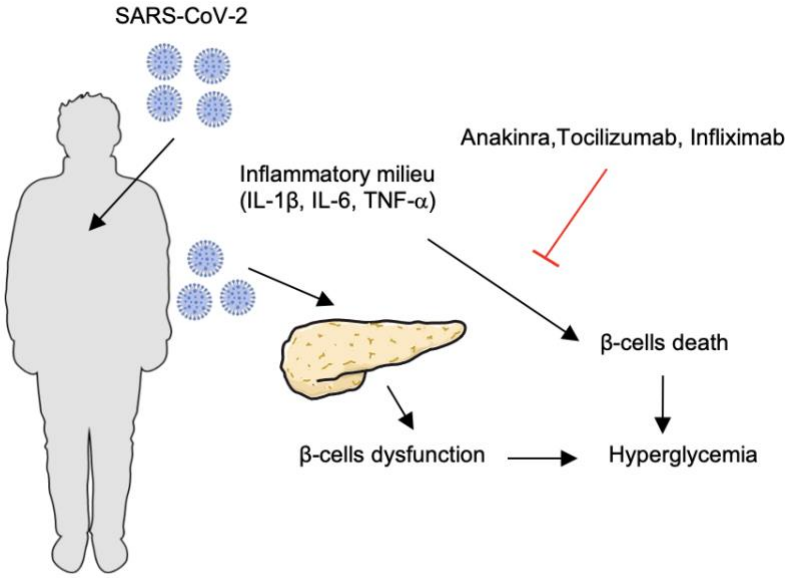

Figure 26 | Working hypothesis for how SARS-CoV-2 infection affects beta-cell function, which states that disfunction of beta-cells and the start of hyperglycemia are caused by the production of pro-inflammatory cytokines and the pancreatic tropism of the virus.

In order to determine whether COVID-19 is the main factor causing a glycometabolic decompensation that lasts for an extended period of time, in vitro investigations were conducted together with observational studies on hospitalized patients for this thesis.

In the first study we conducted, the retrospective analysis of data derived from COVID-19 hospitalized patient records allowed us to first recognize that newly diagnosed diabetic and hyperglycemic patients recorded a clinical score at baseline that was more severe than normoglycemics, as well as a longer discharge time. Additionally, we observed that these patients consistently required more oxygen and ventilatory assistance than normoglycemic individuals did.

We were able to conclude that the episodes of new hyperglycemia discovered were caused by COVID-19 and were not present before the infection, thanks to the baseline normal outcomes of glycated hemoglobin. We were able to show that the COVID-19 infection was associated with increased glycemic variability that endures even months after recovery thanks to the usage of the CGM. We discovered that patients with COVID-19 have developed insulin resistance and beta cell dysfunction that can be found even after they have recovered thanks to fasting serum hormone collection and the arginine stimulation test. Additionally, the examination of the cytokines found in the serum of COVID-19 and post-COVID-19

patients revealed that newly diagnosed hypoglycemia individuals had higher amounts of these cytokines.

In fact, given that glucose levels are decreased in a subgroup of patients after receiving an IL-6 inhibitor, inflammation appears to influence fasting blood glucose levels. We were able to show that beta cells are directly cytotoxic as well as indirectly affected by the general inflammatory status thanks to the findings of the second investigation. The serum of COVID-19 patients and recovered patients in particular revealed to us a cytotoxic effect on the pancreatic islets that caused their death. Furthermore, a toxic effect on pancreatic islets was revealed by the secretome of COVID-19 patients and that of recovered individuals.

We found evidence that the virus had a tropism for pancreatic tissue in the lymph nodes and pancreatic islets of deceased hyperglycemic donors who had the virus detected by RT-PCR. A minor lymphocytic infiltrate within the pancreatic islets was also detected by histological investigation, which further supported the virus' presence in the pancreatic endocrine tissue. By analyzing the pancreatic tissue of a patient who died from COVID-19, the confocal microscopy has also shown that the SARS-CoV-2 spike protein colocalizes with insulin-secreting beta cells and with trypsin-secreting exocrine cells.

The combined analysis of all these findings gives us the first tangible evidence that in certain patients, the SARS-CoV-2 infection can result in the development of hyperglycemia, which can sometimes progress to full-blown diabetes and lasts for months after recovery. But further research and analysis are required to fully understand this association.

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Appendix

Appendix A

Indirect and Direct Effects of SARS-CoV-2 on Human Pancreatic Islets.
Ben Nasr M, D'Addio F, Montefusco L, Usuelli V, Loretelli C, Rossi A, Pastore I, Abdelsalam A, Maestroni A, Dell'Acqua M, **Ippolito E**, Assi E, Seelam AJ, Fiorina RM, Chebat E, Morpurgo P, Lunati ME, Bolla AM, Abdi R, Bonventre JV, Rusconi S, Riva A, Corradi D, Santus P, Clark P, Nebuloni M, Baldi G, Finzi G, Folli F, Zuccotti GV, Galli M, Herold KC, Fiorina P., Diabetes, 2022 Jul 1;71(7):1579-1590. doi: 10.2337/db21-0926.PMID: 35499468

Appendix B:

Acute and long-term disruption of glycometabolic control after SARS-CoV-2 infection.
Montefusco L, Ben Nasr M, D'Addio F, Loretelli C, Rossi A, Pastore I, Daniele G, Abdelsalam A, Maestroni A, Dell'Acqua M, **Ippolito E**, Assi E, Usuelli V, Seelam AJ, Fiorina RM, Chebat E, Morpurgo P, Lunati ME, Bolla AM, Finzi G, Abdi R, Bonventre JV, Rusconi S, Riva A, Corradi D, Santus P, Nebuloni M, Folli F, Zuccotti GV, Galli M, Fiorina P., Nat Metab. 2021 Jun;3(6):774-785. doi: 10.1038/s42255-021-00407-6. Epub 2021 May 25.PMID: 34035524

Appendix C:

Sitagliptin Treatment at the Time of Hospitalization Was Associated With Reduced Mortality in Patients With Type 2 Diabetes and COVID-19: A Multicenter, Case-Control, Retrospective, Observational Study.
Solerte SB, D'Addio F, Trevisan R, Lovati E, Rossi A, Pastore I, Dell'Acqua M, **Ippolito E**, Scaranna C, Bellante R, Galliani S, Dodesini AR, Lepore G, Geni F, Fiorina RM, Catena E, Corsico A, Colombo R, Mirani M, De Riva C, Oleandri SE, Abdi R, Bonventre JV, Rusconi S, Folli F, Di Sabatino A, Zuccotti G, Galli M, Fiorina P., Diabetes Care. 2020 Dec;43(12):2999-3006. doi: 10.2337/dc20-1521. Epub 2020 Sep 29.PMID: 32994187