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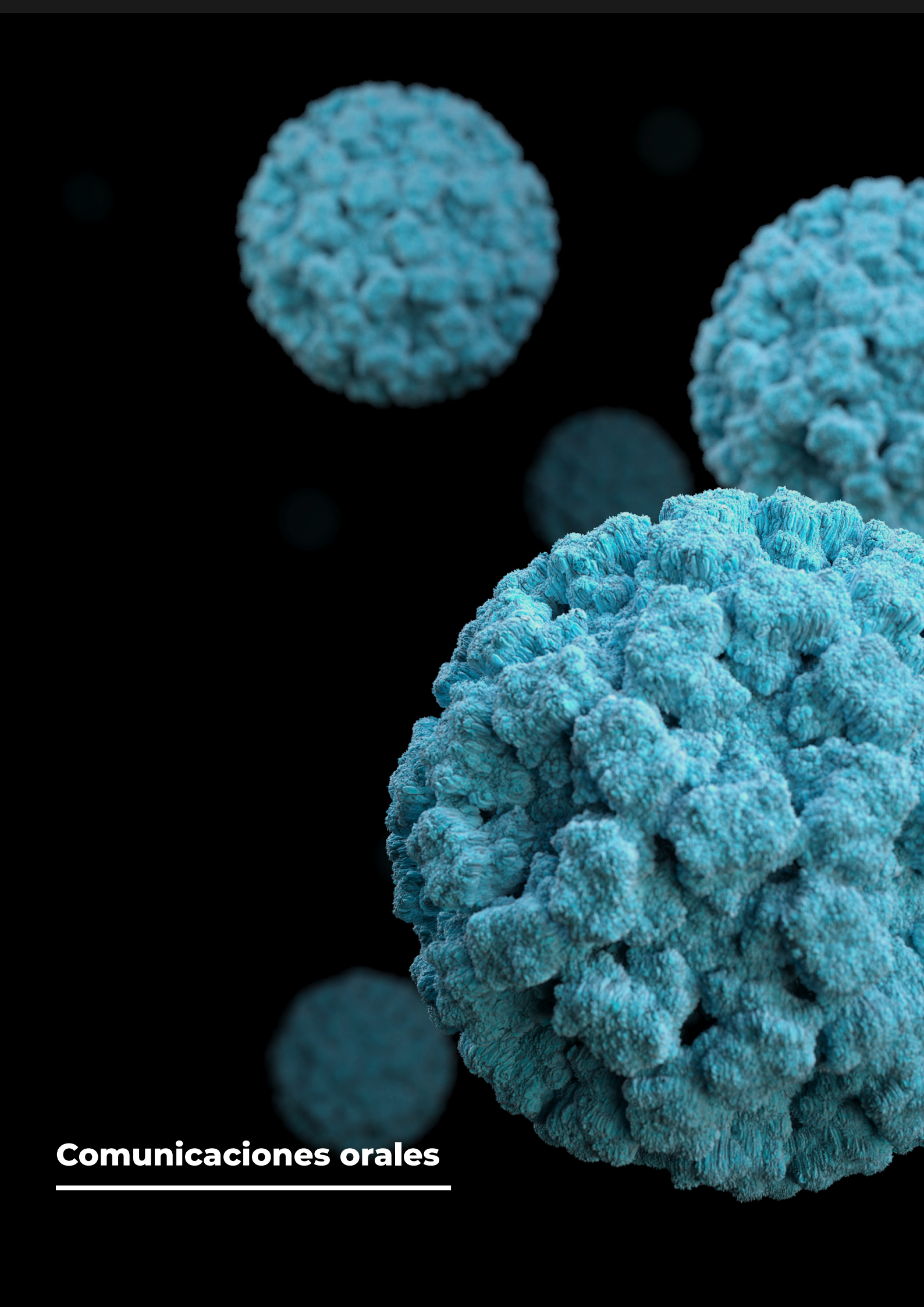
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Comunicaciones orales

Nº1

Discovery of new inhibitors and mechanisms of inflammasome activation: applications in COVID-19

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Background. COVID-19 is a systemic disease caused by the respiratory virus SARS-CoV-2, whose pathogenicity is largely owed to the action of viral factors that prompt imbalanced and untimely immune and inflammatory responses in the host. We have addressed a major mechanism underlying COVID-19-induced hyperinflammation, namely unbridled inflammasome activation, by developing a pipeline for the discovery of new inflammasome inhibitors with therapeutic potential.

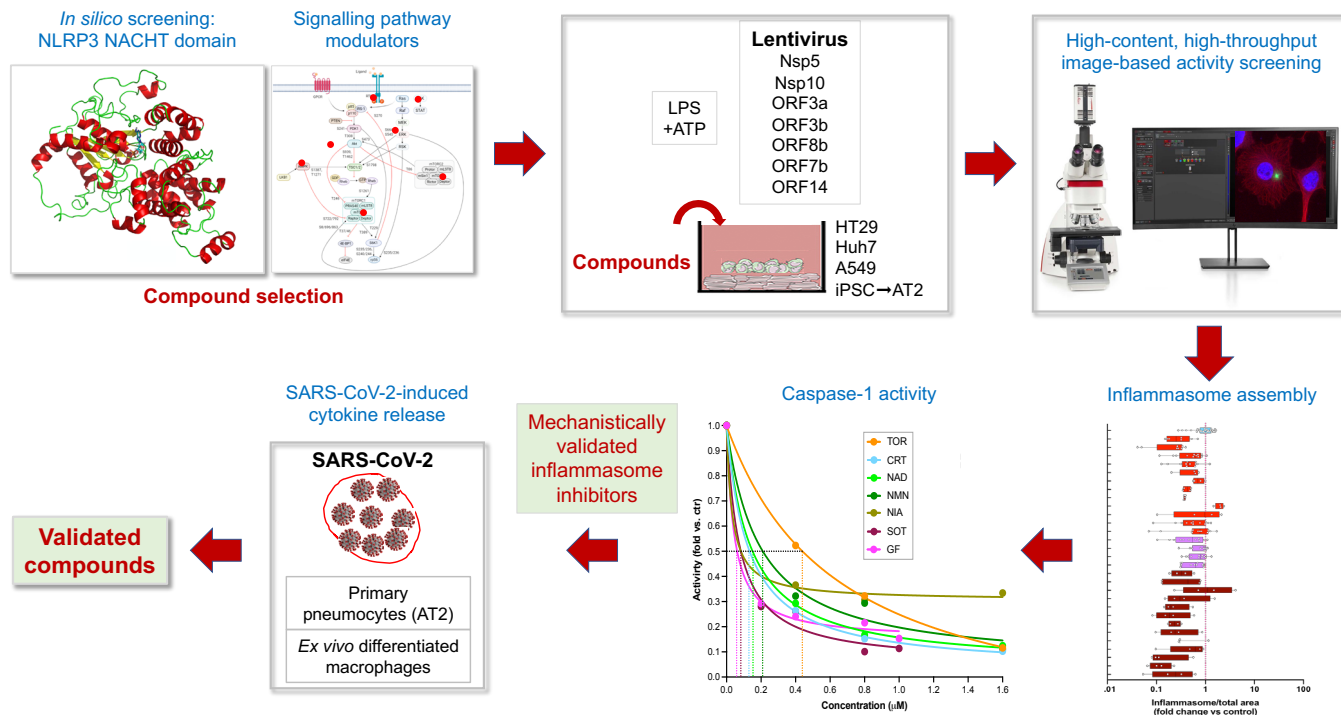
Methods. We have developed a 5-tier Drug Discovery pipeline: 1) In silico screening for compounds with binding potential to the NLRP3 NACHT domain; 2) Generation of cellular models integrating fluorescent sensors for inflammasome assembly; 3) High-content, high-throughput image-based, mechanism-centered screening platform; 4) Functional validations in cell models, macrophages and AT2 pneumocytes; 5) Animal models: toxicity and anti-inflammatory activities, including mitigation of SARS-CoV-2 infection.

Results. A limited number of structures were selected by in silico screening of \approx 1700 compounds predicted to strongly bind the NACHT domain of NLRP3, as potential inhibitors of the NLRP3 inflammasome. Further compounds were selected based on prior known activities on the inflammasome and additional signaling pathways. We generated HT-29 (intestinal), A549 (lung), Huh7 (liver) and THP-1 (monocyte-macrophage) cell lines, stably integrated with a fluorescent sensor for real-time monitoring of inflammasome assembly, and used them to screen selected compounds for inhibition of inflammasome assembly, by means of high-content, high-throughput automated image analysis. Compounds with significant inflammatory assembly inhibitory activity were tested for their ability to inhibit caspase-1 enzymatic activity in response to inflammasome-inducing stimuli. Compounds significantly active in both assays were tested for their ability to mitigate cytokine release from ex- vivo differentiated human macrophages and from AT2 pneumocytes explanted from human donors, exposed to either inflammasome-inducing stimuli or to SARS-CoV-2. These determinations have led to the discovery of hitherto unknown anti-inflammatory activities for several drugs. In this process, we have also unveiled a prominent role played by mTORC2 as a novel and actionable mechanism of inflammasome activation. The most active compounds in in vitro assays were tested in a zebrafish model of viral (rhabdovirus) infection and inflammation, showing significant anti-inflammatory activities in this animal model. The two most active compounds in the entire suit of read-outs are currently being tested in susceptible mice infected with SARS-CoV-2 (ancestral and variant strains).

Conclusions. Thus far, our effort has yielded (1) a seamless drug discovery pipeline, applicable to unveiling compounds and chemical structures active against a broad range of molecular targets amenable to in silico screening and cell- and image-based functional read-outs; (2) the discovery of previously unknown inflammasome inhibitory and anti-inflammatory activities for several compounds, validated in various cell models, donor cell types and animal models; (3) the discovery of a novel mechanism of inflammasome activation, targetable with small molecules. We expect to shortly complete the required pre-clinical proof-of-concept activities, prior to conducting clinical trials.

Nº1

A pipeline for the discovery of new inflammasome inhibitors



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Nº2

Molecular mechanisms related with post COVID19 syndrome

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Post-COVID syndrome is defined as a wide range of chronic symptoms that appear after infection, persist for more than 12 weeks and cannot be explained by an alternative diagnosis. Symptoms can fluctuate or cause relapses; being the most common fatigue, dyspnea, anxiety, depression, etc. Their biological mechanisms are nevertheless unknown.

Hence, cell memory, cytokines and immune phenotyping using flow cytometry, multiplex and mass cytometry respectively were carried out in 83 post-COVID patients and 25 post-FLU, both of them 3 months after hospital discharge, compared with 17 pre-pandemic controls. Several cytokines and immune cell subsets were altered in post-COVID patients, referred to both the post-FLU and the pre-pandemic controls, revealing a post-COVID-specific immune signature (Figure 1). Moreover, post-COVID patients displayed a higher T-cell basal activation which became more evident following CD3/CD28 stimulation confirming that the immune alterations are also functional rendering a more activated immune system in these patients.

Machine learning models (random forest, logistic regression and PCA) were able to classify with good accuracy individuals according to their diagnosis. Additionally, k-means algorithm revealed four different clusters, two of them consisting of post-COVID patients that seemed to differ in age. No differences between these two clusters were found regarding gender, severity (days in hospital and oxygen need) and post-COVID symptoms. In summary, we hereby have demonstrated that: i) Post-COVID patients have immune sequelae 3 months after hospital discharge being those changes specific to SARS-CoV-2 infection; ii) Post-COVID patients have a pro-inflammatory T-cell phenotype, both in resting conditions and after stimulation; iii) Immune changes were independent of post-syndrome symptoms; iv) Post-COVID individuals can be further divided into two major clusters based on the expression of chemokine receptors and the age of the patients.

Nº2

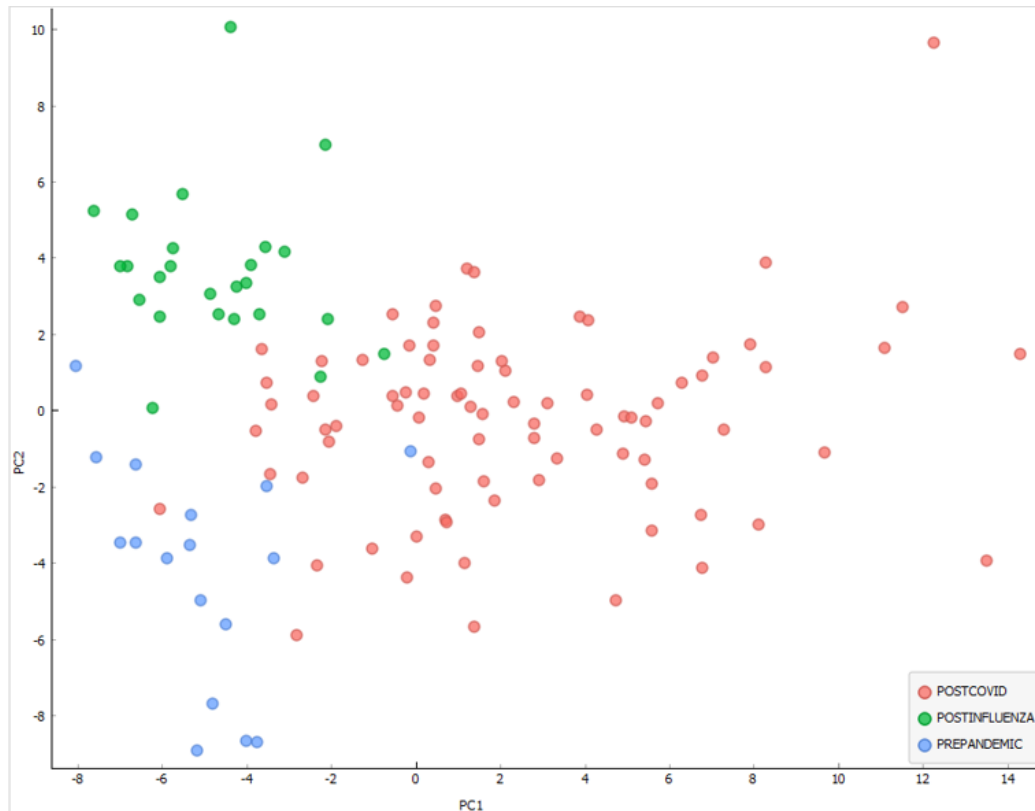


Figure 1 legend. Principal component analysis (PCA) biplot for post-COVID (red), post-Influenza (green) patients and pre-pandemic healthy donors (blue).

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Nº3

SARS-CoV-2 natural immunity to vaccine-induced immunity: Systematic Evaluation of Humoral Response by multipronged functional proteomics approaches

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An unresolved question is to find out whether the hybrid immune model (natural infection plus vaccine administration or vaccine plus infection) can be a reproducible method to boost immunity. In both cases, dynamic integration of multiple components of the humoral immune response is required, among the cellular immunity. Here, we present a comprehensive and systematic analysis of humoral response against SARS-CoV-2 antigens in recovering COVID19 patients and vaccine induced immune response based on multipronged functional proteomic approaches. Immunoglobulin profile was assed against SARS-CoV-2 virus in a full proteome epitope array including the main structural proteins, accessory and non-structural proteins by full-length structure and epitopes peptides. Additionally, the acute phase reactants and antibody profiles were characterized by protein microarrays in order to further investigate the humoral response generated by the SARS-CoV-2 infection. The elucidation of common hallmarks of immunogenic epitope production in vaccinated and naturally protected individuals can bring valuable insights to reveal the immune processes underlying favorable disease progression. Our results can predict the increase in the intensity and breadth of the antibody response after vaccination in people previously infected with SARS-CoV-2 and previously vaccinated and follow infected by SARS-CoV-2

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Nº4

ACE2 serum levels as a potential biomarker to predict infectability and disease subtypes in Covid-19

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Background. The virus SARS-CoV-2 enter to human cells via the interaction between its spike (S) protein with the angiotensin-converting enzyme 2 (ACE2) receptor. ACE2, regulates the renin- angiotensin-aldosterone system (RAS) playing an important physiologic role in the homeostasis of tissue microcirculation and inflammation.

Methods. Human sera were obtained from different cohorts of adults and paediatric volunteers (highly exposed to virus but uninfected, recently infected, infected with persistent symptoms and un- exposed seronegative controls). Soluble ACE2, angiotensin and cytokines were measured by ELISA and antibodies IgM, IgA and IgG anti-S or anti-N by flow cytometry or ELISA. Neutralization assay with S-pseudotyped was carried out.

Results. Significant differences were detected in the serum levels of ACE2 between groups. Seronegative subjects highly exposed to the virus had significantly higher ACE2 levels than seropositive with similar exposure conditions ($p < 0.0001$). Interesting, the levels of ACE2 in those exposed but seronegative cases were always in the upper limit, being higher than 10 ng/ml. Seropositive with persistent symptoms had the lowest ACE2 levels, being lower than 3ng/ml in the 87% of cases. Infected patients were clinically classified according to the type (cutaneous, gastrointestinal or respiratory) and severity of symptoms during the acute infectious process, showing a significant correlation between levels of ACE2 in serum and some symptoms. Infected patients developing cutaneous manifestations had significantly higher ACE2 levels while patients with pneumonia tended to have lower ACE2. In contrast to ACE2 levels, anti-S IgG1s were clearly the lowest in seropositive patients developing cutaneous manifestations and the highest in those with pneumonia. Moreover, sera from highly exposed but seronegative persons can neutralize SARS-CoV-2 infection in cellular assays more strongly than sera from non-exposed negative controls even though they do not have anti-CoV-2 IgG antibodies suggesting that high levels of ACE2 in serum may somewhat protect against an active infection. This was corroborated by showing that ACE in serum has an inhibitory antiviral IC₅₀ of 11ng/ml.

On the other hand, the pediatric cohort with half of them asymptomatic during the acute phase of infection and who remained SARS-CoV2 seronegative afterwards, had very low levels of soluble ACE2 (lower than 3ng/ml in 77% of them). However, they show high, and relatively stable, titer levels of IgM anti -S (but not IgG anti-S). A small group show also detectable anti-N antibodies likely indicating a previous infection with another coronavirus.

Conclusions. In adults, soluble ACE2 could be used as a potential biomarker to predict SARS-CoV- 2 infection risk and to discriminate COVID-19 disease subtypes. Serum ACE2 may act as a decoy receptor to neutralize the SARS-Cov2. Children develop a different immune response than adults; some can be protected by natural antibodies to previous CoV infection or may develop a B-1 IgM- dependent innate immune response to SARS-Cov2.

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Nº5

Simultaneous targeting of IL-1-signaling and IL-6-trans-signaling preserves human pulmonary endothelial barrier function during a cytokine storm

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Background. Systemic inflammatory diseases, such as severe COVID-19 and sepsis, provoke acute respiratory distress syndrome (ARDS) in which the pathological hyperpermeability of the microvasculature, induced by uncontrolled inflammatory stimulation, causes pulmonary edema. Identifying the inflammatory mediators that induce human lung microvascular endothelial cell (HLMVEC) barrier dysfunction is essential to find the best anti-inflammatory treatments for critically ill ARDS patients.

Methods. We have compared the responses of primary HLMVECs to the main inflammatory mediators involved in cytokine storms induced by sepsis and SARS-CoV2 pulmonary infection, and to sera from healthy donors and severely ill sepsis patients. Endothelial barrier function was measured by electric cell-substrate impedance sensing, quantitative confocal microscopy and western blot.

Results. The HLMVEC barrier was completely disrupted by IL-6 conjugated with soluble IL-6 receptor and by IL-1beta, moderately affected by TNF-alpha and IFN-gamma and unaffected by other cytokines and chemokines such as IL-6, IL-8, MCP-1 and MCP-3. The inhibition of IL-1- and IL-6- mediated signaling pathways simultaneously, but not separately, fully prevented lung endothelial hyperpermeability upon exposing HLMVECs to a cytokine storm consisting of eight inflammatory mediators and significantly reduced barrier dysfunction in response to sera from sepsis patients.

Conclusions. These findings strongly suggest a critical role for both IL-6 trans-signaling and IL-1beta signaling in the pathological increase in permeability of the human lung microvasculature during systemic inflammation. Though barely explored so far, simultaneous targeting of these two pathways may be the best therapeutic option to prevent pulmonary edema in response to a cytokine storm.

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Nº6

Identification of IL-6 Signalling Components as Predictors of Severity and Outcome in COVID-19

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Background and objectives. The proposed central role of a cytokine storm in COVID-19 disease escalation prompts the question of whether interleukin (IL)-6 normalization strategies during the maladapted inflammatory response could be useful. Indeed, tocilizumab, an anti-IL-6 receptor monoclonal antibody, was widely used in COVID-19; nevertheless, clinical trials have shown conflicting results. It is important to know that the net biological effect of IL-6 is established by multiple factors beyond its absolute concentration. Moreover, IL-6 can signal by the classical mode with anti-inflammatory and antibacterial activities, or by trans-signalling with proinflammatory effects. The aim of this study was to investigate the ability of IL-6 signalling serum components (IL-6, sIL-6R and sgp130) to discriminate severity and to predict disease course and outcome in COVID-19 patients. We also studied different approaches to analyse potential IL-6 trans-signalling during the disease.

Methods. Serum samples of 366 hospitalized COVID-19 patients were collected at the hospital admission. Clinical and biochemical data were recorded on the day of the blood extraction. Serum concentrations of IL-6, sIL-6R and sgp130 were performed by ELISA assays. To estimate IL-6 trans- signalling, a ratio between the pro-inflammatory binary IL-6:sIL-6R complex and the inactive ternary IL-6:sIL-6R:sgp130 complex (binary/ternary complex), as well as the fold molar excess of sgp130 over sIL-6R (FME) were determined.

Results. Our data demonstrated that high levels of IL-6, sIL-6R, sgp130, binary/ternary complex ratio and low FME were independent predictors of COVID-19 severity in survivor patients (without death). Moreover, the combined analysis of IL-6 + sIL-6R + sgp130 improved the predictive capacity of their individual measurements, exhibiting the most robust specificity, accuracy and Odds Ratio (91%, 89%, and 27 respectively). Notably, patients who needed a longer length of hospital stay were those with increased levels of the three variables. Conversely, in a subgroup of patients with a very poor prognosis, high levels of IL-6 and FME and low levels of sIL-6R, sgp130 and binary/ternary complex ratio were early predictors of death. In this context, the combined analysis of IL-6 + FME + lymphopenia + creatinine had the highest predictive capacity, with 85% sensitivity and 80% accuracy. The lowest survival time corresponded to patients with increased IL-6 and decreased sIL-6R and sgp130 levels.

In conclusion, our study suggests that the screening of IL-6 signalling components at the hospital admission can identify patients at risk of severe COVID-19. Nevertheless, the levels of IL-6, sIL-6R and sgp130 markers have to be evaluated in the clinical context. Altogether, we identify novel biomarkers that may constitute a helpful tool for the early identification, stratification of patients at hospital entry, and prediction of the disease progression in terms of severity and/or death, with clear implications in treatment and clinical decision-making.

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Nº7

Iron oxide and iron oxyhydroxide nanoparticles impair SARS-CoV-2 infection of cultured cells

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Background. Coronaviruses usually cause mild respiratory disease in humans. Nevertheless, some human coronaviruses are able to cause more severe diseases, such as the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which has spread globally resulting in the ongoing coronavirus pandemic. Much of the research related to SARS-CoV-2 has focused on the development of preventive vaccines. While this is certainly important to prevent future outbreaks, it is also clear that additional strategies need to be put in place for the treatment of patients. The repurposing of existing drugs with an established safety profile to treat COVID-19 is an interesting approach.

Methods. In this work we have analyzed the potential use of iron oxide nanoparticles (IONPs) coated with biocompatible molecules such as dimercaptosuccinic acid (DMSA), 3-aminopropyl tri-ethoxysilane (APS) and carboxydextran (FeraSpin™ R), as well as iron oxyhydroxide nanoparticles (IOHNPs) coated with sucrose (Venofer®), and iron salts (ferric ammonium citrate (FAC)), in the treatment and prevention of SARS-CoV-2 infections in Vero E6 cells. Interestingly, some of the nanoparticles used in this work have already been approved for their use in humans as antianemic treatments, such as the IOHNP Venofer®; and in small animals like mice, as contrast agents for magnetic resonance imaging (MRI), such as the IONP FeraSpin™ R.

Results. Used at non-cytotoxic doses, IONPs and IOHNPs impaired virus replication and transcription, and the production of infectious viruses in vitro, either when the cells were treated prior to or after infection, although with different efficiencies.

FeraSpin R, DMSA-IONP-10 and DMSA-IONP-16 can induce significant oxidative stress in Vero E6 cells, activating different antioxidant effects, being DMSA-IONP-10 and DMSA-IONP-16 the NPs that induce the highest levels of oxidative stress. The weaker antiviral activity in the presence of NAC, a ROS scavenger, suggested that the induction of oxidative stress in the cells by these DMSA-IONPs was at least partially responsible for the antiviral effect they produced.

IONPs and IOHNPs also affect the expression of genes involved in iron metabolism and transport in Vero E6 cells. SARS-CoV-2 infection enhances the expression of genes involved in iron metabolism and transport, such as FPN1, DMT1, LCN2 and IREB2. Treatment of cells with FAC, Venofer® or IONPs can counteract the induction of FPN1, DMT1 and IREB2 expression to a greater or lesser extent in the infected cells.

In summary, our results suggest that the treatment of cells with IONPs and IOHNPs affects oxidative stress and iron metabolism to different extents, likely influencing virus replication and production.

Conclusions. Having studied the potential of IONPs and IOHNPs for the treatment and/or prevention of SARS-CoV-2 infection, we conclude that IONPs and IOHNPs have a prophylactic and therapeutic effect against SARS-CoV-2 infection at doses that are not cytotoxic. Our results suggest that IONPs and IOHNPs may be repurposed to be used as prophylactic or therapeutic treatments in order to combat SARS-CoV-2 infection. In fact, our group has filed two repositioning patents based on these results (EP21382973 and EP21382974, October 28th 2021).

Reference. Manuscript submitted to Journal of Nanobiotechnology (ID:c72e3271-95f4-4e7d-a6dc-335cf3e61e2d, under second revision, minor revision).

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Nº8

Fasciola hepatica tegument antigens inhibit SARS-CoV-2 infection efficiency in vitro

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Background: The trematode parasite *Fasciola hepatica* has adapted to its mammalian host during long co-evolution processes by establishing host-parasite relationships that modulate different physiological routes within the host. The regulation of the host immune system by *F. hepatica*, which supports parasite development inside the host, is a paradigmatic example of this. Previous data from our lab showed that *F. hepatica* newly excysted juvenile flukes (FhNEJ) modulate molecular routes in host cells related to vesicle-mediated transport and innate anti-viral responses, which could potentially be relevant during viral infections. Therefore, the aim of the present study was to determine whether FhNEJ molecules can regulate pathways that inhibit SARS-CoV-2 infection.

Methods: We set up an in vitro system based on the use of SARS-CoV-2 pseudotyped viral particles to screen for the potential of FhNEJ whole extracts and specific recombinant proteins to inhibit virus entry into epithelial cell lines, and validated the results using genuine SARS-CoV-2 infections.

Results: FhNEJ tegument and somatic extracts, together with the protein KTSPIDP, inhibited viral infection in the infection screen with pseudotyped viral particles, and antiviral effects were confirmed for the tegument FhNEJ antigenic compartment via infections with live SARS-CoV-2.

Conclusions: FhNEJ express molecules at their tegument surface that are capable of reducing infection efficiency of SARS-CoV-2. Based on previous results of our lab, these molecules may alter endocytic routes or modulate innate antiviral responses in epithelial cells, which results in reduced SARS-CoV-2 cell entry and/or replication. Although very preliminary, our results show that *F. hepatica* could also affect the outcome of the hyperinflammatory response in COVID-19 patients. Altogether, our results shed light into a mechanism of co-evolutionary adaptation between parasites and humans with regard to human interaction with pathogenic viruses, and potentially encourage the use of *F. hepatica*-derived molecules in a safe, synthetic format as therapeutic agents against SARS-CoV-2 and other emerging respiratory viruses.

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Nº9

Time-based quantitative proteomic and phosphoproteomic analysis of A549-ACE2 cells during SARS-CoV-2 infection

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Background. The outbreak of COVID-19, a disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), led to an ongoing pandemic with devastating consequences for the global economy and human health [1]. With the global spread of SARS-CoV-2, multidisciplinary initiatives such as COVID-19 MS Coalition [2] or “Global Health” Interdisciplinary Platform (PTI-CSIC) were launched to articulate different research groups to understand SARS-CoV-2 pathogenesis and explore new diagnostic, therapeutic, and vaccination strategies. In this perspective, proteomics could help to understand the mechanisms associated with SARS-CoV-2 infection and to identify new therapeutic targets for antiviral drug repurposing and/or discovery [1,3].

Methods. In this work, a TMT-based quantitative proteomics approach was applied to compare the proteome of human lung alveolar cells transduced to express human ACE2 (A549-ACE2) after infection with SARS-CoV-2 (3, 6, 9, and 16 hours) with mocked-infected cells. For phosphoproteomics analysis, 200 µg of the TMT-labeled sample were enriched using titanium dioxide (TiO₂). Tryptic peptides and phosphopeptides were analyzed by nano-LC-MS/MS coupled to an Orbitrap Exploris™ 240 Mass Spectrometer and the obtained data were analyzed with Proteome Discoverer 2.5.

Results. A total of 6802 protein groups (10 SARS-CoV-2) were identified (FDR<1%), of which 91, 115, 261, and 417 (q-value<5%) were found differentially regulated after 3, 6, 9, and 16h of infection. Regarding phosphoproteomics analysis, 7986 modification sites were identified, of which 6428 have been assigned with a high probability score (100%). According to the functional analysis of these (phospho)proteins, A549-ACE2 cells undergo changes in the cytoskeleton organization, cell-cell adhesion, calcium signaling, and estrogen signaling within 3h after infection, which could be consistent with the induction of cornification in the host cell. Inflammatory and acute-phase response signaling is triggered in host cells after 6h and sustained after 9h/16h, as suggested by the significant increase in the levels of coagulation factors, adhesion molecules, and inflammatory and acute-phase proteins. Our results also suggest that SARS-CoV-2 induces host cellular senescence and controls key processes such as autophagy, cell cycle, and translation/transcription machinery (e.g. Spliceosome). Several proteins related to virus-host interaction, inhibition of host mRNA processing and RNA silencing, viral replication, and exportation of viral RNA from host cell nucleus were found differentially expressed in SARS-CoV-2 infection compared to mock-infected cells. Regarding the SARS-CoV-2 proteome, 8 protein groups were found differentially expressed from the 6h of infection. The abundance of SARS-CoV-2 proteins increases with the infection time, except for non-structural protein 8, which was an abundance peak at 9h. Furthermore, we detect several phosphorylation sites with a high probability score in nucleoprotein (n=6), replicase polyprotein 1a (n=1), and membrane protein (n=2). Although the majority of these were previously described in the literature, two new phosphorylation sites were found in the nucleoprotein (S410, S416) in this work.

Conclusions. Our results suggest that SARS-CoV-2 leads to a hijacking of several key processes in the host cell, which could be regulated by changes phosphoproteome landscape of A549-ACE2/SARS- CoV-2. Therefore, targeting the protein kinases responsible for these phosphorylation changes could offer an alternative strategy for COVID-19 treatment.

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Nº10

Wastewater based epidemiology beyond SARS-CoV-2

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Background. Wastewater based epidemiology (WBE) has already been long-time implemented with success on the tracking of chemical pollutants, drug spread within communities, antibiotic resistance genes and waterborne viruses. The concept of WBE could be applied to a wide range of viruses beyond the confirmed waterborne viruses.

Methods. Implement molecular methods to concentrate, isolate and quantify viruses of emerging concern, including SARS-CoV-2, influenza virus and respiratory syncytial virus (RSV). Several virus concentration methods and RNA extraction strategies has been evaluated. Furthermore, wastewater samples have been analyzed for the analysis of SARS-CoV-2 viral diversity combining WBE together with high-throughput sequencing techniques (Illumina and Minion).

Results. Along this project different concentration methods have been evaluated, and further validated being currently used as a reference method in the Spanish COVID-19 wastewater surveillance project (VATar COVID-19) for the detection of SARS-CoV-2 and its variants. Furthermore, surveillance of sewage drains has served as an early warning system for isolated cases or outbreak declaration of SARS-CoV-2 infection at nursing homes.

Conclusions. WBE has proven to be an effective tool for epidemiological surveillance of SARS-CoV-2 to complement public health surveillance in Spain and these methods can be applied for other emerging viruses

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Nº11

SARS-CoV-2 surveillance in the air: a long-term study in education centres

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Aerosols produced by an infected patient, as well as small droplets, have been established as the main transmission pathway of SARS-CoV-2. However, few studies describing reliable detection of viruses in the air, especially in non-hospital places, have been published. Moreover, studies evaluating the transmission level of different variants of SARS-CoV-2 in infants and toddlers are lacking. We present a study performed between June 2021 and May 2022 in 8 centres from 4 different educational levels: nurseries, elementary schools, high schools and university centres. Airborne viruses were captured for a few hours with an air sampler developed by CSIC, consisting of an air pump coupled to filters, in different locations of the education centres. The sampling times were coincident with different waves of incidence in Madrid, Spain. Among the sampled locations, we prioritized classrooms and dining rooms for students and/or teachers. The indoor CO₂ concentration was also monitored every 5 minutes, along with temperature and relative humidity.

After RNA isolation from the filter, specific identification of SARS-CoV-2 was performed in triplicate by RT-qPCR specific for the nucleoprotein gene. A total of 1053 air samples have been collected and analysed. Obtained C_q ranged from 27 to 39, and a synthetic SARS-CoV-2 RNA was used for calibration. We observed a relevant increase in the level of copies of viral RNA by m³ of air in January 2022, when SARS-CoV-2 Omicron started to be the prevalent variant in Spain. In May 2022, just after the removal of the masks indoors, we also detected high amounts of virus in the air. Detection of SARS-CoV-2 in aerosols was higher in centres with 0-3 and 3-12 years-old students, corresponding to the age range of non-vaccinated people. We were able to detect very relevant levels of SARS-CoV-2 in the environment in a coincident moment of confirmed positive cases of COVID-19 among the centre's staff. Finally, we sequenced the complete genome of SARS-CoV-2 captured from the air in the filters by an amplicon-based protocol (ARTIC). This study illustrates the application of airborne virus capture methodology as a tool for monitoring virus presence and anticipate viral outbreaks.

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Nº12

Catalytic filters for virus inactivation

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Respiratory infectious diseases take first place in the ranking of the burden of disease measured by years lost through death or disability, according to the World Health Organization (WHO). Lower respiratory tract infections represent the third leading cause of death in the world. The COVID-19 pandemic has been responsible for more than 533 million cases and 6.3 million deaths [1]; airborne transmission is the main propagation vector [2-4]. Our objective is to develop catalytic filters, which will be placed in indoor air-cleaning device, which will inactivate virus by oxidative stress of the virus membrane by heating the filter selectively at mild temperatures (up to 37 °C).

Two families of filters were prepared at ICP: ceramic and polymeric filters. Ceramic filters were prepared by immersion of pre-shaped discs or monoliths into a solution whereas polymeric filters were prepared by impregnating commercial air conditioning or fan filters using an airbrush. To facilitate pre-screening, a new methodology, based on acellular assays, has been developed to evaluate the oxidative potential of the filters; it uses organic molecules probe test.

For modelling purpose, deposition of nanoparticles with fine-tuned properties over ceramic or polymeric filters has also been evaluated in order to assess the role of the oxidation state, of the particle size and of the composition of the nanoparticle. This controlled deposition of nanoparticles is performed STARDUST rig at ICM.

Virus inactivation was determined by plaque assays at CBMSO. Human coronavirus (HCoV-229E) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were used for evaluating the efficacy of the filters. Different inoculation times and process temperatures were evaluated.

Some 100 filters have been prepared spanning different supports, active phases, preparations and concentrations, among other variables. Good inactivation potential for HCoV-229E is delivered by several filters, even at room temperature. For inactivation of SARS-CoV-2, mild temperature (37°C) was needed. A good correlation between oxidative capacity and inactivation potential is established. For the air cleaning evaluation, a system to generate aerosols was designed and commissioned to feed the reactor with virus-containing air and evaluate its viral inactivation capacity. We are currently integrating the aerosol chamber and the reaction system (microwave-assisted reactor).

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Nº13

New material with virucidal activity against SARSCoV2 and other viruses

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Respiratory pathogens kill more people than any other infectious agent each year worldwide. The COVID-19 pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection [1] has experienced a catastrophe both economically and socially, with more than 6.22 million people dead and 500 million people infected worldwide. Development strategies such as vaccination have mitigated this problem, [2] however, currently the existence of new variants with greater transmissibility are causing an extension in infections and an increase in hospitalizations.

Airborne transmission of the COVID-19 virus between humans occurs primarily through respiratory droplets. A recent study suggests that the use of masks could reduce the severity of the disease, since they can reduce the viral load. [3] Virucidal coatings could be applied to the surface of the mask, improving both protection and durability. Historically, metallic nanoparticles have been shown to display a wide range of virucidal and bactericidal activities, [2] in particular copper (Cu) has shown more potent activity than other metals, such as silver or zinc.

However, most of the commercial materials composed of copper, for example in masks, has focused on the use directly of metal threads, compounds based on mixtures of copper species at bulk or micromolecular structure. This has the problem of loss of material in each use in addition to presenting a lower efficiency. In addition, these materials have been mainly described with antibacterial activity and only few against COVID-19. [4]

In this sense, we describe here for the first time a nanostructured material composed of very small crystalline copper nanoparticles of a single species, synthesized based on a new biohybrid technology that employs the use of a biological agent for its formation, creating a stable material in aqueous medium and r.t., with high efficiency against SARS-CoV-2 and other viruses. [5] In addition, this new material has been successfully demonstrated to have multiple applications as coating agent on different surfaces of different compositions (cotton, polyester, cellulose, paint, etc.) maintaining the virucidal efficiency against different viruses and also through a mechanism of 100% efficiency where there is no loss of material in each use, also allowing its reuse.

In this way, this material, also optimized for large-scale production, allows access to the manufacture of a new type of anticovid masks with the advantage of being able to be implanted in all types of masks (surgical IIR, FFP2, textile) with high efficiency allowing to develop a new type of EPIS with an economic cost that is also adequate to reach the whole society. Therefore, this represents a new type of material in the fight against infectious diseases, particularly viruses, since this type of coating agent is also efficient against other viruses and its efficiency is potentially extensible to many others.

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Nº14

Green procedure for textile functionalization with reactivable virudical groups: surface modification, characterization and activity against human H-CoV 229E and SARS-CoV-2 coronaviruses

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Background. SARS-CoV-2 and other human coronaviruses can survive for up to 9 days on contaminated environmental and inanimate surfaces. Contaminated surfaces are a potential source of viral transmission, especially in healthcare settings and nursing homes, where high viral load and/or vulnerable individuals can be expected. Reducing viral load on inanimate surfaces is therefore a critical factor to minimize viral transmission. This is especially important on textile materials that are used for Personal Protective Equipment (PPE), bedlinen and workwear.

The objective of this work is the development of a green, rapid, non-toxic and economic procedure to functionalize cotton and polyester fabrics to obtain textiles with very high virucidal activity. Moreover, the functional group should be reactivable under laundering conditions, thus providing the textiles with a long-term rechargeable virucidal activity.

Methods. Fabric samples: commercial cotton (Ribes y Casals) and polyester fabrics (ANTEX) were functionalized by a pad-dry procedure using water as solvent. The extent of functionalization was measured by titration. Stability and rechargeability of the functional group were studied under wet (immersion) and dry (storage in dark) conditions. Virucidal activity was studied against coronaviruses (HCoV- 229E GFP, SARS-CoV2 MAD6 strain), influenza virus H1N1 (A/WSN/33 strain) and rhinovirus (HRV- 14). For the infectivity assays against the last three viruses Vero-E6, Mdck and H1 HeLa cell lines were respectively used and the efficacy of the fabrics to inactivate the viruses was quantified by lysis plate counting. The amount of GFP found in HUH7 cells served as an indicator of HCoV-229E GFP infection. To determine the cytotoxicity of the fabrics, C166-GFP endothelial cells were exposed to liquid extracts resulting from textile release experiments in aqueous media.

Results. The procedure developed for cotton chemical modification involves the impregnation of the textile by spraying with or immersion in an aqueous solution of a precursor of the functional group, followed by an ironing step under domestic conditions. Polyester textiles need a pre-treatment step of immersion in a basic solution. Activation of the precursor takes place at room temperature by immersion in aqueous solution of commercial bleach.

Both commercial cotton and polyester fabrics show great inactivation efficacy against all enveloped viruses (coronavirus, influenza virus) in less than 30 minutes of incubation, while they require overnight contact to inactivate non-enveloped HRV-14.

Conclusions. The process for functionalization of common cotton and polyester fabrics results in a high degree of modification via a pad-dry method at room pressure and temperature, and in water media. The functional group is stable under laundering conditions and fully rechargeable by 2 hour immersion in aqueous solution of diluted commercial bleach. The modified fabrics show great inactivation efficacy against envelope viruses SARS-CoV 2, HCoV 229E and H1N1 in less than 30 minutes. They are also capable of inactivation the non-enveloped virus HRV-14 after overnight contact. Indirect contact of the fabrics with endothelial cells did not cause changes in cell morphology or a decrease in cell viability.

So, this process seems very promising to be used for commercial and even domestic modification of pre-existing textiles to acquire high virucidal activity.

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Nº15

Development of Nanofiber Based Filters for Inclusive, Reusable, Kids, Biodegradable and Compostable Antiviral Masks and for Air Filtration Applications to Fight the COVID-19 Pandemic

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This presentation will show our efforts to develop different filtration materials based on nanofibers to fight the COVID-19 crisis, which were converted into certified masks and air cleaning products that reached commercial phase via partnership with various companies.

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Nº16

A convolutional neural network for early detection of Covid-19 using chest X-rays

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Background. During the COVID-19 pandemic, chest radiography has played a very important role in the detection and monitoring of lung disease, especially at the beginning of the pandemic. However, even in the hands of expert radiologists, the sensitivity of a chest X-ray for COVID-19 diagnosis is modest. Early detection of disease using a tool such as a chest X-ray could be useful in resource-poor settings. Thus, the ability to early detect COVID-19 using artificial intelligence tools could anticipate timely care and isolation for patients.

In this work, a deep learning artificial intelligence (AI) model has been developed taking the first clinical encounter of patients as an input to detect the risk of COVID-19 lung disease. The model has then been compared with a group of radiologists with different years of experience in diagnostic chest imaging.

Methods. A set of four deep convolutional neural networks have been trained using images from chest radiographs from COVID-19 positive and negative patients. To avoid possible selection and group attribution bias we only used images from the first clinical encounter of the patients. The reference standard for positive patients was a positive real-time polymerase chain reaction (RT-PCR) test taken within one day of the chest X-ray. Negative patients did not show a positive result in any of the RT-PCR or antigen tests. The performance of the algorithm was compared with that of 5 radiologists with different years of experience on an independent smaller subset of test patients. The performance of the deep model training was also validated using the large public COVIDx dataset.

Results. The convolutional neural network model achieved an AUC of 0.85 using images from the first clinical encounter. In comparison, the consensus of the five radiologists achieved an AUC of 0.71. Compared to other state-of-the-art models, the performance of a single model in our ensemble achieved non-significant differences in the public COVIDx dataset.

Conclusions. The performance of our convolutional neural network model under these challenging conditions is considerably superior compared to a consensus of five radiologists. However, using images from the first clinical encounter significantly decreases the detection performance of COVID-19. This is a reasonable result considering that using images from later encounters may bias the model by focusing it on information that is not relevant for early detection, such as a lung condition that is too obvious or even the appearance of tubes or other medical instruments that are used when the disease is in an advanced stage. We believe that artificial intelligence can help detect lung affections due to COVID-19 early and thus anticipate appropriate treatment for patients.

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Nº17

Computer aided diagnosis of pneumonia in COVID-19

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Background. Pneumonia is the principal complication in COVID-19, and its detection and quantification at early stages gives valuable information for patient treatment. Moreover, knowing affectation level of the lungs is useful at all stages of the disease, from monitoring the treatment response during hospitalization, to follow-up after discharge.

Lung echography is a promising technique for diagnosis of pneumonia, as it is a non-invasive and radiation-free technique that can be applied at patient bedside. Nevertheless, interpretation of lung ultrasound images is a difficult task, mainly because it is based on acoustic artifacts that are not always evident to the operator. Being a relatively new technique, its use during the pandemic was restricted to few highly trained physicians, and its potential could not be fully released for the whole medical community.

Contribution / Methods. The motivation of this work was to democratize the access to lung echography by contributing to simplify the lung echography exam. Our approach was to develop an intelligent ultrasound scanner, able to guide the physician during the examination and to provide an automated diagnosis of pneumonia. This could hopefully reduce the learning curve of the technique, making it accessible to more physicians and medical services and, therefore, to more patients.

An image processing algorithm was developed to measure the lung affectation level. From a set of ultrasound videos registered in 12 regions of the thorax, the algorithm calculates a lung score between 0 and 36 points, that quantifies the lung compromise level.

A fully functional scanner was developed, and a clinical trial with 30 patients with COVID-19 was performed in two hospitals in Madrid. The objectives were to evaluate the performance of the algorithms and to test the usability of the equipment in the field.

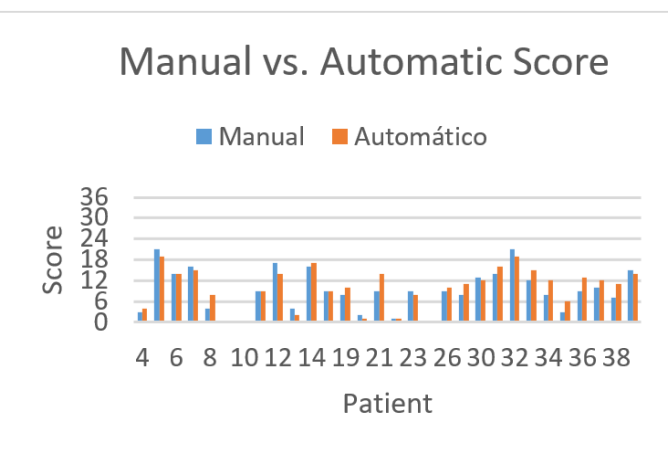
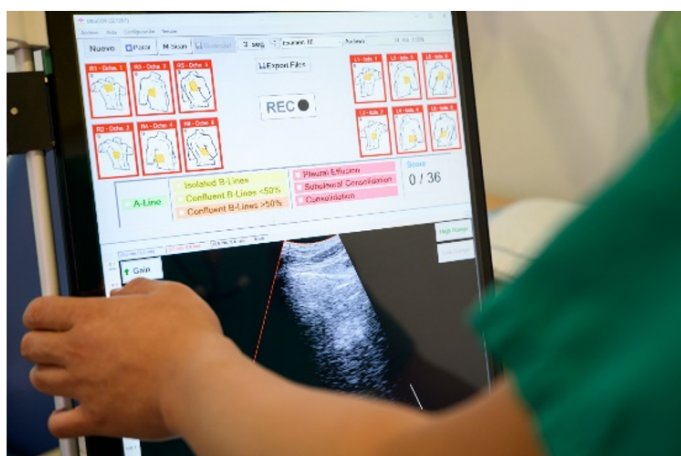
Results. The coincidence between the automated diagnosis algorithm and the expert physician was 88.0 % for B-Lines and 93.4% for consolidations, the two principal artifacts associated with pneumonia. When analyzing the global lung score, the standard deviation of the difference between the expert and the algorithm was ± 2 points over 36. With regard to the scanning time, the average with the developed scanner was 5.3 minutes per patient, while with a conventional scanner without aided diagnosis tools, it was 12.6 minutes.

Conclusions. The principal conclusion is that developing an automated algorithm for detection and quantification of pneumonia by ultrasound is feasible. The clinical trial revealed that the concordance between the algorithm and an expert is high, in particular with regard to the lung score. Furthermore, the scanning time was reduced less than half when compared with a conventional scanner, which could have a positive impact on high demand services as emergency or primary care.

Current work involves implementing these algorithms in strict real-time at high frame-rates inside the scanner, to show the physician the detected indications while it moves the probe during the scan. Future work includes extending these results for the diagnosis of pneumonias of other origins, in particular for pediatrics, where ionizing radiation limits hinder the use of X-Ray or CT.

Nº17

ULTRACOV



(Left) Image of the developed scanner (Right) Comparison between the automatic and manual lung score.

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Nº18

Dynamics of humoral immunity response during SARS-CoV-2 infection: Prognostic factors disease evolution and long-covid19

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The humoral immune response remains a key element in the diagnosis and follow-up of patients infected with SARS-CoV-2. Specifically, the autoimmune response, the immunoglobulin (Ig) neutralizing profile against the virus and other soluble factors such as acute phase reactants (APR) can serve as a basis for determining a dynamic in patients with SARS-CoV-2 infection. In-depth characterization of humoral response of more than 400 COVID-19 patients with different symptoms and severity was performed. Multiplex high-throughput analysis has led to more than 62,000 immunoassays that have been used to characterize the humoral dynamics of patients.

The Ig production against SARS-CoV-2 proteins drew distinct profiles at different severity levels of COVID-19 infection as well as APR and autoantibodies (AAB) profiling reveals key players of dynamic humoral response at different COVID-19 disease stages. Monitoring of humoral response against SARS-CoV-2 infection convalescence reveals potential long-COVID-19 biomarkers and widespread changes in the functional coordination of these factors in COVID-19 patients. Several humoral response proteins, as prognostic biomarkers, have been found to be significant and allow differentiation of the severity and evolution of infected patients in ICU as well as provide clues about the follow-up of patients in post-acute sequelae of SARS-CoV-2 infection (PACS).

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Nº19

A compact fluidic electrochemical biosensor platform for SARS-CoV-2 RNA detection

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Background. The main objective of this study was the development of a compact fluidic electrochemical biosensor platform for quantitative detection of specific sequences of SARS-CoV-2. Our aim was to develop a cost-effective, user-friendly, and timely analytical tool that could be implemented at the point-of-care.

Methods. The biosensor platform comprises the following main components:

1. A reusable gold two-electrode electrochemical cell.
2. A disposable fluidic component comprising a paper channel sandwiched between two polyvinyl layers.
3. A home-made poly (methyl methacrylate) cartridge to integrate and align the electrochemical cell and the fluidic component that also includes a neodymium magnet.

The performance of the biosensor requires the use of magnetic nanoparticles (MNPs) functionalized with different capture sequences for sample pre-treatment outside the device. This was a one-step incubation with the sample and a reporter sequence conjugated to a peroxidase enzyme (HRP), to trap and preconcentrate the target sequence. Then, MNPs were cast on the sample addition area of the platform. They flowed on the paper channel by capillary action and were trapped in an area over the magnet inserted in the cartridge. The biosensor response was recorded by chronoamperometry following the HRP reaction, which took place by adding a buffered solution containing H₂O₂ substrate and ferrocenemethanol mediator. For this, a compact, low-power instrumentation was used. The overall analysis time was around 40 min.

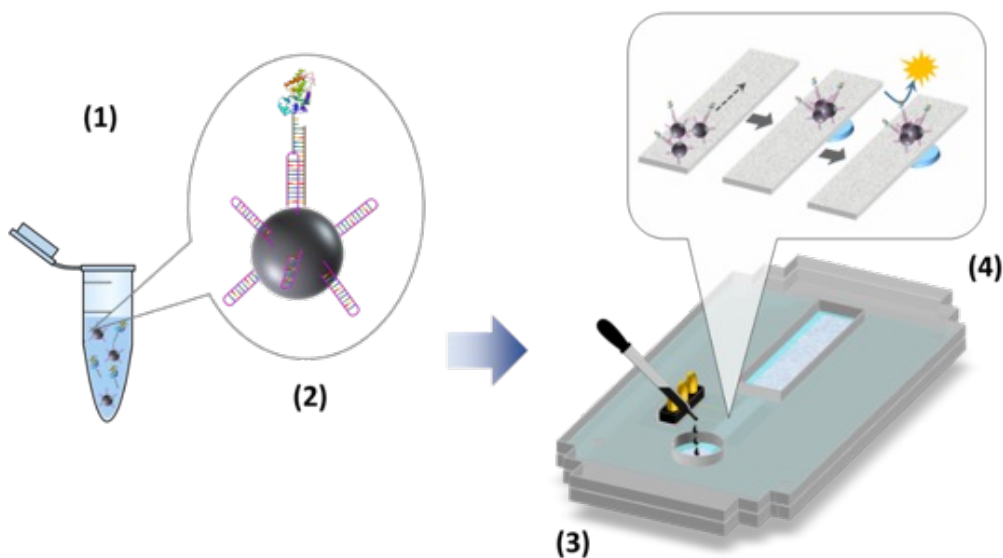
Results. The HRP label catalyzed the reduction of H₂O₂ using the ferrocenemethanol as electron donor, generating in-situ the ferrocinium-methanol cation. This cation was reduced back to ferrocenemethanol at the electrochemical cell, and the recorded current was directly proportional to the concentration of the target sequence in the sample.

The cathodic current increased with the target concentration, 0.01 nM (1 fmol) being the lowest concentration that unambiguously produced a signal different from that of the blank solution. The Polypurine Reverse Hoogsteen CC1 (PPRH-CC1) formed a triplex giving the best results and, therefore, was chosen for the analysis of 58 nasopharyngeal swab samples provided by the IGTP in a retrospective study. Considering the RT-PCR results as true positives and negatives, the electrochemical biosensor showed an 86% of both sensitivity and specificity.

It was observed that samples with low Ct values (< 20) produced lower amperometric signals than samples with medium to high Ct values (< 35) and this effect seemed to be more intense when the viral load increased. Such behaviour might be related to the secondary structures of the viral RNA and the intermolecular interactions taking place among RNA strands that may hinder the accessibility to the specific target sequence. This was indicated by studies carried out using an RNA fragmentation kit and measurements recorded with serial dilutions of some of these samples.

Conclusions. An electrochemical biosensor platform for detecting specific SARS-CoV-2 RNA sequences in nasopharyngeal swab samples, showing good sensitivity and specificity and a response time below 1 h, has been developed. The biosensor performance is controlled by a portable instrument. All these features make the presented analytical tool to be of potential application for decentralized analysis at the point-of-care.

Nº19



GRAPHICAL ABSTRACT: Scheme of the device platform and the analytical procedure: 1 – Sample addition to a solution containing functionalized MNPs and the reported sequence labelled with HRP enzyme; 2 – Hybridization assay on the MNP surface; 3- Pretreated sample added to the electrochemical device; 4 – Steps taking place in the electrochemical device, including MNP flow, trapping and concentration, and electrochemical detection.

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N°20

Photothermal Lateral Flow Point of Care test for SARS-CoV-2 RNA Detection

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The outbreak severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) received global attention from 2020 because it quickly turned into a global pandemic. Laboratory diagnosis played an important role in the disease and outbreak management. After Chinese health authorities posted the full genome of SARS-CoV-2 several laboratories developed assays to detect COVID-19. RT-PCR was used as reference standard methodology for diagnosing of COVID-19, however, this method has time consumed, because its average duration is around 30 minutes to 2 hours, and also has higher expertise and technical skills limitations. Early diagnosis might produce better prognosis, prevention and pandemic control. With that propose rapid and simple techniques based on the qualitative detection of SARS-CoV-2 structural proteins were developed (point-of-care, POC, tests). These tests provide results within minutes and may also extend testing to communities and populations that cannot readily access care. On this basis, specific viral RNA detection could be used as an innovative biomarker for early diagnosis of this disease. Combining a conventional POC, how is the Lateral Flow technology, with inorganic nanoparticles, as Gold Nanoprisms (AuNPRs), a novel ultrasensitive Photothermal Lateral Flow Assay (PLFA) was developed. Gold nanoprisms (AuNPrs) biofunctionalized with a specific DNA oligonucleotide which recognize the viral genetic material will be used as thermal transducers for the biosensor. Due to their optical properties, AuNPs can convert light into heat. Viral RNA is recognized by the AuNPRs and also by the capture biomolecule deposited on the nitrocellulose strip, the subsequent no visible test line irradiation with a NIR laser will generate a visible spot in a thermosensitive paper than could be further quantified. With this novel nanobiosensor, we are able to detect genetic material of COVID-19 with high specificity and sensitivity, overcoming the classical limitations of LFIA tests (low sensitivity and lack of quantification). Nasopharyngeal samples collected in inactivated viral transport media (VTM) have been analyzed and samples with a very low viral load (35 Cts) have been also detected. Further steps will be devoted to detect different RNAs or DNAs from different organisms and attempt to unravel a possible biomarker for cancers or degenerative diseases.

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Nº21

Assessment of human IgG response with a fluorescent peptide immunoarray after SARS-CoV 2 infection

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Background. In the context of the recent pandemic, SARS-CoV-2 was identified as the causative of coronavirus disease 2019 (COVID-19). Evidence suggests that host immune response plays a crucial role throughout the disease course as asymptomatic, mild, or severe. The analysis of IgG response after SARS-CoV-2 exposure provide accurate information about the immune state of the patients and may be used as predictor of disease progression. The motivation behind the project consisted on the development of a multiplexed epitope fluorescent microarray chip based on viral peptides and proteins to assess IgG response in human serum samples after infection. Our aim is to establish correlations between serological profiles with clinical outcomes, comorbidities among other parameters such as sex or age.

Methods. Initially, a rational selection of the most antigenic peptide sequences was performed taking into consideration their antigenicity, accessibility, selectivity and chemical viability. 22 sequences belonging to the spike glycoprotein and 6 to the nucleocapsid protein were chosen from different domains of each protein. Then, the synthesis and purification of the peptides was performed by solid phase synthesis using a Fmoc/Boc strategy (MS4N group, IQAC-CSIC).

Afterwards, the peptides and full-length proteins were spotted in triplicate over previously epoxy derivatized glass slides. A matrix containing 35 different epitopes was printed using a piezo dispense arrayer allowing the assessment of 24 samples per slide. Only 10 µl of human serum was required to perform the assay and after sample incubation, an anti-Human IgG-FITC was utilized for fluorescent readout. Finally, a biostatistical analysis was carried out to study the significance of each epitope over the classification of seropositive patients and to establish correlations between serological profiles and clinical outcomes using univariate and multivariable analysis tools (IBEC).

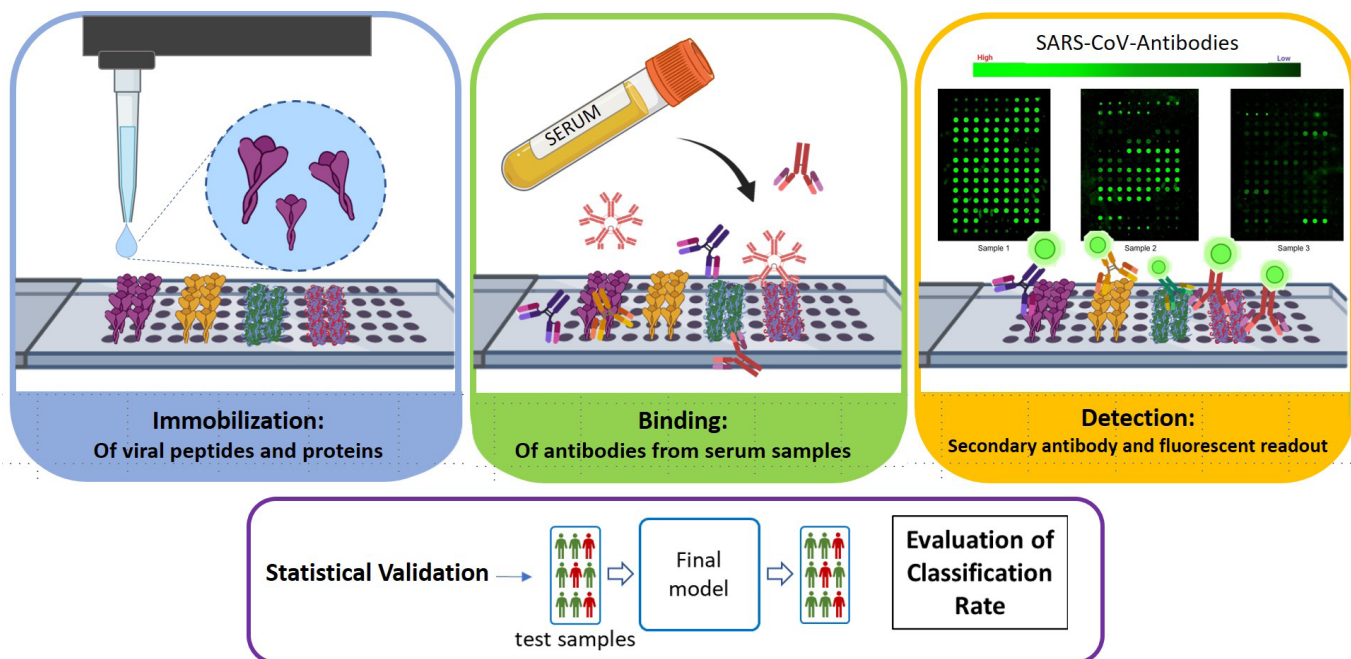
Samples were collected from different locations (IACS, IGTP and PSMar) and the vast majority included relevant clinical data to perform the biostatistical analysis such as UCI admission, Hospitalization and Exitus.

Results. A total of 755 human serum samples (Prepandemic, PCR negative and PCR positive) were analysed with the immunoarray in less than 2 hours in three different days.

The univariate analysis revealed that from 35 epitopes under study, 27 were significant towards detection of seroconversion after day 10 post symptoms onset. Peptide 10 showed the most significant values with almost 90% of correlation. On the other hand, the multivariate analysis improves considerably the classification rate up to 98% discriminating serologically positive patients.

Conclusions. A multidisciplinary effort lead to a successful development of a multiplexed immunoarray based on SARS-CoV-2 peptides and proteins to detect IgG response. More than 750 human serum samples were analysed with the platform established. Multivariate analysis performed better than univariate, reaching up to 98% of classification rate and predictive capacity of this tool is under evaluation. This platform allows multiple applications for the analysis of several parameters associated with viral immune response.

Nº21



GRAPHICAL ABSTRACT

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Nº22

Toward a platform for data-driven computation

Fredreric Bartumeus¹; Alvaro Lopez²; **Jose Javier Ramasco**³; Diego Ramiro⁴; FACE Collaboration FC⁵

Since the beginning of the 2000, we have suffered the continuous arrival of epidemics produced by different emerging diseases. These include the initial SARS epidemic, the H1N1 influenza pandemic of 2009, the Zika propagation in the Americas, the Ebola epidemic in West Africa, the COVID-19 pandemia and the current spread of monkeypox. All these cases have shown the direct need of efficient data management and of standardised computational tools to assess the effects and efficiency of public policies to contain the disease spreading.

In the WP3, we have as aim the creation of such tools in the form of the FACE platform. For this, we have divided the work in several tasks, from the creation of a computational architecture, databases with different access levels and privacy guarantees, flexible machine learning tools, the collection of data of both human and vectors and the construction and testing of epidemic models.

Here we will review the state of the WP3, showing very briefly the advances achieved and that will be more extensively explained in posters by the members of the Collaboration.

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Nº23

Genomic surveillance and impact of SARS-CoV-2 mutations

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Background. Deciphering the impact of SARS-CoV-2 mutations in immune evasion is essential to understand the dynamics of the epidemic. We aim to better understand the potential of different SARS-CoV-2 variants for impacting transmissibility and virulence.

Methods. We employ a complex workflow starting with mutation detection and epidemiological investigation, to further characterise the impact of mutations in the structure of the SARS-CoV-2 proteins and in the biology of a pseudovirus system in vitro (Figure 1). Mutation surveillance has employed different datasets including a global dataset with more than seven million viral sequences, and three local datasets up to ten thousand sequences paired with detailed hospitalisation and vaccination host information.

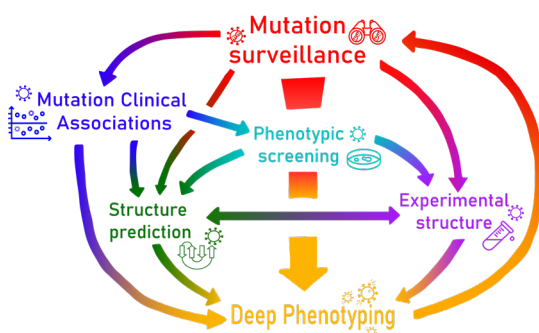
Results. First we have identified cluster 1163.7, characterised by spike mutations S:D1163Y and S:G1167V inside cluster 20E and we concluded that the two spike mutations impacted syncytia formation and infectivity in vitro. However, they did not impact thermal stability or neutralisation by antibodies except from those infected in the first Spanish epidemic wave.

Second, we have studied which combinations of S1 terminal domain spike mutations present in two variants of concern are more transmissible in different epidemiological settings.

Third, we have studied the diversity and migration dynamics of variants of concern.

Finally, we have used different statistical approaches to find associations between viral genomic variants and breakthrough infections and/or hospitalisation.

Conclusions. We have characterised SARS-CoV-2 variants circulating globally, with special focus in Spain, and studied the association of specific viral variants with vaccine breakthrough, hospitalisation, and transmission. Our study provides an effective pipeline for the characterization of biological fitness driven by the early identification of mutations of relevance in SARS-CoV-2, and followed by in vitro viral characterization and protein structure elucidation.



GRAPHICAL ABSTRACT

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Nº24

Biophysical, biochemical and structural characterization of SARS-CoV-2 spike variants

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Coautor(es): IBV-Covid¹⁹-Pipeline ¹; Francisco Del Caño ¹; Guillaume Dim ¹; Mónica Escamilla-Aguilar ¹; Alonso Felipe-Ruiz ¹; Francisca Gallego ¹; Alba Iglesias-Ceacero ¹; Susana Masiá ¹; Lidia Orea-Ordoñez ¹; Borja Sáez de la Fuente ¹; Carla Sanz-Frasquet ¹

Background. Most SARS-CoV-2 variants of concern present mutations on the spike (S) protein associated with higher infectivity and/or resistance to vaccines and antibody therapies. Many key mutations map to the Receptor Binding Domain (RBD) and are involved in the interaction with the host receptor ACE2 or with antibodies. Although most mutations are well-characterized, these studies often rely on isolated RBDs or in proline-substituted stabilized spikes, which could mask long distance effects of mutations outside the RBD but affecting the degree of exposure of the latter. Our aim was to develop an experimental platform for rapid production and characterization of spike variants “as natural as possible” to better understand the effect of certain mutations in the stability and interaction with ACE2 or antibodies.

Methods. 1) Mutagenesis and cloning of S variants in appropriate expression vectors. 2) Production of wild-type and mutated S, RBD and ACE2 proteins using baculovirus/insect and mammalian cultures. 3) Monitor stability of S variants by thermofluor and negative-staining electron microscopy (EM); 4) Analysis of protein conformation in solution by size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) and native-red electrophoresis. 5) Biolayer interferometry protein-protein interaction assays. 6) Structural characterization by cryo-EM. 7) Molecular dynamics (MD) simulations of conformational changes associated to certain mutations.

Results. We developed a pipeline for evaluating biophysically and structurally the impact of different mutations on the SARS-CoV-2 spike protein. We produced >30 S protein variants, most of them without stabilizing prolines and few with intact furin site. We also produced 9 RBD variants and >70 variants of monomeric and dimeric ACE2. Combining different techniques, we analyzed the stability of the recombinant proteins and showed that the spike variants are in an equilibrium between at least two conformations, that is influenced by temperature and by certain mutations. We also measured how the mutations affect the binding kinetics of the interaction between the spike or RBD variants with ACE2. Using cryoEM and MD simulations we provide detailed information on the impact of mutation A222V (characteristic of the 20E (EU1) and the Delta subvariant AY.4.2) on the transmissibility of the virus.

Conclusion. This collaborative consortium of several CSIC groups resulted in a pipeline for the fast characterization of known and future spike variants, and of protein ancestors. We observed mutation-driven changes in the stability and binding of the S variants, a conformational equilibrium of the spike, and a different behavior with artificially stabilized proteins. The results support our working hypothesis that the use of “as natural as possible” S proteins is important to accurately understand the molecular mechanisms governing SARS-CoV-2 infectivity.

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*Autores y Co-Autores por orden alfabético. Los Co-Autores pertenecen a la IBV-COVID19-Pipeline

Nº25

The role of SARS-CoV-2 genetic background in the emergence and success of spike mutations: the case of the spike A222V mutation

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Background. Most SARS-CoV-2 variants of concern present mutations on the spike (S) protein associated with higher infectivity and/or resistance to vaccines and antibody therapies. Many key mutations map to the Receptor Binding Domain (RBD) and are involved in the interaction with the host receptor ACE2 or with antibodies. Although most mutations are well-characterized, these studies often rely on isolated RBDs or in proline-substituted stabilized spikes, which could mask long distance effects of mutations outside the RBD but affecting the degree of exposure of the latter. Our aim was to develop an experimental platform for rapid production and characterization of spike variants “as natural as possible” to better understand the effect of certain mutations in the stability and interaction with ACE2 or antibodies.

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Conclusion. This collaborative consortium of several CSIC groups resulted in a pipeline for the fast characterization of known and future spike variants, and of protein ancestors. We observed mutation-driven changes in the stability and binding of the S variants, a conformational equilibrium of the spike, and a different behavior with artificially stabilized proteins. The results support our working hypothesis that the use of “as natural as possible” S proteins is important to accurately understand the molecular mechanisms governing SARS-CoV-2 infectivity.

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Nº26

Preclinical characterization of the SARS-CoV-2/COVID-19 vaccine candidate MVA-CoV2-S: Robust immunogenicity and full efficacy against SARS-CoV-2 in mice, hamsters and rhesus macaques

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Background. To control the coronavirus disease 2019 (COVID-19) pandemic and the emergence of different variants of concern (VoCs), novel vaccines with wide and long-term action against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are needed.

Methods. We have generated a COVID-19 vaccine candidate based on the modified vaccinia virus Ankara (MVA) vector expressing a human codon optimized full-length SARS-CoV-2 spike (S) protein (termed MVA-CoV2-S), and we have analyzed its immunogenicity and efficacy in several animal models: mice, hamsters and rhesus macaques.

Results. In C57BL/6 mice, two doses of the MVA-CoV2-S vaccine candidate induced robust, broad and polyfunctional adaptive and long-term memory S-specific humoral and T-cellular immune responses, even at 6 months after immunization. Remarkably, one and two doses of MVA-CoV2-S protected K18-hACE2 transgenic mice from a lethal dose of SARS-CoV-2, preventing virus replication in the lungs, reducing lung pathology, and diminishing levels of pro-inflammatory cytokines. High titers of IgG antibodies against S and receptor-binding domain (RBD) proteins and of neutralizing antibodies were induced against parental virus and VoCs, markers that correlated with protection. Similar SARS-CoV-2-specific antibody responses were observed at prechallenge and postchallenge in the two-dose regimen, while the single-dose treatment did not avoid vaccine breakthrough infection. All vaccinated mice survived infection and were also protected from SARS-CoV-2 reinfection. In hamsters, immunization with one or two doses of MVA-CoV2-S also elicited high titers of S- and RBD-binding IgG antibodies and neutralizing antibodies against parental SARS-CoV-2 and several VoCs. After SARS-CoV-2 challenge, vaccinated hamsters again showed a significantly strong reduction of viral RNA, infectious virus and lung histopathology compared to the control group.

Finally, in rhesus macaques, two doses of MVA-CoV2-S were well tolerated and induced S and RBD- binding IgG antibodies and neutralizing antibodies against SARS-CoV-2 and several VoCs. S-specific IFN γ , but not IL-4, -producing cells were also elicited. After SARS-CoV-2 challenge, vaccinated macaques showed a significant strong reduction of virus loads in bronchoalveolar lavages (BAL) and decreased levels in throat and nasal mucosa. Remarkably, MVA-CoV2-S also protected macaques from fever and infection-induced cytokine storm. Moreover, computed tomography and histological examination of the lungs showed reduced lung pathology in MVA-S-vaccinated animals.

Conclusions. The robust T-cell and humoral immunogenicity and full efficacy induced by the MVA- CoV2-S vaccine candidate in several animal models supports its use as a potential vaccine for SARS- CoV-2/COVID-19 in human clinical trials.

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N°27

The antibiotic resistance-free vaccine based on the non-replicative pPAL vector is fully protective against SARS-CoV-2 in the murine model

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Background. The main objective of this work is the development of a DNA vaccine against the SARS-CoV-2 virus based on the non-replicative antibiotic resistance marker gene-free the plasmid vector pPAL.

Methods. We designed pPAL-Sfs and pPAL-structural protein constructs. A PCR cloning procedure was carried out to obtain the pPAL-based recombinant vaccine and laboratory-scale batches of pPAL-based SARS-CoV-2 vaccine constructs were produced. Transfection was performed on the human HEK293 cell line with the pPAL-based recombinant vaccine. Expression was evaluated by Western blot. Evaluation of protection experiments against a lethal dose of 105 pfu of SARS-CoV-2 (Wuhan-Hu-1 and Delta strains) in K18-hACE2 female mice vaccinated intramuscularly with a prime/boost regimen was carried out by assessing both humoral and cellular immune responses. ELISA was used to evaluate humoral immunity, namely total IgG, as well as IgG1 and IgG2c subclasses. The cellular immune response was evaluated by quantifying the rate of IFN- γ producing splenocyte clones used ELISpot. In addition, characterization of the cellular response was carried out by intracellular staining (ICS) to identify of the rate of IFN- γ and TNF- α producing TCD4+ lymphocytes, as well as the proportion of TCD8+ lymphocytes. Determination of viral load in the main target organs was done by RT-PCR (lungs, heart, and brain). Virus replication capacity was also evaluated in target organs tissues. In vitro assays were performed out to determine the levels of neutralizing antibodies against SARS-CoV-2 virus.

Results. The results show 100% protection of vaccinated animals in terms of symptomatology, animal weight, level of neutralizing antibodies against the virus and the rate of IFN- γ and TNF- α producing splenocyte clones. The analysis of IgG subclasses shows a predominance of IgG2c over IgG1, indicating the activation of a specific and cytotoxic Th1 protective cellular immune response and immunological memory. Finally, a reduction of viral load has been observed in vaccinated animals, with a clear reduction of virus replication in the main target organs. Furthermore, there is a synergistic effect increasing protection using the two plasmids p-PALSfs + pPAL-structural protein (under patent).

Conclusions. The DNA vaccine pPAL-Sfs + pPAL-structural protein is fully protective in the mouse model in terms of maintenance of body weight, absence of significant clinical signs, viral load clearance in target organs and immune response. The immune response included neutralizing antibodies, predominance of IgG2c over IgG1 ratio, a Th1 response, and a multifunctional cytotoxic cellular response.

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N°28

Attenuation of SARS-CoV-2 derived RNA replicons for the development of safe and effective vaccine candidates

Isabel Sola¹; Melissa Bello-Perez¹; Jesus Hurtado-Tamayo¹; Ricardo Requena-Platek¹; Diego Muñoz-Santos¹; Jorge Ripoll-Gómez¹; Ana Esteban¹; Pedro J Sánchez-Cordón²; Sonia Zuñiga¹; Luis Enjuanes¹

Coronavirus RNA replicons are promising candidates for next generation vaccines against human coronaviruses (CoV) with pandemic potential. Self-amplification of RNAs in host cells generates more potent immune responses with significantly reduced amounts of RNA delivered. Propagation deficiency makes them highly safe as vaccines. Another essential requirement for the design of CoV-derived RNA replicons is the identification and deletion of virus virulence genes to prevent pathogenic responses in vaccinated individuals. Moreover, the characterization of their mechanisms of virulence provides information for the rational design of antivirals.

Coronaviruses encode a variable number of accessory genes that are not essential for virus replication, but are involved in the regulation of virus-host interactions and in *in vivo* virulence. We have provided a proof of concept for the development of safe and highly effective RNA replicon vaccines against deadly human CoVs using MERS-CoV replication-competent propagation-defective RNA replicons. Deletion of MERS-CoV accessory proteins 3, 4a, 4b and 5 attenuated the RNA replicon in a mouse model of infection. Protein 4b was demonstrated to be a virulence factor *in vivo* and its deletion from the viral genome completely attenuated MERS-CoV in a humanized mouse model, resulting in no mortality. Attenuation in the absence of 4b was associated with a significant reduction in lung pathology and chemokine expression levels, indicating that 4b contributed to the induction of lung inflammatory pathology. 4b protein regulated autophagy in the lungs of mice, leading to upregulation of BECN1, ATG3 and LC3A mRNA. Autophagy activation in the absence of 4b correlated with downregulation of a pathogenic inflammatory response, thus contributing to attenuation of MERS-CoV-Δ4b.

We are currently developing replication-competent propagation-defective RNA replicons derived from SARS-CoV-2 as safe and more efficient vaccine candidates, which induce mucosal sterilizing immunity against SARS-CoV-2 infection by intranasal immunization. The combined deletion of four accessory genes 6, 7a, 7b, 8 significantly attenuated SARS-CoV-2, resulting in a reduction of lung inflammation and 80% survival of infected mice. Attenuation was associated with a decrease of IFN-β and pro-inflammatory responses in the lungs over infection, as compared to WT virus. Gene 8 was a main determinant of SARS-CoV-2 pathogenesis. Deletion of gene 8 significantly increased the interferon (IFN-β) and pro-inflammatory responses in the lungs of mice at early times post infection. These results suggested that early IFN and pro-inflammatory responses were protective *in vivo*, while delayed innate immune responses contributed to SARS-CoV-2 virulence.

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N°29

Oligosaccharide-based nucleic acid delivery for next-generation gene therapy

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Background. Viruses cunningly achieve their own transport and target infection by subverting the cellular structure of the host cell and its signal transduction pathways. Infection and propagation cycles are then accomplished through concerted multistep processes encompassing hierarchical self-assembly, defined tropism, specific cellular binding and uptake and opportune uncoating. All of these stages are tightly synchronized in time and space. Nonviral vectors (such as cationic lipids and polymers) exhibit superior safety and biocompatibility, but have enhanced difficulty in overcoming biological barriers while keeping the virus's amazing infectivity potential. In the last years, a variety of intelligent supramolecular systems overlaying different levels of organizations and environmental stimuli responsiveness have been proposed to overcome this critical bottleneck [1]. A residual limitation is the polydisperse character of the vector components: differently from virus capsids, which are made of one or a few perfectly defined proteins, conformationally indefinite constituents and/or polymeric materials are most often found in artificial virus emulators. Nanosized molecular entities exhibiting persistent shape and volume (molecular nanoparticles; MNPs) represent an interesting alternative. MNP platforms enable the preorganization of functional elements to encode precise supramolecular behaviors, the generation of libraries of discrete architectures and the implementation of structure/activity relationship studies, offering unprecedented opportunities for vector optimization [2].

Methods. Oligosaccharides are privileged MNP scaffolds for vector prototyping given their inherent biocompatibility and low toxicity. They offer a well-defined pattern of reactive centers dictated by configurational and conformational bias and can be addressed through precision chemistry strategies. We have developed MNP vectors for nucleic acid delivery using three paradigmatic carbohydrate cores: cyclodextrins (CDs), cyclotrehalans (CTs) and α,α' -trehalose [3]. Emphasis is placed in the opportunities to encrypt topological information at the molecular level that translates into defined internal orders and topologies (sizes and shapes) at the nanoscale, upon co-assembly with the nucleic acid cargo, which further results in distinct cell selectivities and in vivo tropisms.

Results. Instilling segregated cationic and lipophilic domains with precise dispositions into an oligosaccharide scaffold allows engineering amphiphilic MNPs leveraging directional interactions that emulate those controlling self-assembling processes in viral capsids. Sequential levels of stimuli responsiveness can further be implemented by combining computational design, selective chemistry and programmed host-guest interactions.

Conclusions. The data spotlight the advantage of (macro)molecular diversity-oriented strategies compatible with strict control over the structural parameters to modulate the self- and co-assembling behaviors. The remarkable organ selectivity differences observed within diverse series of prototypes emphasize the compelling effect of both the vector architecture and the nanocomplex topology on the biological activity. Taking together, the ensemble of results inform a disruptive technology uniquely suited for nonviral gene therapy applications, including vaccines.

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Nº30

The sweet taste of mRNA delivery

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Introduction. The approval of the first two mRNA-based vaccines against COVID-19 virus by the regulatory agencies in 2021 and their worldwide commercialization marked the beginning of a new era in the Pharmaceutical Industry. Their development in less than a year could only be achieved thanks to disruptive technological advances in the mRNA field [1] in combination with the use of lipid-nanoparticle (LNP) delivery systems, previously clinically validated with the approval of ONPATTRO® [2]. However, to fully harvest all mRNA nanomedicine potential therapeutic interventions, purpose-customized carriers and tailored formulations need to be developed. BioNTech entered a research collaboration with the group of Jose Manuel Garcia Fernández at IIQ-CSIC and Carmen Ortiz Mellet at University of Seville, in order to explore the potential of carbohydrates to fully develop novel tailored carriers. In this work, an original molecular vector platform based on oligosaccharides (paOs) was investigated and optimized for mRNA vaccination. In contrast with previously investigated polycationic amphiphilic cyclodextrins (paCDs) (3), that poorly deliver mRNA, the use of a linear oligosaccharide played a key role in the activity. Further, the impact of the cationic head in the self-assembly process and the biological performance of these systems was investigated.

Methods. Candyplexes (CPLXes) were assembled from modRNA and different paOs variations. Characterization of the delivery vehicles included mRNA content, hydrodynamic diameter, binding affinity and size. Further structural and morphological information was obtained from SAXS. Biological activity was first evaluated in vitro and then in vivo.

Results. The amphiphilic character of paOs, comprising segregated cationic and lipophilic domains built onto a linear oligosaccharide core, was proven to be extremely privileged for mRNA delivery. Upon formulation with the RNA, paOs self-assembled to globular nanoparticles where a distinct oligolamellar internal organization could be revealed from X-ray scattering measurements. It was found that certain combinations of ionizable cationic elements and H-bonding moieties in the structure of the paOs lead to unprecedented strong protein translation and immune response. On this basis, a lead-candidate was identified with outstanding performance and excellent vaccination potential that was comparable or even outperforming LNP benchmarks.

Conclusions. Our results clearly demonstrate that paOs are a powerful platform for mRNA delivery, orthogonal to the current “gold standard” LNP technology. The relative dispositions of the cationic or lipophilic domains over the linear oligosaccharide scaffold allows sublime control over the supramolecular arrangement of the complexes with mRNA and thus the transfection efficiency. In summary, paOs technology enables elaboration of RNA nanomedicines for vaccination, as well as a wide variety of further therapeutic interventions.

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Nº31

Artificial Intelligence to evaluate the impact of COVID-19 disease on the elderly

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Background. Artificial intelligence and, particularly, machine learning provide us with algorithms that are specifically programmed to autonomously learn from features, patterns and correlations in data. Machine learning algorithms are designed to be able to predict, classify or cluster future data that has not been previously seen by the model, along with several other capabilities. Branyas project applies artificial intelligence techniques to detect and evaluate individual risks of COVID-19 disease. The ultimate goal is to establish risk profiles associated with COVID-19 in the elderly and, if possible, expand them to the entire population.

Methods. Machine learning is a branch of artificial intelligence which uses data to train statistical algorithms to solve certain tasks, for example, to perform classifications or predictions. Branyas project works with multiple data sources to characterize elderly residents as accurately as possible. Thus, sociological, demographic, biological, immunological, medical, genome-wide association studies (GWAS) and epigenome-wide association studies (EWAS) data from the residents are being collected to provide a complete risk profile, discovering factors and correlations that may have remained hidden in small-scale studies. Our research with machine learning methods is currently focused on two main branches, immunology and gut microbiota. Specifically, we want to provide explanations and interpretability to the results that are obtained within these substudies, and to relate them with the global dataset that is being collected in Branyas project.

Results. Artificial intelligence methods are being applied to different immunology experiments performed in the Centro de Biología Molecular Severo Ochoa (CBMSO), as well as to the gut microbiota analysis carried out in the Instituto de Investigación en Ciencias de la Alimentación (CIAL). We aim to discover which immunity variables play a key role in SARS-CoV-2 infection and to study the immune response induced by SARS-CoV-2 vaccines. The results will determine which variables, within the demographic, GWAS and EWAS data, immunology experiments and gut microbiota analysis play key roles in the COVID-19 disease, in order to finally provide individual risk profiles.

Conclusions. Artificial intelligence tools applied in Branyas project are intended to shed light on risk factors and hidden correlations between the wide range of variables that are being gathered. As a result of the different separate analyses carried out with machine learning models, we will be able to identify among all the variables, the ones which are most involved in SARS-CoV-2 infection and, ultimately, prevention.

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Nº32

Longitudinal study of the immune response after SARS-CoV-2 infection and vaccination in elderly care homes

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Background. The correlates of immune protection against severe COVID-19 in the elderly remain undescribed. Here we report a longitudinal follow-up study based on the evaluation of the different arms of the immune response (innate and adaptive, humoral and cellular) in the Branyas cohort of aged individuals from care homes, who were vaccinated against SARS-CoV-2, while monitoring for new SARS-CoV-2 infections and COVID-19 severity. Our goal is the identification of the correlates mediating immune protection in the vaccinated elderly and the establishment of risk profiles to identify vulnerable individuals.

Methods. A cohort of 196 individuals (median age 86, 73.5% Females, 98% vaccinated) housed in care homes were monitored for SARS-CoV-2 events since the COVID-19 pandemic outbreak, with the collaboration of the Branyas care home managers and residents. Immune responses were evaluated using plasma, serum and peripheral blood mononuclear cells (PBMC) isolated from fresh samples, collected at different time-points after vaccination. For innate immunity, the phenotype of monocytes and NK cells was analyzed by flow cytometry. Plasma antibodies against N and Mpro viral proteins were detected by ELISA. Anti-SARS-CoV-2 neutralizing antibody titers were determined in serum by flow cytometry using a GFP-expressing S-protein pseudotyped lentivirus. CD4⁺ and CD8⁺ T-cell responses were evaluated from PBMCs stimulated with SARS-CoV-2 peptide pools, soluble proteins and autologous cells infected with recombinant vaccinia viruses (rVACV) expressing S, N or M SARS-CoV-2 proteins. Soluble proteins and peptide pools were used to analyze activation-induced markers (AIM); cytokines and degranulation markers were analyzed following coculture with rVACV infections and peptide pools.

Results. From the onset of the COVID-19 pandemic until May 2021, 32% of the individuals had overcome SARS-CoV-2 infection according to the care homes. Of them, 21% showed undetectable antiN and antiMpro antibodies. Also, we detected 16 new positive individuals who were unnoticed, which account for 20% of the total SARS-CoV-2 cases. Concerning innate immunity, we observed altered percentages of monocyte and NK cell subpopulations and phenotypic changes over time after each vaccine dose. Regarding the neutralizing antibody titers, we observed a strong increase shortly after the third vaccine dose compared to samples extracted around 5 months after the second dose. CD8 responses were best detected using rVACV-infected autologous target cells. Up to 50% of vaccines showed CD8⁺ T-lymphocyte responses against S, which increased to 75% among those who were also infected. AIM assays were better for detecting CD4 T-cell responders. Overall, over 70% of the Branyas uninfected individuals showed cellular responses against S protein after the second dose.

Conclusions. Most of the vaccines developed adaptive immunity against SARS-CoV-2. We performed a thorough study including innate, humoral and cellular immune responses that will allow us to determine correlates of protection against severe COVID-19 and risk profiles in elderly vaccinated individuals. Remarkably, we developed a system based on rVACV that improves the capacity to detect anti-SARS-CoV-2 CD8 T-cells compared to the widely-used peptide pools. This allowed us to detect that a high fraction of the elderly individuals in our Branyas study developed cellular immune responses after vaccination.

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Nº33

Foro Social: Social sciences and humanities contribution in dealing with the Pandemic

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Foro Social comprises 5 humanities and social sciences projects within WP11 in PTI+ Salud Global. The oral submission will present findings from 3 of these projects.

The first **ComplyCOVID** (Designing compliance for COVID-19 public policies; IP Carlos Closa IPP) examines two issues: citizens' compliance with COVID measures and public authorities' design of public policies to contain the spread of the virus. Findings of a survey asking about citizens' reasons for complying and interviews with decision makers asking about the rationale for the measures adopted will be presented.

Secondly, **MADCOVID-CSIC** (Investigación y diseño de material y actividades de divulgación científica sobre COVID-19 destinados a los sectores juveniles de la población Española, IP Mercedes Jiménez, CIB Margarita Salas). MADCOVID is an outreach project aimed at young people concentrating on the aspects of the pandemic ranging from contagion, disease, vaccines, duration of the pandemic, misinformation, fake news, emotional impact. In the talks in schools with teachers and students, emotional management is treated preferentially, through collaboration with a cabinet of psychologists. Youngsters learn to recognize and name emotions, normalizing them and discarding emotional garbage, favoring more emotionally proactive, tolerant and healthy attitudes.

The third project, **RESPONTRUST** – Uncertainty, trust and responsibility. Keys to counteracting disinformation, infodemic and conspiranoia during the COVID-19 pandemic; IP Astrid Wagner, IFS) explores the epistemic and ethical dimension of trust and responsibility in face of the profound uncertainty that COVID-19 health emergency has caused in our society. Infodemic, disinformation and post-truth discourse changed patterns of common sense and rationality and hindered the implementation of measures to contain the virus, provoking instead an increase of conspiranoia, denialism, anti-science and anti-vaccine movements, as well as anti-democratic attitudes.

Research teams

ComplyCOVID: Carlos Closa, IP (IPP); Salvador Parrado (UNED); Marta Fraile (IPP); Roberta Perna (Lleida/UCM); Manuel Pereira Puga (IPP), Alberto Mercado (IPP).

MADCOVID: Mercedes Jiménez; IP (CIB Margarita Salas); Luisa Ma Botella; Nuria Campillo; Ma del Carmen Fernández; Begoña García; Yolanda González; Miguel Ángel Robles; Marta Sobrinos and Alberto Rodríguez (all CIB Margarita Salas-CSIC); Matilde Cañelles (CTS-IFS-CSIC); Valle Palomo (IMDEA Nanociencia); Ángel Cuesta (UCM)

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Nº34

West Nile virus ecology and surveillance after the 2020 outbreak in Andalucía

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West Nile virus is transmitted by several species of mosquitoes, and birds are its main natural reservoir. Most infections in humans are asymptomatic but less than 1% of cases result in severe disease and even death. This virus has been endemic in Spain for almost 20 years, but until 2019 only 6 cases of disease in humans were registered. In 2020 there was a major outbreak that affected areas of Seville, Cádiz and Badajoz, causing 77 serious cases and 8 deaths. In response to this outbreak, we developed an intensive entomological surveillance program that allowed us to evaluate changes in the abundance of mosquitoes, determine which species of mosquito are involved in the amplification of the virus, and how the virus spread from natural areas to the urban areas of several villages.

The results of the entomological samplings were incorporated in real time to the webpage <http://mosquitos.ebd.csic.es>. The genome of the detected viruses confirms that the outbreak was caused by a variant of the virus that have been circulating in Spain for several years. *Culex perexiguus* was the species of mosquito that most contributed to the amplification of the virus, although lower numbers of infected *Culex pipiens* and *Culex modestus* were also detected. The proliferation of *Cx. perexiguus* in the rice fields near the villages probably facilitated the amplification and subsequent introduction of the virus in the urban areas. Virological surveillance in mosquitoes allowed detecting the presence of the virus four weeks before the first human infection was detected in 2021. The significant presence of antibodies in urban birds (e.g. in *Turdus merula* and *Passer domesticus*) confirms that the virus circulated significantly, in urban areas, both in 2020 and 2021. The important mosquito control measures adopted in 2021 probably explain the low number of human cases detected that year and highlight the need to adopt vector surveillance and control programs in areas affected in recent years by this virus. This type of surveillance makes it possible to reinforce and adapt mosquito control programs based on the estimated risk of virus transmission, reducing the possible impact of these viruses on public health. This project shows how the rapid transfer of results to administrations and society can contribute to improving public health and the response to emerging infectious diseases of zoonotic origin.

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Nº35

Monitoring network of biodiversity and ecological processes in mountain National Parks

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The recent pandemic has revealed the importance of monitoring the health of natural ecosystems, as well as the need to make society aware of the role of science as the best way to solve environmental problems, and the interest of involving citizens in extensive monitoring.

The “Natural Patrimony” project aims to promote networked ecological monitoring in 6 mountain National Parks. These places are some of the richest, most pristine and best preserved places on the European continent, especially sensitive to environmental deterioration resulting from global change and climate change in particular. For this purpose, teams from CSIC and Universities have been connected to monitor changes and trends in a variety of indicators based on abiotic and biological variables. Among them we highlight ozone and particulate matter in air, temperature and water composition in the scarce and threatened wetlands, in permanent water bodies (lakes), oscillations in rain precipitation in caves, the volume of the southernmost glacier in Europe, as well as the effect of climate and extreme weather events on the dynamics of micro- and macrophytes, of sensitive amphibians and fish in flowing waters, the productivity of grasslands and forests, habitats and landscapes, and of flora and fauna species. This work is mainly based on the use of new technologies, the installation or reinforcement of sensors for continuous environmental surveillance, and long-term studies based on biological or photographic archives.

On the other hand, some activities are addressed to raise social awareness of the role of science and the rigor with which researchers acquire contrasted information. These include the installation of stands and videos inside the visitor centers, documenting the abundant but often invisible research carried out by scientists. Finally, through the use of apps for the participation of volunteers (Citizen Science), visitors, environmental agents and naturalists are being involved in the quantification of biodiversity, in order to make them aware of its magnitude, so that they feel it as a complex puzzle where each piece counts, and the need to preserve its integrity.

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Nº36

CSIC Antiviral Screening and Chemical Library Platforms

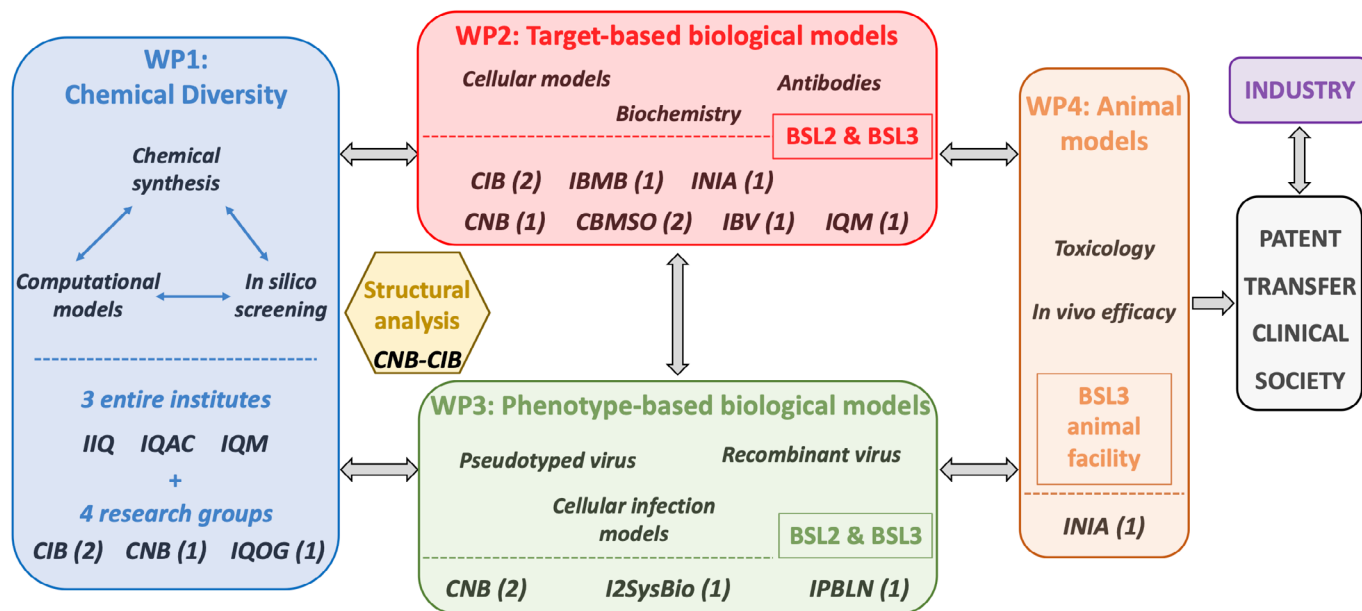
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The COVID-19 pandemic has revealed the need for increasing our preparedness to fight new viral pathogens causing human, animal and environmental problems. In March 2020 and in response to the pandemic, an institutional call to gather and screen compounds as antivirals against SARS-CoV-2 was initiated at the Spanish National Centre for Biotechnology. The response to that call was outstanding and in few months several thousands of compounds belonging to many different scientific groups and chemical synthesis institutes were available for screening. This unprecedented situation created the seed of what is known today as CSIC Antiviral Screening and CSIC Chemical Library Platforms, a collaborative network of chemists, biochemists, computational biologists, cell biologists, virologists and technology transfer specialists, working together. The objective of this initiative is to create and maintain a permanent and transversal structure dedicated to the identification, optimization and characterization of new antiviral compounds against emerging and re-emerging viral pathogens relevant to human health. To do so, researchers have been grouped into four complementary and interconnected working packages covering areas of: chemical diversity, target-based biological models, phenotype-based biological models, and animal models. Two technical transversal units are also part of the initiative. On one hand, the structural analysis unit provides the means for compound structure determination and on the other hand, the CSIC knowledge and technology transfer office gives support on patentability, product development and interaction with industry. A workflow has been established where computational modelling, in silico screening and de novo unbiased chemical synthesis approaches are combined. The need to provide screening platforms with collections of compounds in a standardized way, led to the creation of the CSIC Chemical Library Platform. This platform is currently centralized in three institutes (Instituto de Investigaciones Químicas, Instituto de Química Avanzada de Cataluña and Instituto de Química Médica) and will provide a unique and diverse collection of organic compounds to other researchers for screening of antivirals in in vitro and in cellulo biological models. Once antiviral compounds are identified medicinal chemistry programs for Hit to Lead optimization are conducted between the chemists who developed the Hit and biologists in order to find a drug candidate. This effort has already led to the discovery of several Lead compounds susceptible of Intellectual Property (IP) protection or patentability and whose in vivo toxicology and efficacy testing are being conducted. In our talk we will present the CSIC Chemical Library Platform, the general working model that the Antiviral Platform follows and a specific example of the identification and optimization of a family of compounds with a selective antiviral activity against SARS-CoV-2.

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Nº36

CSIC Antiviral Screening and Chemical Library Platforms



GRAPHICAL ABSTRACT

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N°37

Antiviral activity of synthetic heparan sulfate mimics

Julia Revuelta¹; Alfonso Fernández-Mayoralas¹; Ron Geller²; Miguel A. Martín-Acebes³

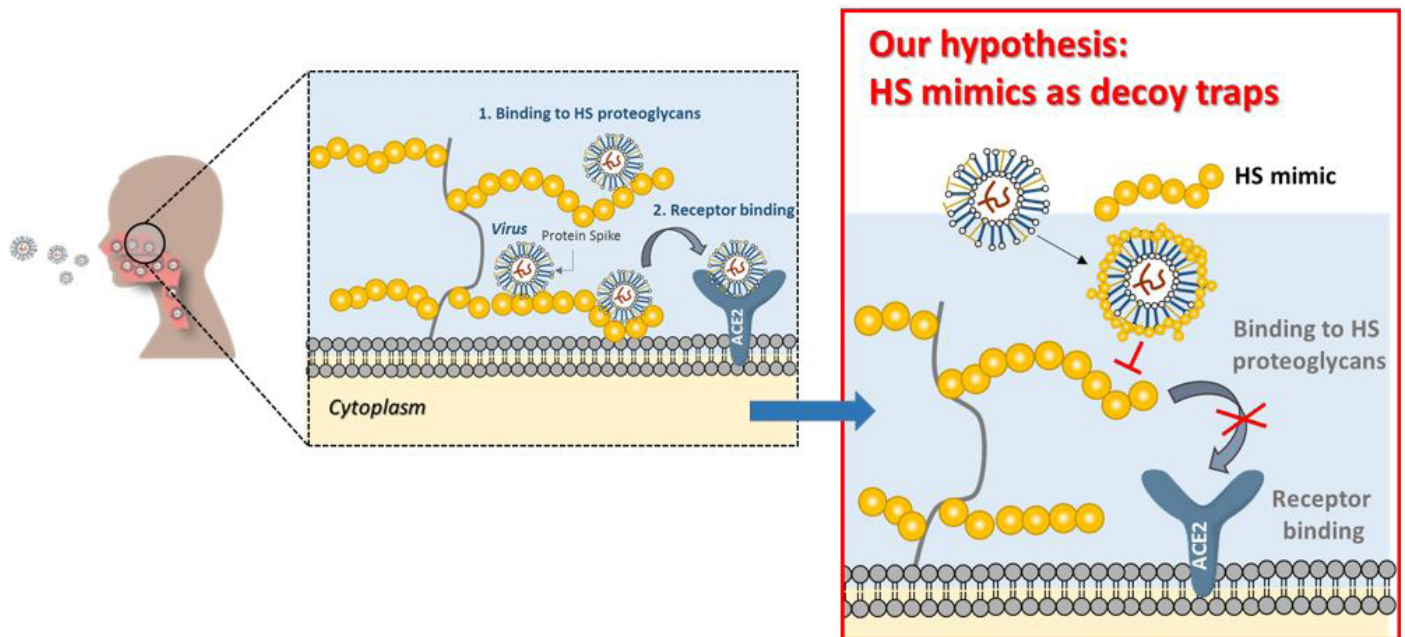
The glycocalyx that surrounds the cell membranes is the first point of contact for many pathogens that infect animal cells. Some viruses from different virus families have evolved to utilize heparan sulfate proteoglycans (HSPG) displayed on the host cell surfaces as the initial interaction with host cells [1]. In the case of SARS-CoV-2, it has been established [2] that the first step for uptake is the interaction of the S-protein of SARS-CoV-2 with HSPG on the cell surface, which is followed by binding of S-protein to the angiotensin converting enzyme 2 (ACE2) (Figure). Inhibition of viral adhesion by exogenous HS or HS mimics is proposed as a promising approach to mitigate viral infection.

In 2018, we reported that chitosan sulfate (ChS), easily obtained from readily available chitosan polysaccharide, can be used as HS mimics [3]. In the context of the COVID-19 pandemic, with the available data on the mechanism of SARS-CoV-2 infection, we decided to evaluate the capacity of ChS to act as decoy traps that interact with the spike protein of the virus and block the binding of the virus to target cells (Figure). We synthesized more than 100 differently functionalized ChS derivatives, with variations in chain length, degree of sulfation and functionalization. In vitro screening to test the capacity of the compounds to inhibit viral infection by SARS-CoV-2 and RSV was carried out. One of the compounds (ET36) was selected to evaluate its toxicity and antiviral effect against SARS-CoV-2 in animals. No adverse effects were observed even when the compound was administered intranasally at the limit of solubility (100 mg/kg) for four daily administration. To test antiviral activity, mice were infected with SARS-CoV-2 and treated with ET36 starting prior to infection (prophylactic) or one day after infection (therapeutic). In all cases, viral loads in lungs harvested three days after infection were significantly reduced in mice treated with ET36 as compared to the control group. Likewise, the expression levels of key pro-inflammatory cytokines in the lung of infected mice were significantly reduced in animals treated with ET-36 compared with control mice. However, in a survival experiment under prophylactic and therapeutic conditions, the treatment did not protect from lethality.

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Nº37



Figure

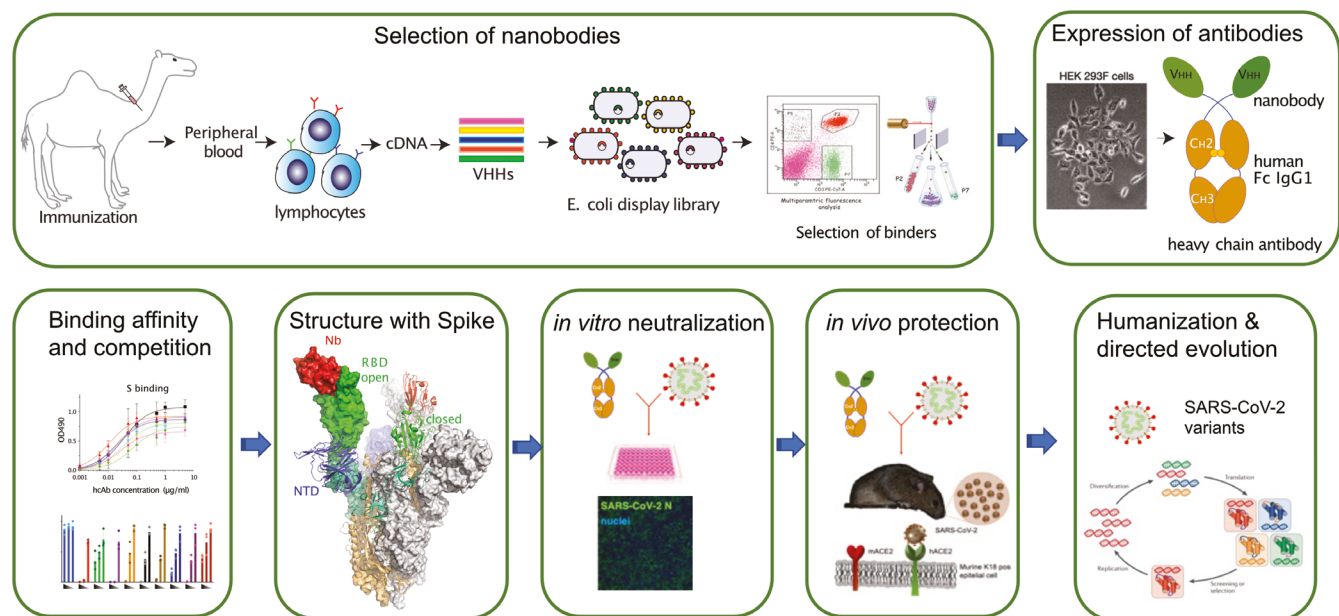
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N°38

Nanobodies protecting mice from lethal SARS-CoV-2: Selection, characterization, humanization and evolution toward escaping virus variants.

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The emergence of SARS-CoV-2 variants that escape from immune neutralization are challenging vaccines and antibodies developed to stop the COVID-19 pandemic. Thus, it is important to establish therapeutics directed toward multiple or specific SARS-CoV-2 variants. We selected a panel of camel nanobodies (Nbs) and engineered heavy chain antibodies (hcAbs) with human IgG1 Fc domain that targeted the receptor-binding domain (RBD) of the envelope SARS-CoV-2 spike (S). These hcAbs bound to S with very high affinities and some inhibited virus cell entry, neutralized SARS-CoV-2 infection *in vitro* and prevented the progression to lethal COVID-19 in infected humanized mice. S-binding cross-competition and structures of S-Nb complexes identified two distinct Nb-binding regions, which either covered or laid outside receptor binding motifs. We also evaluated mono and bispecific hcAb binding to the RBD of SARS-CoV-2 variants, identified variant-specific and molecules that recognized all virus variants of concern but omicron. Our current research efforts focus on directed evolution of Nbs for binding escaping variants of concern like omicron. In addition, more potent neutralizing Nbs have been fully humanized and are being tested *in vitro* and *in vivo*. Our work demonstrated the high potential of Nbs as therapeutics to prevent SARS-CoV-2 infections.



GRAPHICAL ABSTRACT

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Nº39

Scipion-Chem: a traversal tool for the development of antiviral drugs

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Background: Virtual Drug Screening (VDS) is a powerful and efficient tool to identify in an efficient way new antiviral drugs from chemical libraries and a detailed knowledge of the structure of the target. VDS allows to save time and money since the experimental work is only focused on the most promising ligands. In this communication, we present a computational platform for virtual drug screening that combines several widely used software suites for this purpose.

Methods: Scipion-chem: Scipion is a workflow engine [1] particularly well-suited for structural studies of biological macromolecules (Fig. 1 top). It includes structure determination by single-particle analysis in CryoEM, atomic modelling, and now, VDS using a library of candidate compounds. We have integrated the most common programs used in the field for this task: Schrödinger, AutoDock4, AutoDock Vina, P2Rank, FPocket, OpenBabel, RDKit, Amber, and Rosetta (Fig. 1 bottom). We have also integrated some tools to design drugs based on ligand properties.

Results: Scipion-chem: We show that by combining the output of various programs we are able to reduce the computational requirements in the VDS and the number of compounds to be experimentally tested in the wet laboratory.

Conclusions: In this abstract, we show how this computational tool, Scipion-chem, can be beneficial for the design of antiviral drugs.

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Nº39

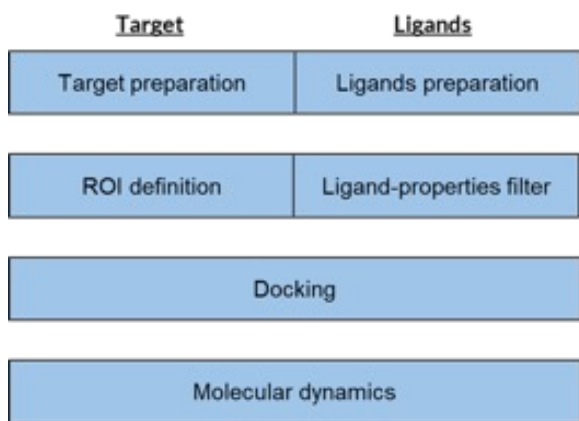
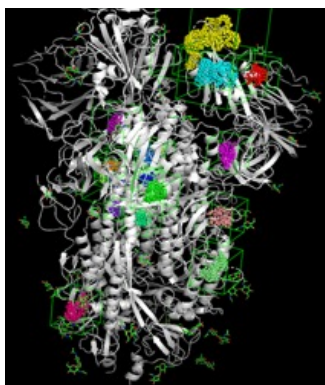


Fig. 1: (Top) Example of the structural analysis of SARS-CoV2 Protein S, in particular, its known variants are represented in the structure. These variants have been collected from Uniprot using Scipion-Chem. (Bottom) Schematic representation of the different steps needed for computational drug screening and some of the programs integrated for each step.

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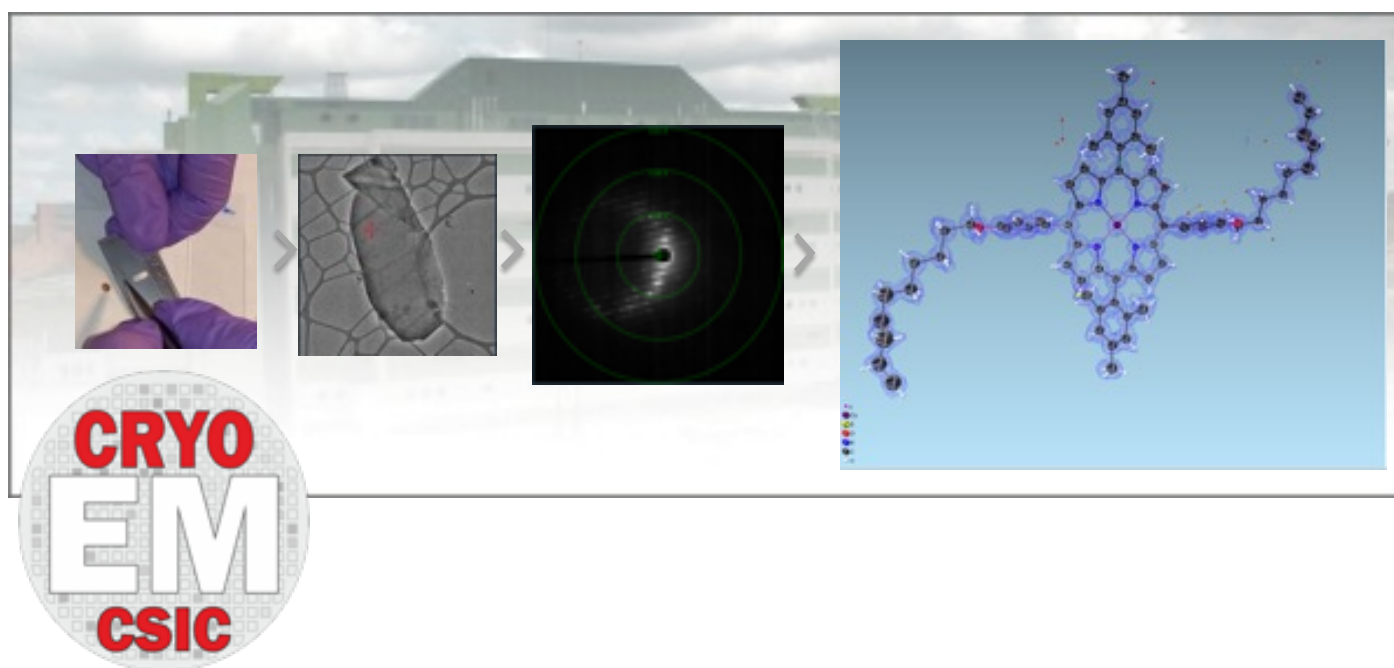
Nº40

Microelectron diffraction implementation at the CNB cryoelectron microscopy facility

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Cryoelectron Microscopy (Cryo-EM) has emerged as one of the most powerful tools in the field of structure biology since the advent of the resolution revolution in the field. New software on image analysis and specially the new hardware used for data collection has positioned the technique at the front of the techniques in molecule structure resolution. A variant of Cryo-EM is the microelectron (MicroED) diffraction of nano crystals, in which the transmission electron microscope is used to diffract crystals of the complete range of molecules that could be crystallized: from small compounds to macromolecules. The flexibility and power of this technique allows us to solve the structure of the crystallized molecules with almost no sample used and in a very short period of time.

We have implemented a robust protocol for MicroED at CNB's CryoEM facility that allows us to solve the structure of a varied range of samples from different users. We are convinced that the established workflow would be of great help to assist researchers from a wide range of scientific concerns, in solving molecules crucial in their investigations.



GRAPHICAL ABSTRACT

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Nº41

Cell entry inhibitors for SARS-CoV-2 based on targeting the SPIKE-ACE2 interaction

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Background. The interaction of the SARS-CoV-2 spike (S) protein (and its Receptor Binding Domain, RBD) with the membrane-bound ACE2 receptor is crucial for viral infection. Based on this interaction we used the following strategy to develop viral entry inhibitors: 1) Identification of inhibitory compounds by in silico screening of public drug libraries and a proprietary chemical collection. 2) Chemical synthesis to improve the validated hits. 3) Production of decoys: soluble ACE2 forms thermally stabilized and having enhanced affinity for the virus. 4) Design of a preclinical validation pipeline.

Methods. 1) Pure wild-type and mutant forms of glycosylated soluble ACE2, as well as RBD or S (variants of concern) were produced using baculovirus/insect cells and mammalian cells. 2) Biophysical affinity monitoring: surface plasmon resonance, biolayer interferometry and microscale thermoforesis. Protein stability monitored by thermofluor. 3) Replication-deficient pseudotyped engineered vesicular stomatitis virus expressing the SARS-CoV-2 S protein variants was used to monitor interference with viral cell entry by the decoys or drugs/compounds. 4) Genuine SARS-CoV-2 virus was used in final cell entry inhibition assays under BSL3 confinement. 5) Molecular dynamics simulations for identifying surface pockets in RBD. 6) In silico docking of compounds to these pockets for identifying putative RBD binders. 7) Chemical synthesis to improve in vitro the validated positive hits and to establish Structure/Activity-Relationships (SAR) studies.

Results.

A. We implemented a pipeline for assessing viral entry inhibition by any compound through: 1) production/purification of a panel of RBDs and S-proteins representing most viral variants of concern; 2) in silico screening; 3) chemical synthesis; 4) biophysical testing; and preclinical testing 5) with pseudotyped virus and 6) with genuine SARS-CoV-2 virus; 7) we also implemented a cryoElectron Microscopy protocol for structural determination.

B. In silico screening of 4586 compounds identified seventeen generic used drugs and thirteen own compounds. Eighteen of these compounds were validated as having affinity for RBD (biophysical tests), and four of them were promising enough to reach final assays against genuine SARS-CoV-2.

C. We validated various multivalent tryptophan derivatives as SARS-CoV-2 entry inhibitors with therapeutic potential, and performed SAR studies.

D. Monomeric and dimeric ACE2 decoys with up to eight amino acid substitutions exhibited up to two-order-of-magnitude enhanced affinity for RBD/S-protein, assessed in biophysical binding assays, and showed even higher enhancement in the inhibition of cell entry of pseudotyped and genuine viruses. The affinity and inhibitory potency of the decoys is similar for all important RBD/S protein variants tested. Thanks to the stabilizing mutations, these decoys have reasonable thermal stability. We prepared enzymatically active and inactive decoy variants.

Conclusion. This collaborative consortium of four CSIC groups has allowed generation of the pipeline as a valuable addition to our armamentarium for the discovery of antiviral agents. The patenting and pre-patenting findings of the present project are promising, heralding potential therapeutic use of the developed decoys, compounds and drugs. The structural data provide deep insight into S protein recognition by ACE2 decoys

Author order. Last 8 authors are IPs in alphabetical order. Other authors are in alphabetical order. Their order should have been per sub-work-package:

1.5 JG-M,SM-S.

2.3 Alphabetical Order: AA,GD,ME-A,CE,AF-R,AF-N,FG,NG,RG-R,MdPH-S,AI-C,MLL-R,CM-M,SM,LO-O,ARdC,RR-P,BSdIF,CS-F,LV-B,SZ-C.

Alphabetical Order, IPs:JB,JLL,AM,SR-M,VR.

2.4 MG,OM-M,EQ,MJC,AS-F,MJP-P.

3.3 CF-G,RG.

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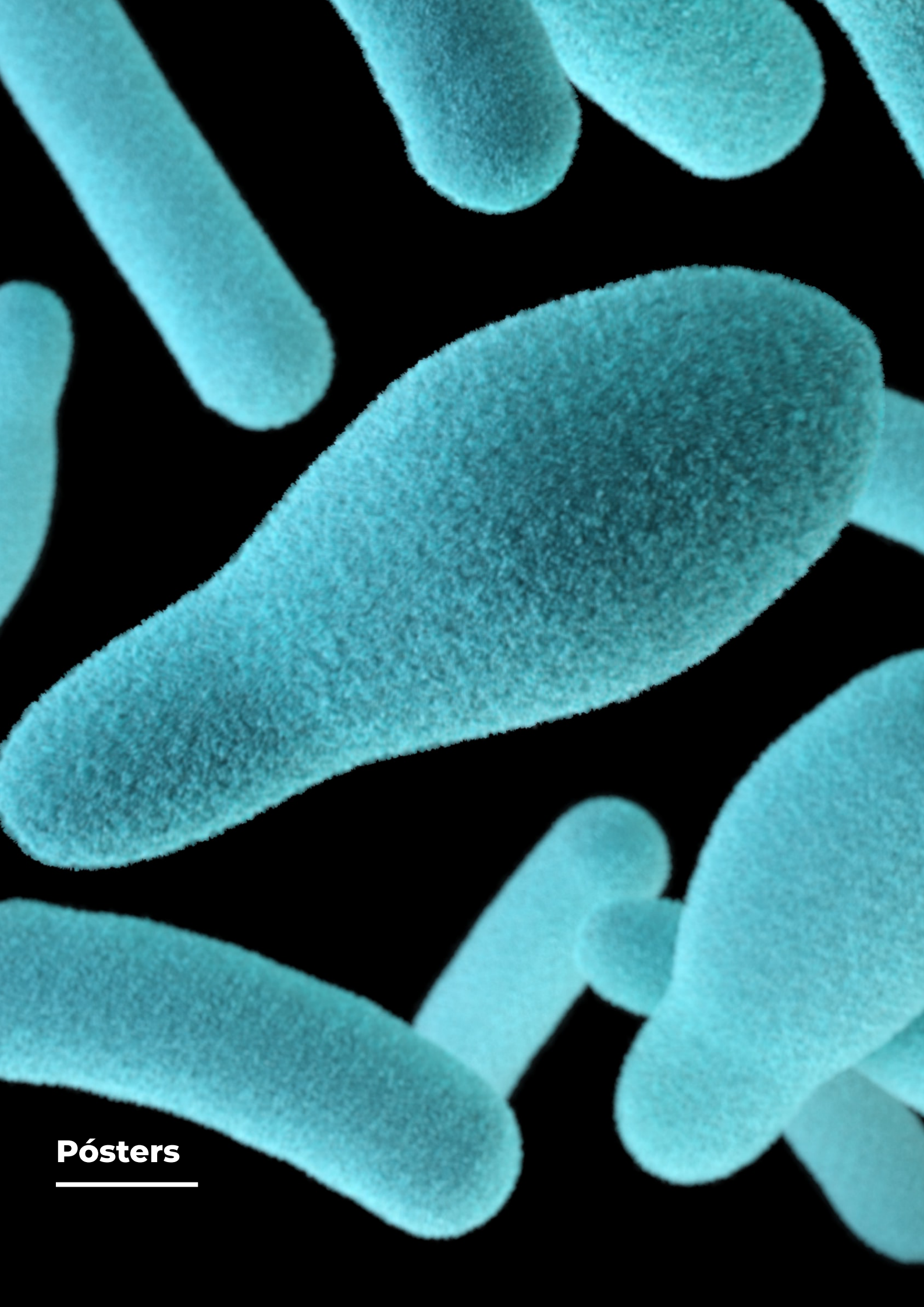
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⁵ Centro de Investigaciones Biológicas



Pósters

Spatial immunization to abate disease spreading in transportation hubs

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Proximity social interactions are fundamental in the transmission of infectious diseases. Crowded agglomerations pose serious risk of triggering superspreading events and, despite mitigation measures, there are occasions where maintaining inter-personal distances can be challenging as, for example, in the transportation hubs. These locations were designed to optimize logistic efficiency, not to reduce crowding, and are characterized by a constant in and out flow of people.

In this work, we consider the paradigmatic example of the busiest European airport by passenger traffic, London Heathrow. Thanks to a dataset of anonymized individuals' trajectories, we are able to model the spreading of different diseases, study the contagion hotspots and to propose a spatial immunization policy targeting such hotspots with disinfection interventions (e.g., surface cleaning, air filtering, UV lights, etc.). By knowing the destinations statistics for each terminal, we are able to detect the most vulnerable destinations to contagions at the airport and quantify the benefits of the spatial immunization technique. This method is immediately generalizable to train, metro and bus stations and to other facilities such as commercial or convention centers.

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Big Data and demographic changes in the different phases of the COVID-19 pandemic: estimation of mobility and population in Spain from cell phone data

Joaquín Osorio Arjona¹, Julia de las Obras-Loscertales Sampérez ¹; Diego Ramiro Fariñas ¹

This work seeks to use cell phone data as an alternative, fast, and high-volume data source to analyze the demographic patterns occurring in Spain during different phases of the pandemic caused by the COVID-19 virus, with the aim of visualizing both mobility flows and population changes occurring in different parts of the country. For that objective, open cell data provided by Spanish National Institute of Statistics (INE) has been used. In comparison with other works, this one offers the mapping of the obtained results during four working days that correspond to four different periods of the COVID-10 pandemic (first state of alarm, “new normality” period, second state of alarm, and normality period after the implementation of vaccines). Also, this investigation uses a spatial scale based on population cells provided by INE, allowing the visualization of mobility and population growth patterns homogeneously in different areas of the territory.

The methodology used has been the elaboration of Origin-Destination matrices to analyze the mobility in Spain during the four days selected for this study, and the calculation of the population of each spatial cell having in account the number of mobile phones entering and leaving the population cells to map the percentage of phones over the population stock. The total population was estimated by weighting the number of phones in each cell with a correction value based on percentages of mobile phone users at provincial level. Then, the percentage of population growth over the official data was calculated. Lastly, the obtained results were validated through mapping of determination coefficients and Ordinary Least Squares residuals values.

The results showed a generalized decrease in both long-distance mobility and estimated population during the first state of alarm. In the subsequent period defined as “new normality”, mobility increased significantly, especially to areas in the north of the peninsula, while the population continued decreasing, especially in the interior of the peninsula. During the second state of alarm, the population growth remained stable but mobility decreased again as a result of the new restrictions established. Finally, in the period after the second state of alarm, an increase in both mobility and population flows was seen particularly in the metropolitan areas of the country. The results obtained are similar to the phenomena observed in reality during the different phases of the pandemic in Spain. In addition, the adjustment of the INE population with the data obtained from mobile phone data has presented high values of coefficients of determination, especially during the alarm phases of the pandemic. Therefore, it can be concluded that cell phone data are a valid and reliable source of data for monitoring and analyzing the continuous changes in demographic and mobility patterns that occur during the different phases of a health crisis.

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What are the vital areas of Spain made up during the COVID-19 pandemic? Elaboration of functional areas using cell phone data

Joaquín Osorio Arjona¹; Sebastián Ruiz Santacruz ¹; Julia de las Obras-Loscertales Sampérez ¹; Diego Ramiro Fariñas¹

This work aims to visualize and identify functional and vital areas in Spain during different phases of the pandemic caused by the COVID-19 virus. Those parts could help to establish zones that serve as areas of action in the event of a new pandemic situation, especially in aspects such as daily mobility and confinement, because the living space beyond the administrative limits is not always known. For that goal, open cell data provided by Spanish National Institute of Statistics (INE) has been used due to value and potential to provide constantly updated information at almost-real time.

This research used population cells from INE as spatial base units. The employed methodology has used Origin-Destination matrices from four working days that correspond to four different periods of the COVID-19 pandemic (first state of alarm, “new normality” period, second state of alarm, and normality period after the implementation of vaccines). The functional areas have been calculated having in account the number of mobile phones belonging to a trip flow between an origin cell and a destination cell. Thus, social network analysis tools were used, in this case algorithms that used the amount of number of cell phones in each OD trip to divide the total area of Spain into modules also called clusters, or communities.

The results showed small functional areas usually coincident with the provincial structure in Spain during the first state of alarm. The small size of the functional areas and the orbit of mobility on the province capitals are indicators that show reduced mobility throughout the country. However, during the “new normality” period after the first state of alarm, big functional areas were obtained, usually at a similar size to Spanish autonomous communities. As result, it can be interpreted there was an increased and active mobility in the country during that time. During the second state of alarm, the size of functional areas was again reduced at a quasi-provincial level, but the size in some areas was considerably larger than during the first state of alarm, indicating a higher degree of mobility. Lastly, during the new normality period after the second state of alarm, the size of functional areas was increased again, but in a lesser degree in some areas of Spain (particularly in Andalusia).

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Mosquito alert: developing a citizen-based early warning system for mosquito-borne diseases

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COVID-19 pandemic has highlighted the importance of strengthened relationship between health authorities and citizens to monitor and contain disease spread. Mosquito Alert shows the potential of citizen science in another field of public health: mosquito-borne diseases. Those diseases are emerging and re-emerging worldwide because of exotic mosquito introductions but the non-scalability and flexibility of traditional surveillance methods limits its monitoring. Mosquito Alert combines digital technologies (smartphones, Internet) to bring together citizens, scientists, managers of public health and environment to fight against disease vector mosquitoes. It combines authoritative data with citizen science methodologies for data quality assessment and modelling, enabling large-scale estimates of mosquito population dynamics and human-mosquito encounters. Data collection is done using the app Mosquito Alert (Android, iOS) that allows citizens to provide geolocalized reports and images of mosquitoes, breeding sites and biting patterns. Mosquito images are mostly independently validated by three entomologists in a private platform (Digital Entomological Laboratory) with a community of 50 experts identifying mosquito species from photos, aided with deep learning methods, allowing a continuous and large-scale data acquisition.

The system fully operates in Spain since 2014, and since 2020 has been escalating as a surveillance tool in Europe. From 2014 to 2021 Mosquito Alert collected 58,844 mosquito reports, 16,948 bite reports, and 12,400 breeding sites, establishing a new paradigm for mosquito surveillance that takes advantage of the existing global mobile network infrastructure and the willingness of citizens to participate in such a cause. During the project life span, Mosquito Alert has demonstrated that citizen science surveillance costs less than traditional surveillance methods and provides early warning information of comparable quality but with larger spatial and temporal coverage.

It has proven to be a useful tool to update the distribution of the tiger mosquito (*Aedes albopictus*) populations in Spain, and has been the primary source for the first discovery of the invasive species *Aedes japonicus*. The system is continuously providing updated evidence on the distribution and densities of disease vector mosquitoes at the micro-scale. The time series of vector data collected since 2015, combined with meteorological, sociodemographic and land use data, is allowing the development of a near real-time early warning system with unprecedented spatial resolution based on citizen science, which can be used by public health actors as a tool to improve surveillance and control programs at different scales. Mosquito Alert is establishing a long-term citizen science culture on mosquito surveillance through a hand-on approach, which aligns with the Vector Control Response outlined by WHO, where community engagement and mobilization are pillars to tackle mosquito-borne diseases globally.

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The many roles of Artificial Intelligence in Mosquito Alert: Automating a Citizen-Based Early Warning System for Mosquito-Borne Diseases

Joan Garriga¹; Daniel Guerrero¹; Roger Eritja²; Isis Sanpera-Calbet³; Alex Richter-Boix³; John Palmer³; Frederic Bartumeus¹

Mosquito Alert is a cooperative citizen science observatory coordinated by different public research institutions. Its main objective is to monitor five mosquito species of most concern in Europe as vectors of global diseases like dengue, Zika or West Nile fever. By means of the app Mosquito Alert citizens provide geolocalized images of mosquitoes and an international team of entomologists inspect the images to check for a possible finding of any of the targeted species. Historically, the inspection of images has been completely man-crafted. The project started in 2014, mainly active in Spain, and focused only on *Aedes albopictus* receiving about 600 reports/year. In 2016, the project included the monitoring of *Aedes aegypti*. Since 2020, Mosquito Alert has scaled to Europe, and expanded the set of targeted species to include *Aedes japonicus*, *Aedes koreicus* and also *Culex* spp receiving around 30,000 reports/year.

Thereafter, the project has been overwhelmed by the growing number of uploaded reports and the manual inspection of images has become a bottleneck for real-time information delivery. Machine learning, and in particular deep learning techniques, have proven successful for the task of image classification and can help reduce the labor-intensive image labelling process. A basic classification system involves three main steps: sorting out off- topic reports from the validation pipeline, cropping the images appropriately, and finally labelling them. Multi-species classification of imbalanced and non-professionally qualified datasets, like the ones contributed by citizens, need strong preprocessing to boost machine learning. Thus, the final labelling step is one important AI challenge in the system. Indeed, the role of machine/deep learning in Mosquito Alert goes beyond the single task of image classification. We also deploy “intelligence” to the system by (i) controlling report flow fluctuations and (ii) rising adjustable, predefined alerts. If input data surges occur the system can switch to an emergency-mode, meaning that we entrust the automated classification of reports with the highest accuracy, and direct the expert’s attention only to a subset of them, the most difficult to classify. Based on stakeholders’ knowledge and risk criteria we can raise alerts for the presence of targeted species in specific areas (for example, an area where it is not yet established) thus, adding to traditional surveillance programs an effective early warning system.

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Zernike3D: A novel approach to study molecular motions from CryoEM data

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Introduction: Understanding how macromolecules change among different three-dimensional structures is very significant as it reveals how proteins and other biological machines behave when performing their function in their environments. The study of these continuous motions (usually known as continuous heterogeneity/flexibility) has been already approached in the past in CryoEM (Dashi et al., 2014; Jin et al., 2014), although there are still many challenges to be solved. Besides, molecular flexibility is recently attracting considerable attention from the CryoEM community, as proved by the many publications appearing in recent months (Chen and Ludtke, 2021; Zhong et al., 2021).

Methods: Although most of the newly developed approaches try to solve the problem using Convolutional Neural Networks, we decided to follow a different perspective. Thanks to a mathematical basis composed of Zernike Polynomials and Spherical Harmonics, we can analyze conformational movements at different frequencies in a simplified and automatic manner, avoiding the need for user supervision as much as possible. Moreover, the Zernike3D approach can be applied to several data types (maps, particles, and atomic structures), translating them to a shared space to merge different heterogeneity studies.

In addition, we proposed a new reconstruction approach called ZART, which considers the structural information of each particle to undo conformational changes during the reconstruction process. In this way, it is possible to correct the motion blur artefacts arising during the reconstruction to increase the resolution of the maps.

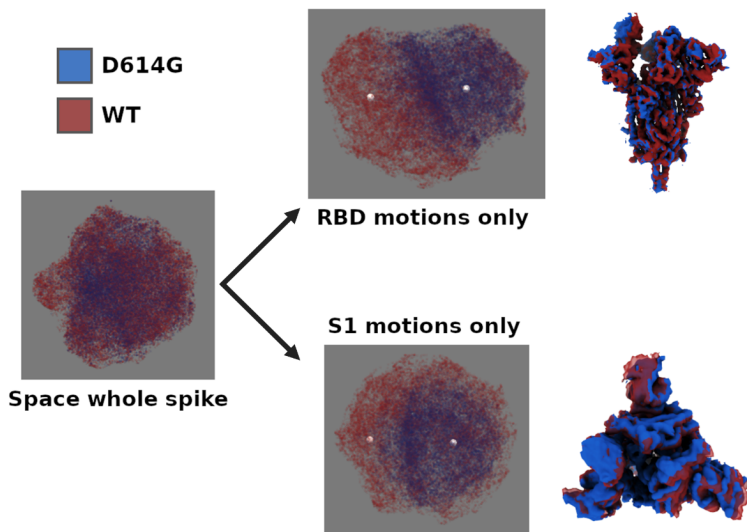
Results: This new tool has been proven to be very versatile in analyzing flexibility in different types of CryoEM data by estimating a deformation field that can be used to reproduce the motion undergone by a molecule when it changes its conformation among different states. The algorithm has been tested on various systems (CCT, ribosomes, COVID-19 spike, and Her2), unveiling the underlying continuous motions by estimating as many conformations as particles, information which can be posteriorly summarized to extract all these structural changes. Furthermore, thanks to the previous analysis, it was possible to complete the structural information obtained through 3D classification, which could be added to the conformational landscape to get more informative results.

Conclusions: The Zernike3D tool, described previously, introduces new and exciting possibilities to better understand continuous heterogeneity from CryoEM data. Thanks to the estimation of per-particle conformation changes, it is possible to define richer conformational landscapes of our molecules, which can be posteriorly analyzed to extract new structural states or improve the resolution of CryoEM maps through the application of our new ZART reconstruction algorithm

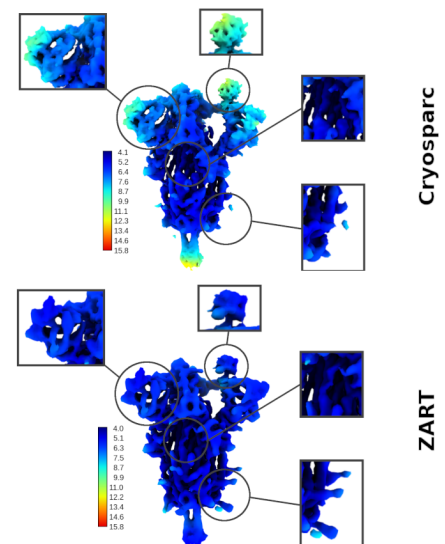
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Flexibility analysis



ZART reconstruction



Graphical Abstract: Zernike³D: A novel approach to study molecular motions from CryoEM data

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Spike mutations and syncytia formation

Marçal Vilar¹; **María Luisa Franco**¹; Ron Geller²

Cell fusion (syncytia formation) has been found in the pneumocytes from the lungs of deceased people infected with SARS-CoV-2. Syncytia form when the spike, S, protein expressed on the surface of infected cells interacts with the ACE2 receptor on neighboring cells. The role of the different mutations in the S protein in the syncytia formation is important to understand the pathological outcomes of present and future SARS-CoV-2 variants. Here we characterized the syncytia formation of the so-called Spanish variant SARS-CoV-2 20E. In the case of the 20E variant, we found that the formation of syncytia is lower than the original Wuhan strain. When analyzed independently the two mutations present in the 20E have different effects on cell fusion. The mutation D614G reduces the formation of syncytial, but the mutation A222V is silent to this property, suggesting that the mutations affect differently to this process. We complete our study comparing the cell fusion properties of the different S protein mutants from the SARS-CoV-2 variants appearing in the different pandemic waves like; alpha, beta, gamma, delta and omicron with the idea to correlate the formation of syncytia with specific mutations and the putative pathological consequences of the different variants.

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Evaluation of the impact on viral assembly of existing and novel mutations on SARS-CoV-2 N protein using a VLP system

Brayan Grau Argente¹ ; Ron Geller ¹

Despite the SARS-CoV-2 acquired knowledge and development of vaccines, the emergence of new variants is constantly threatening the effectivity of available treatments making the ability to predict the effect of mutations on viral replication, spread, and immune-evasion of chief importance. Most efforts to date have focused on the Spike (S) surface glycoprotein due to its clear relevance for vaccine development and antibody responses while other essential viral proteins, such as the nucleocapsid (N) protein, have received less attention, largely due to the lack of suitable systems to study these proteins. Recently, a system has been described that allows for assessing the function of several key proteins in viral assembly and infection. This system is based on the co-expression of the S protein in cell culture together with the nucleocapsid (N), matrix (M), and envelope (E) proteins, which form virus-like particles (VLP) that successfully assemble and bud from transfected cells. Importantly, this system allows for the selective packaging of an RNA (e.g. a reporter RNA for luciferase or GFP) into the VLP, which allows for easy quantification of VLP formation, also by RT-qPCR analysis. By adapting the system, a selective packaging of the SARS-COV-2 N RNA into the VLP can be achieved while that same RNA is translated, along with S, M, and E, to form VLP. This variation enables to perform a deep mutational scanning (DMS) analysis to understand how mutations in different regions of the N protein affect the interaction with RNA and the assembly of the viral particle.

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Bioinformatics analysis of mutations in SARS-CoV-2 and clinical phenotypes

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Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), initially reported in Wuhan (China) has spread worldwide. Like other viruses, SARS-CoV-2 accumulates mutations with each cycle of replication by continuously evolving a viral strain with one or more single nucleotide variants (SNVs).

However, SNVs that cause severe COVID-19 or lead to immune escape or vaccine failure are not well understood. We aim to identify SNVs associated with severe clinical phenotypes.

Methods: In this study, 27429 whole-genome aligned consensus sequences of SARS-CoV-2 were collected from genomic epidemiology of SARS-CoV-2 project in Spain (SeqCOVID) [1]. These samples were obtained from patients who required hospitalization and/or intensive care unit admission (ICU), excluding those registered in the first pandemic wave. Besides, 248 SARS-CoV-2 genomes were isolated from COVID-19 hospitalized patients from Gregorio Marañón General University Hospital (GMH) of which 142 were fully vaccinated. Bioinformatics tools using R and Python programming languages were developed and implemented comparing those to SARS-CoV-2 Wuhan-Hu-1 (reference genome).

Results: Using a selection threshold mutational frequency 10%, 27 SNVs were expected to have association with hospitalization and ICU risk. The reference haplotype differing at the SNV coding for lysine at the residue 203 (N:R203K) was found to have negative association with COVID-19 hospitalization risk ($p = 5.37 \times 10^{-04}$). Similarly, a negative association was observed when the residue at 501 is replaced by tyrosine (S:N501Y) ($p = 1.33 \times 10^{-02}$). The application of a Chi-square test suggested that SNV-haplotypes coding for mutants residues such as (S:A222V, N:A220V, ORF10:V30L) and (ORF1a:T1001I, ORF1a:I2230T, S:N501Y, S:T716S, S:S982A, ORF8:Q27*, N:R203K, N:S235F) have negative associations with COVID-19 hospitalization risk ($p = 6.58 \times 10^{-07}$ and $p = 2.27 \times 10^{-16}$, respectively) and COVID-19 ICU risk ($p = 1.15 \times 10^{-02}$ and $p = 2.51 \times 10^{-02}$, respectively). Focusing on the SNV-haplotype coding the mutations (S:A222V, N:A220V, N:D377Y, ORF10:V30L) were observed to increase the risk of COVID-19 hospitalization ($p = 2.71 \times 10^{-04}$). Results from SARS-CoV-2 genomes analysis from GMH showed 63 coding SNVs which met the established threshold value. Applying a Chi-square test, the SNV-haplotype carrying coding variants for mutant residues in 5 ORF proteins and surface and membrane glycoprotein and nucleocapsid phosphoprotein was significantly associated with vaccine failure in hospitalized COVID-19 patients ($p = 7.91 \times 10^{-04}$).

Conclusions: SNV-haplotypes carrying variants lead to non-synonymous mutations located along SARS-CoV-2 whole-proteome may influence COVID-19 severity and vaccine failure suggesting a functional role in the clinical outcome for COVID-19 patients.

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Analyzing the biophysical properties of the ectodomains from natural SARS-CoV-2 spike variants

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Background: The fast spreading of the SARS-CoV-2 during 2020 required the rapid structural and biophysical characterization of the viral particle, including particularly the spike (S) protein, main player in the infection process. The “artificial” stabilization of the S protein through proline-substitutions of key residues was crucial to solve the structure, to analyze the interactions of the virus with human host cell receptors and even to develop the Pfizer and Moderna vaccines. However, to fully understand the mechanisms governing SARS-CoV-2 infectivity, studying the native S protein variants is a must. Our aim is to produce the ectodomains from natural S variants characterizing and comparing their biophysical properties specially with those of the artificially stabilized S proteins.

Methods:

1. Spike variants constructions were made using fragment cloning strategies.
2. Glycosylated S proteins were expressed in mammalian cells (Expi293) and purified through Ni- affinity standard procedures followed by size-exclusion chromatography.
3. Protein stability was assessed by differential scanning fluorimetry (DSF) and negative staining electron microscopy (NSEM)
4. Detection of conformational populations of S proteins was performed using native red electrophoresis (NRE), DSF, dynamic light scattering (DLS) and NSEM.
5. Determination of the affinity between S proteins and the host cell receptor ACE2 was made using bilayer interferometry (BLI)
6. Monitorization of spike:ACE2 complexes formation through

Results: The first part of the IBV-CSIC pipeline for assessing and understanding SARS-CoV-2 spreading potential and for testing candidate antivirals, is the production and biophysical characterization of the ectodomains from spike variants of concern and other useful variants. The non-proline stabilized variants produced are relatively stable and were obtained in sufficient amounts for analyzing their biophysical and biochemical properties. From our data, the ectodomains from non-artificially- stabilized S protein variants appear to oscillate between at least two conformers, whose respective populations are strongly influenced by temperature. Determination of the binding affinities of different S variants for the ACE2 host receptor led us to detect significant differences in the formation of the spike:ACE2 complex at different temperatures, possibly explained by the temperature-dependent conformational transitions observed in natural S protein variants.

Conclusions: A platform involving 5 different IBV-CSIC groups achieves rapid in vitro production and thorough molecular characterization of spike variants closely resembling these variants in nature: non-proline stabilization, mammalian glycosylation pattern, and even retained furin site in some of them. An unanticipated effect of temperature on binding kinetics of S variants to ACE2 soluble receptor, led us to hypothesize that such notable effect results from temperature-dependent changes in the conformational landscape of the S protein. These results stress the need to characterize natural S protein variants rather than artificially stabilized variants for full understanding of the mechanisms underlying SARS-CoV-2 infectivity. The applicability of this pipeline, exceeds the realm of SARS-CoV-2, being potentially useful for speeding responses to the threat posed by epidemics or even pandemics involving other infectious agents.

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Real-time surveillance of SARS-CoV-2: surveying mixtures of lineage- defining markers

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Background: Genomic surveillance has become a powerful asset to monitor SARS-CoV-2 evolution in real-time and detect the emergence and spread of lineages of concern. In 2021 alone, sudden global takeover by novel variants happened three times, starting with the Alpha variant, then Delta, and lastly Omicron. Interestingly, these three variants belong to different clades that diverged early in the pandemic. In fact, Alpha and Delta only share one substitution on the S gene, which gathers the most diversity, hence the need to understand the generation of variability to tackle the current state and future progression of the pandemic. Despite having a relatively low mutation rate, SARS-CoV-2 genomes acquire, on average, 2 substitutions per month. Additionally, many human betacoronaviruses are suspected to be a result of recombination between other coronaviruses involving the S gene, including MERS-CoV, SARS-CoV and SARS-CoV-2.

Methods: Following the global surveillance efforts, we performed a worldwide survey that revealed the co- occurrence of mutational markers located at the N-terminal end of the S gene from the Alpha variant (B.1.1.7 and Q. lineages) on samples assigned to the Delta variant (B.1.617.2 and AY. lineages). To further investigate these events, we defined sixteen genotypes resulting of the segmentation of the S gene into three sets of lineage-defining substitutions and deletions around the region of interest, plus the rest of the S gene as background –an approach that circumvents some of the issues arising from the low genetic diversity of the virus. Then, we identified each haplotype-matching sample on the global dataset, and assessed the abundance of each genotype, their spatiotemporal distribution, and their phylogenies in their local geographic context. To address the impact of each combination of markers on their fitness, we estimated the minimum number of independent emergences and their implication in transmission events. Due to the sheer number of available samples, two approaches were applied to this end: a reduction strategy based on k-mer clustering, and a phylogenetic placement of the target genomes on a comprehensive reference phylogeny.

Results: The performance of certain mixed genotypes does not fit neutrality when considering their relative abundance, the expected emergence rate, and genetic distance. Strikingly, some combinations of mutations have never been recorded. However, we have identified one genotype whose combination of markers is associated with an increased success. Both strategies for estimating the success of each group converge in their results. Regarding their mechanism of emergence, evidence suggests at least two independent recombination events between the Alpha and Delta variants as the most plausible explanation, although verification is challenging due a poor phylogenetic signal.

Conclusions: We have identified thousands of viral samples displaying a mixture of Alpha and Delta variant- defining genetic markers on their Spike N-terminal end. The lack of some of the mixtures point to strong epistatic interactions between said markers. The most abundant genotypes have emerged multiple times, and been implicated in transmission events, with one of them clearly outperforming the rest. Our framework is ready to be re-implemented for any other context, genomic region, or variants of interest.

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Co-evolution of the SARS-CoV-2 genome and sequencing approaches in the PTI+ Global Health Genomic Surveillance Platform

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Since 2020, the PTI+ Global Health Genomic Surveillance Platform has received thousands of samples that have made it possible to track the pandemic. Genome-wide monitoring of the SARS-CoV-2 sequence has enabled us to see the phylogenetic evolution and geographic transmission of the virus in real time. In this way, it has been possible to characterize lineages of the coronavirus as it has evolved. Some of these have been of particular relevance and have therefore been classified as variants of concern. In the same way, the platform has adapted to the evolution of the virus by implementing new sequencing and bioinformatics analysis protocols.

This adaptation has taken place at two levels. On the one hand, we have studied over time the detection limit of the genomic sequencing technique used in the laboratory (based on a panel of amplification primers known as ARTIC). On the other hand, this same panel has had to adapt as the virus has evolved due to the appearance of some variations in its target sequences that compromise its specificity to bind and amplify the viral target regions. Moreover, this loss of specificity directly interferes with the quality of the sequencing and promotes errors in the identification of genomic variants. For all these reasons, since the pandemic began in 2020, the sequencing technique has been adapted with refining the ARTIC panel primers used from its first appearance, designed on the basis of the Wuhan-01 reference genome sequence, to the current V4.1 version; solving the problems of characterization of new variants appearance such as Delta or Omicron at all times. Around 35000 clinical samples and 350 non-clinical samples, sewage waters mostly, have been analyzed since 2020. New primer panels incorporation has allowed the identification of the different variants as they appeared. Limit detection was initially set at samples with Ct values <33 in clinical samples for whole genome sequencing and has gradually been refined and improved somewhat by the incorporation of the new primer panels. The aim of this study is to 1. study the detection limit of genomic sequencing to determine which samples can be sequenced and which are not; 2. compare the quality of the sequences and the detection limits of the variants using different versions of the ARTIC primers throughout the different waves; and 3. present the Genomic Surveillance Platform available to PTI+ Global Health members and CSIC in general.

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SARS-CoV2 genomic statistical analysis to study hospitalization and vaccine failure

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Background: The study of the genome properties of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a very relevant topic to provide a better understanding of the evolution of the pandemic. The availability of sequenced genomes of SARS-CoV-2, including its different mutations, has led to an attempt to characterize the mutations that may be associated with infection severity or vaccine failure. Most of the existing research is centered on individual mutations, with a minor focus on models that account for interactions between mutations.

Methods: We developed a framework for processing and studying genomic data to characterize the effect of individual and pairs of mutations in the spike protein on SARS-CoV-2 infection severity and vaccine failure. To this end, we employed different datasets with the relevant information available: we used over 25.000 sequences obtained from patients with information about the acuteness of the infection (hospitalization, ICU admission, and death of the patient). On the other hand, we studied the prevalence of certain mutations or pairs of mutations depending on the vaccination status of almost 5.000 patients using their respective SARS-CoV-2 sequences. These analyses were conducted fitting a hierarchical group Lasso model that allows for the presence of individual effects and pairwise interaction terms. In addition, individual studies of the relevant mutations and interactions were conducted using propensity score matching to control for possible confounding factors.

Results: In both cases, we identified some of the most prevalent individual positions already present in the literature, which serves as a good benchmark. Moreover, we found other relevant individual mutations, as well as significant interaction effects between them, which sometimes play a more important role in the model than previously studied individual mutations. We constructed a graph representing the pairwise interactions selected by the model that provides insight into the disjoint community structure for important mutations. Finally, through a detailed individual study, we extracted further information about the relevance of the selected mutations and interactions, which are of major importance in vaccine failure studies.

Conclusions: Our model allows for the identification and characterization of novel individual mutations and interaction terms that are associated with infection severity and breakthrough infection. The results show different structures of important mutations and interactions in the SARS-CoV-2 spike protein. These have not been reported in the literature yet and, in some cases, have stronger effects on the final outcome for the patient than the individual mutations.

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SARS-CoV-2 infectivity is effectively treated and prevented using a combination of MEK and p38 inhibitors

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Keywords: SARS-CoV-2, COVID-19, MAPKi, MEKi, p38i.

Background: The recent emergence of the new pathogen SARS-CoV-2 (COVID19) virus in China and its rapid pandemic spread, posed an unprecedented health emergency that demanded an urgent response. In the absence of specific treatments, the current therapeutic arsenal offers a plethora of drugs, approved for other uses, amenable for repurposing against COVID-19, based on their mechanisms of action. Previous findings have demonstrated that the signaling pathways mediated by ERK and p38 Mitogen-Activated Protein Kinases (MAPK) are involved in COVID-19 multiplication at different stages. As such, inhibitors targeting these pathways block COVID-19 replication. Moreover, p38 inhibitors have also proven effective against the inflammatory response elicited by COVID-19 infection. The MEK inhibitors (MEKi) Trametinib/Selumetinib/Cobimetinib are FDA/EMA-approved drugs for metastatic melanoma treatment; whereas the p38 inhibitor Losmapimod is currently in phase II trials for chronic obstructive pulmonary disease. Herein, we show a drug class-effect MEK and p38 inhibitors in suppressing SARS-CoV-2- virus infection of human cells. We aim at establishing these MAPK inhibitors' potential as antivirals in comparison to the most effective therapeutics currently utilized in the clinic against COVID-19, namely: chloroquine, the antiretroviral Remdesivir, and the IL6 inhibitor Tocilizumab.

Methodology: SARS-CoV2 is classified as a Level 3 risk pathogen, all procedures involving viruses, including infection and lysis before RNA extraction, were carried out in our physical containment level 3 (BSL-3) laboratory at IBBTEC (UNE 171400-1).

Vero E6 (monkey, kidney epithelial); cancerous A549 (human, lung epithelial) and H1299 (human, lung epithelial) cells were infected at MOIs 0.5-2. At 24h, 48h, 72h, and 96h after treatment, cells were harvested for RNA analysis. We also performed 3D-organoids, which present many advantages over conventional cell cultures, such as complex cytoarchitecture and a more physiological microenvironment.

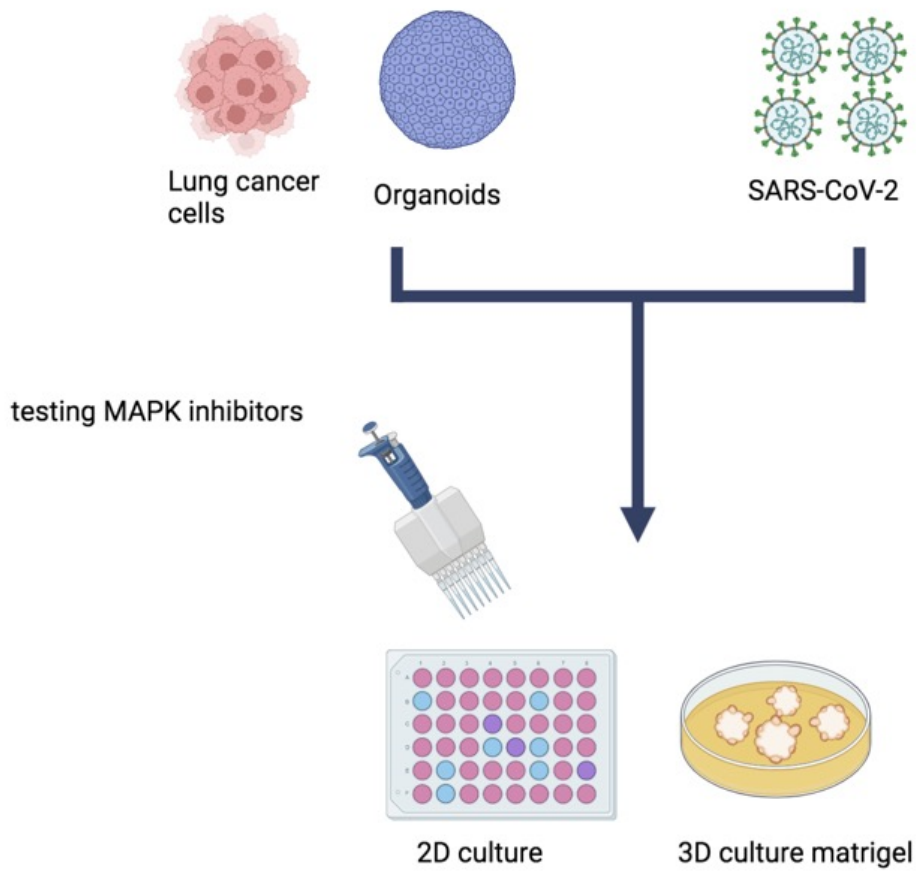
Cells were observed for SARS-CoV-2 specific cytopathic effects (CPEs). Cellular and supernatant viral RNA were purified using an RNA Pure Kit (Roche Diagnostics) and quantified by real-time PCR to amplify an 81-bp fragment of the nucleoprotein gene, using ABI-7000 Prism (Applied Biosystems) with specific primers.

MAPK activation status was evaluated by western blotting using monoclonal antibodies specific for phospho-p38; p38; phospho-ERK and ERK, from Cell signaling.

Results: First, we evaluated the cytotoxicity of the stated drugs by performing a dose-response assessment of their effects on cell viability. Then, we tested non-toxic doses, Trametinib (1 μ M), Losmapimod (5 μ M), Remdesivir (2 μ M) and Hydroxychloroquine (5 μ M) to uncouple the observed molecular effects on infectivity from the direct cell-killing effects of the drugs on target cells.

Our results show that therapeutic and prophylactic administration of Trametinib and Losmapimod in combination markedly inhibited SARS-CoV-2 replication *in vivo*, and thus has considerable potential for COVID-19 treatment and prevention. Moreover, we observed that SARS-CoV-2 infection of human bronchial epithelial cells provide an experimental model to discover or test therapeutics with potential to block coronavirus infection.

Conclusions: Our results support the idea that MEK and p38 inhibitors could be tested in the clinic to suppress early COVID-19 infection and, when used in combination, they may lessen viral infection spread.



Graphical Abstract: SARS-CoV-2 infectivity is effectively treated and prevented by combining MEK and p38 inhibitors

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Recovery of serum testosterone levels is an accurate predictor of survival from COVID-19 in male patients

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Background: SARS-CoV-2 infection portends a broad range of outcomes, from a majority of asymptomatic cases to a lethal disease. Robust correlates of severe COVID-19 include old age, male sex, poverty and co-morbidities such as obesity, diabetes and cardiovascular disease. A precise knowledge of the molecular and biological mechanisms that may explain the association of severe disease with male sex is still lacking. Here we analyzed the relationship of serum testosterone levels and the immune cell skewing with disease severity in male COVID-19 patients.

Methods: Biochemical and haematological parameters of admission samples in 497 hospitalized male and female COVID-19 patients, analyzed for associations with outcome and sex. Longitudinal (in- hospital course) analyses of a sub-cohort of 114 male patients, analyzed for associations with out- come. Longitudinal analyses of immune populations by flow cytometry in 24 male patients, studied for associations with outcome.

Results: We have found quantitative differences in biochemical predictors of disease outcome in male vs. female patients. Longitudinal analyses in a subcohort of male COVID-19 patients identified serum testosterone trajectories as the strongest predictor of survival (AUC of ROC = 92.8%, $p < 0.0001$) in these patients among all biochemical parameters studied, including single-point admission serum testosterone values. In lethal cases, longitudinal determinations of serum luteinizing hormone (LH) and androstenedione levels did not follow physiological feedback patterns. Failure to reinstate physiological testosterone levels was associated with evidence of impaired T helper differentiation and augmented circulating classical monocytes.

Conclusions: Recovery or failure to reinstate testosterone levels is strongly associated with survival or death, respectively, from COVID-19 in male patients. Our data suggest an early inhibition of the central LH-androgen biosynthesis axis in a majority of patients, followed by full recovery in survivors or a peripheral failure in lethal cases. These observations are suggestive of a significant role of testosterone status in the immune responses to COVID-19, and warrant future experimental explorations of mechanistic relationships between testosterone status and SARS-CoV-2 infection outcomes, with potential prophylactic or therapeutic implications.

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Persistent SARS-CoV-2 detection by PCR in healthy adults is not associated with an impaired immune response

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Background: Throughout the SARS-CoV-2 pandemic, it has been observed that the duration of viral detection by PCR from the onset of symptoms is highly diverse. There are cases in which viral detection persists for several months, but the clinical meaning and underlying immune response associated with this persistence is still uncertain. Although this has been observed mostly in immunodeficient individuals, there are numerous cases in healthy individuals. Our goal was to analyze antiviral immune responses in a cohort of healthy adults that had a persistent detection of SARS-CoV-2 by PCR in order to identify any immunological impairment that could explain this phenomenon. We focused on the main responses for elimination of infected cells and infectious virus: T-lymphocytes and neutralizing antibodies, respectively.

Methods: Our cohort of infected adults was divided in two groups: control and persistent PCR. Those for which the virus was detected for up to 4 weeks by PCR were assigned to the control group, and those with longer viral detection were defined as persistent PCR. The control group was composed of 10 individuals (70 % female, median age 53), and the persistent PCR group was composed of 21 individuals (57 % female, median age 48). Additional information regarding general health, severity of COVID-19 disease and vaccination against SARS-CoV-2 was collected. Antibody and T-lymphocyte responses were analyzed in serum and peripheral blood mononuclear cells (PBMCs) isolated from fresh blood samples.

CD4⁺ and CD8⁺ T-cell responses were evaluated from PBMCs stimulated with SARS-CoV-2 peptide pools, soluble proteins or autologous cells infected with recombinant vaccinia viruses (rVACV) expressing S, N or M SARS-CoV-2 proteins. Peptide pools and soluble proteins were used to analyze activation-induced markers (AIM); cytokines and degranulation markers were analyzed following co-culture with peptide pools or rVACV infections. Anti-SARS-CoV-2 neutralizing antibody titers were determined in serum by flow cytometry using a GFP-expressing, S-protein pseudotyped lentivirus.

Results: The activation of T lymphocytes, measured in the case of CD8⁺ as the percentage of cells that produce cytokines (IFN γ , TNF and IL-2) after stimulation with the different SARS-CoV-2 antigens, was equivalent for the group of controls and persistent PCR, observing response in all individuals. In the case of CD4⁺ T cells, where expression of activation markers (CD69⁺, CD134⁺ and CD137⁺) was studied after the stimulus, a response of a similar magnitude was equally observed in all individuals.

Concerning neutralizing antibody titers, we observed a tendency towards higher titers in the persistent PCR group. Therefore, no defects were detected in the neutralizing antibody response. Moreover, similar titers were detected when comparing patients who suffered from severe, moderate or mild disease, or had an asymptomatic infection.

Conclusions: COVID-19 patients with persistent viral detection by PCR included in our study have a normal anti-SARS-CoV-2 immune response, regarding CD4⁺ and CD8⁺ T lymphocytes and neutralizing antibodies. Our results indicate that this persistence in healthy people may not be associated with a worse immune response and potentially not with a

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Immune response of mRNA COVID-19 vaccines in elderly adults and patients with lymphoid malignancies.

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COVID-19 affects the population unequally with a higher impact on aged and immunosuppressed people. Hence, we aimed to characterize the immune response triggered by mRNA vaccines in three different cohorts: 25 healthy adults (under 70); 28 institutionalized elderlies (over 70 years); and 48 oncohematologic patients untreated or treated with B-cells antiproliferative drugs (lenalidomide, ibrutinib and rituximab). Peripheral blood mononuclear cells (PBMCs) were obtained pre- vaccination and 3 months after immunization.

Humoral and cellular memory towards SARS-CoV-2 was also determined before and after vaccination, together with the phenotype of the PBMC by spectral cytometry (40-plex spectral cytometry panel) and the plasma cytokine profile (13-plex panel). While healthy adults and institutionalized elderlies developed a serologic response, that was much lower in the hematooncologic cohorts (untreated: <70%; lenalidomide-treated: <90%; ibrutinib-treated: 60%; rituximab-treated: <10% response). Nevertheless, all cohorts (excluding patients treated with ibrutinib) developed a T-cell mediated immune response. T-SNE analysis of the spectral cytometry characterization revealed all cohorts grouped independently of each other (Figure 1). Indeed, while healthy adults, together with untreated and rituximab patients changed their immune profile following vaccination, elderly adults and patients treated with lenalidomide or ibrutinib were not affected by vaccination. Indeed, Post/Pre-vaccination ratio for every immune subset was also analyzed referred to healthy adults revealing that untreated hematologic patients had a significant increase in monocytes, NK, CD4+ and CD4+CD8+ T-cells and T regs coupled with a decrease on ILCs. On the other hand, elderly adults had an increase in NK, and CD8+ T-cells. In addition, the NK subset was reduced on rituximab patients. Cytokine analysis showed significant increased CXCL10 levels in older adults and untreated patients, as well as increased CCL2 in patients treated with rituximab and elderly prior to immunization. No differences were nevertheless observed due vaccination. In summary, here we have shown that these cohorts, especially oncohematologic patients treated with ibrutinib, display a reduced immune response to mRNA COVID vaccines, suggesting that they may be less protected against infection.

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Autoimmune mechanism in long COVID patients

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Background: The new disease called “long-lasting Covid” or “persistent Covid” has been recently defined by the World Health Organization (WHO) as those individuals with Covid-19 who experience symptoms for more than 4 weeks after diagnosis. Moreover, there is a high number of infected people who continue to manifest symptoms, even more than 20 months later. These long-term effects affect systems as diverse as the pulmonary, cardiovascular, osteoarticular, and nervous systems, also having psychological consequences that chronically affect their personal and work life. The presence of these persistent symptoms was not associated with the severity of the acute Covid-19 illness and females were significantly more likely to suffer it. The mechanisms by which these symptoms are produced and maintained are not at all defined, nor can they be similar in the different types of clinical manifestations of persistent Covid. An understanding of this mechanism(s) is the first step in effective treatment of these conditions.

Methods: Human sera were obtained from 4 different cohorts of adults volunteers suffering persistent symptoms compatible with long-COVID, infected people that recovered with no further symptoms and either vaccinated or not in both groups. Soluble ACE2 and angiotensin were measured by ELISA and antibodies IgM, IgA and IgG anti-S by flow cytometry. Autoantibodies against ACE2 and others (anti-IFN, anticytokines) in the serum were measured by ELISA and in saliva by a modified Multiplex test.

Results: Significant differences were detected in the serum levels of ACE2 between groups. Long Covid patients present much lower ACE2 levels (in many of them close to the lower detection limit) than healthy groups and infected but recovered groups. This indicates a long time and continuous alteration of the RAS/angiotensin system in those Long Covid patients. In addition, we detected mostly IgM anti ACE2 autoantibodies in serum of long COVID patients. Although the implications and its relationship with persistent Covid symptomatology are yet to be determined those alteration in ACE2 explain the chronicity of some symptoms.

In saliva samples, in a small preliminary study, individuals with vascular symptoms present anti-ACE2 autoantibodies in saliva while those with respiratory or neurological symptoms do not induce this response, but instead present a greater amount of anti-protein S antibodies of the virus. We are comparing it with the presence of the same antibodies in serum. Vaccinated individuals not exposed to the virus do not seem to induce any type of response in saliva.

Conclusions: Altogether our results indicate the induction of persistent alteration of the RAS/angiotensin II system, mostly ACE2, after viral infection that may explain the continuous presence of symptoms.

In addition, the presence of autoantibodies may lead to continuous inhibition of ACE2 prolonging the durations of those symptoms. More important, it will offer clues to the clinical management, including treatment, for this new disease.

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Assessment of SARS-CoV-2 neutralizing antibody titers in breastmilk from convalescent and vaccinated mothers

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Breastmilk contains antibodies that could protect breastfeeding infants from infections. In this work, we examined if breastmilk contained antibodies that could neutralize SARS-CoV-2 in >100 breast milk samples from women that were either vaccinated (Pfizer, Moderna, or AstraZeneca), infected with SARS-CoV-2, or both infected and vaccinated. The neutralization capacity of these sera was tested using pseudotyped vesicular stomatitis virus carrying either the Wuhan, Delta, or Omicron spike proteins. We find that neutralization correlates positively with levels of IgA in breastmilk. Moreover, natural infection resulted in higher neutralizing titers. Finally, significant differences in the capacity to produce neutralizing antibodies were observed between the mRNA-based vaccines and the AstraZeneca vaccine. Overall, our results indicate that breastmilk from naturally infected women or those vaccinated with mRNA-based vaccines induces SARS-CoV-2 neutralizing antibodies that could potentially provide breastfeeding infants protection from infection.

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Breast Milk and Passive Immunity during the COVID-19 Pandemic: SARS-CoV-2 antibodies after natural infection and vaccination

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Background: Breast milk is a vehicle to transfer bioactive components, among them protective antibodies, from the lactating mother to the neonate. It is considered the gold standard in infant nutrition. In the beginning of the COVID-19 pandemic there were great concerns about a possible mother-to-infant transfer of SARS-CoV-2. When starting vaccination programs, limited data were available on the impact of different COVID-19 vaccine types in lactating women and their safety.

Methods: Two prospective, multicenter longitudinal studies with 60 mothers with SARS-CoV-2 infection and/or who have recovered from COVID-19 and also, 86 mothers vaccinated with mRNA-based vaccines (BNT162b2, mRNA-1273) and adenoviral-vectored vaccines (ChAdOx1 nCoV-19) were collected longitudinally from baseline up to 30 days after the second dose at seven or eight time points (depending on vaccine type).

Breast milk samples were collected and analyzed in order to identify the levels of SARS-CoV-2 RBD-specific IgA, IgM and IgG, in both infected women and vaccinated mothers. Furthermore, in COVID-19 women we also assessed the presence of SARS-CoV-2 RNA targeting the N1 region of the nucleocapsid gene and the envelope (E) gene.

Results: After SARS-CoV-2 infection, no SARS-CoV-2 RNA was detected in breast milk samples. 82.9% (58 of 70) of milk samples were positive for at least one of the three antibody isotypes, with 52.9% of these positive for all three Igs. Positivity rate for IgA was relatively stable over time (65.2%– 87.5%), whereas it raised continuously for IgG (from 47.8% for the first 10 days to 87.5% from day 41 up to day 206 post-PCR confirmation).

Vaccination elicited also a strong induction of virus-specific antibodies, mainly observed after the 2nd dose and higher in IgG when compared to COVID-19 infected and/or recovered women. The presence and persistence of specific SARS-CoV-2 antibodies in breast milk were dependent on the vaccine type, with higher IgG and IgA levels in mRNA-based vaccines when compared to the adenovirus-vectored vaccine, and on previous virus exposure. High intra- and inter-variability were observed, being relevant for IgA antibodies. Women with previous COVID-19 increased their IgG antibodies levels after the first dose to a similar level observed in vaccinated women after the second dose.

In conclusion, our work confirms the safety of breastfeeding and highlights the relevance of virus-specific SARS-CoV-2 antibody transfer, both after COVID-19 infection and/or vaccination.

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IL11 involvement in inflammatory and pro-fibrotic alterations via STAT3-WNT5A signaling activation by SARS-CoV-2 accessory proteins

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SARS-CoV-2, the cause of the COVID-19 pandemic, possesses eleven accessory proteins encoded in its genome. Their roles during infection are still not completely understood and several of them have been mutating into the different variants of the virus. WNT5A dysregulation signaling has been implicated in the development of various pathological conditions in humans such as inflammation and fibrosis. Interleukin-6 (IL6) family members induce WNT5A expression in various cell types, highlighting a critical role for WNT5A in immune responses. Expression of Interleukin-11 (IL11), a member of IL6 cytokine family, correlates with the extent of fibrosis and its signaling induced fibroblast activation via TGF β . In this study, A549 were transduced with lentivirus expressing individual viral accessory proteins ORF6, ORF8, ORF9b or ORF9c from SARS-CoV-2 (Wuhan-Hu-1 isolate) and their interaction with cellular responses were analyzed. Firstly, transcriptomic analysis revealed that both WNT5A and IL11 were significantly up-regulated in all transduced cells. Some IL11 signaling-related genes, such as STAT3 or TGF β , were differentially expressed. IPA software analysis showed that both WNT5A and IL11 were involved in pulmonary fibrosis idiopathic disease. Subsequently, bioinformatics and functional assays revealed that these four accessory proteins were implicated in both inflammatory and fibrotic responses. While overexpression of ORF8 and ORF9c appear to trigger a STAT3-dependent cellular response mediated by IL11, ORF6 and ORF9b seem to provoke a cell profibrotic response mediated by TGF β through WNT5A. Our results suggest that ORF6, ORF8, ORF9b and ORF9c could be involved in inflammatory and fibrotic responses in SARS-CoV-2 infection. Thus, these accessory proteins could be targeted by new therapies for COVID-19 disease.

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Novel mechanism of cross-talk between Interferon signalling and pro-inflammatory cytokine production

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Background: previous work from our group has shown that patients deficient for the IRF9 transcription factor show marked susceptibility to viral infection, as consequence of impaired IFN $\alpha\beta$ signalling, but also suffer from hyper-inflammatory reactions after infection.

Methods: Bioinformatics analyses identified candidate genes not previously known to be regulated by IRF9 and this putative regulation was confirmed biochemically. The functional consequences of this interaction were investigated using techniques of gene silencing and overexpression followed by analysis of cytokine production and Interferon-stimulated gene (ISG) expression.

Results: IRF9 can regulate the expression of subunits of the linear ubiquitin chain assembly complex (LUBAC) and IRF9 silencing leads to increased production of pro-inflammatory cytokines, including IL-1 β and IL-6, by stimulated monocyte/macrophage cells.

Conclusions: Cross-talk between the interferon and NF- κ B pathways at the level of linear ubiquitin assembly complex (LUBAC) can influence the balance between immune activation for pathogen control and exaggerated responses that result in auto-inflammation.

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Elevated levels of cell free NKG2D ligands modulate NKG2D expression and reduce NK cell function in SARS CoV 2-infected individuals with severe COVID-19 disease

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Background: We have searched for differences in the phenotype and function of NK cells in SARS-CoV-2 infected individuals who developed either very mild, or life-threatening COVID-19 disease.

Methods: To rigorously define features of NK cell populations that differ significantly between mildly affected COVID-19 patients and those with life-threatening disease we performed an automated, unbiased comparison of multi-parameter flow cytometry analyses of NK cells in these two patient groups using the CITRUS algorithm. These phenotypic studies were complemented by assays of NK cell cytotoxicity and cytokine production.

Results: Although NK cells from patients with severe disease appeared more activated and the frequency of adaptive NK cells was increased, they were less potent functionally. Further analysis of peripheral blood NK cells in these patients revealed that a population of NK cells that had lost expression of the activating receptor NKG2D were a feature of patients with severe disease and this correlated with elevated levels of cell free NKG2D ligands, specifically ULBP2 and ULBP3 in the plasma of critically ill patients.

Conclusions: These observations of reduced NK function in severe disease are consistent with the hypothesis that defects in immune surveillance by NK cells permit higher levels of viral replication, rather than that aberrant NK cell function contributes to immune system dysregulation and immunopathogenicity.

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Therapeutic Interleukin-6 Trans-signalling Inhibition Improves SARS-CoV-2 Infection Pulmonary Pathology in K18 human ACE2 Transgenic Mice

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Background and objectives: IL-6 is a pleiotropic cytokine produced in response to infection and tissue damage. Although IL-6 classic signalling is thought to represent a defence mechanism (e.g. against pathogens), trans-signalling has been suggested as an important patho-mechanism conveying pro-inflammatory effects. We have recently published that IL-6 signalling components (IL-6, sIL-6R and sgp130) and indicators of potential IL-6 trans-signalling are accurate predictors of COVID-19 severity and outcome with clear implications for treatment and clinical decision-making. On the other hand, the inhibition of JAK1/2, one of the main pathways activated by gp130, has resulted to be the best immunomodulatory treatment with efficacy to reduce COVID-19 mortality. Nevertheless, side-effects such as secondary infections and venous thrombosis have been reported. We hypothesize that a therapeutic approach specifically targeting IL-6 trans-signalling could specifically block the pro-inflammatory milieu without interfering with physiologic and host defence activities involving classic IL-6 signalling. The **aim of this study** was to investigate the potential benefit of specific inhibition of IL-6 trans-signalling during SARS-CoV-2 infection.

Methods: Mice were obtained from the Jackson Laboratory (SN34860-B6.Cg-Tg(K18-hACE2)2PrImn/J) and distributed in three groups: Control (non-infected, n=4), SARS-CoV-2-infected (n=17) and SARS-CoV-2-infected-treated mice (n=18). IL-6 trans-signalling specific inhibitor was administered intraperitoneally twice per week. Mouse weight and health were monitored daily, up to 15 days post infection (end of experiment) or until they reached end-point criteria. Biochemical, histopathological and molecular studies were performed in lung and liver tissues. Luminex and RNA-qPCR were performed for measuring quantification and expression of selected variables.

Results: Infected mice showed multifocal foci of inflammation in lungs, which were reduced in animals treated with the IL-6 trans-signalling specific inhibitor. Importantly, this treatment decreased COVID-19 related mortality 20% compared to untreated mice. Moreover, COVID-19 related symptoms were moderated from the 7th day after infection in treated mice. Histological and immunohistochemistry studies in lung showed the efficacy of the treatment. Pro-inflammatory cytokines and senescence associated secretory phenotype were significantly diminished by the treatment.

Conclusions: Our data suggest that blockage of IL-6 trans-signalling could holds promise for the therapy of SARS-CoV-2 infection.

Acknowledgements: These experiments are being completed in collaboration with Dr. J. Millán (Centro de Biología Molecular Severo Ochoa, Madrid), member of the PTI Salud Global of CSIC. His group has a broad experience on the trans-activation of endothelial cells, and we have designed experiments to study the mechanisms by which SARS-CoV-2 alters the physiology of endothelial cells inducing during the infection. Moreover, a series of experiments have been designed to study the impact of the complexes IL-6/IL-6R and IL-11/IL-11R (involved in sgp130 signalling) on endothelial cells trans-activation.

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The GSK3b-MAFB axis controls the pro-fibrotic gene profile of pathogenic monocyte-derived macrophages in severe COVID-19

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MAF and MAFB are members of the “large MAF” transcription factor family that shape the transcriptome of anti-inflammatory and pro-tumoral human macrophages. We have now determined the MAF- and MAFB-dependent gene profile of M-CSF-dependent monocyte-derived macrophages (M-M ϕ), and found that both factors exhibit overlapping transcriptional outcomes during monocyte-to-M-M ϕ differentiation, but differentially affect macrophage effector functions like production of monocyte-recruiting chemokines, T-cell activation and immunosuppression. Remarkably, MAFB was found to positively regulate the expression of the genesets that define the pathogenic monocyte-derived pulmonary macrophage subsets in COVID-19, as evidenced through siRNA-mediated silencing and analysis of MAFB-overexpressing M-M ϕ from a Multicentric Carpotarsal Osteolysis (MCTO) patient. MAFB silencing downregulated the expression of genes coding for biomarkers of COVID-19 severity, and genome-wide mapping of MAFB-binding elements in M-M ϕ identified biomarkers of COVID-19 severity (CD163, IL10, HGF and CCL2) as direct MAFB targets. Further, and in line with the GSK3b-dependent expression of MAFB, GSK3b inhibition in M-M ϕ significantly boosted the expression of genes that characterize pathogenic macrophage subsets in severe COVID-19, an effect that was primarily dependent on MAFB. In addition, we have demonstrated that a large number of MAFB-dependent genes, as well as GSK3b-dependent expression of MAFB genes were modulated by SARS-Cov-2 infection on human macrophages. Globally, our results demonstrate that the GSK3b-MAFB axis controls the transcriptome of pathogenic pulmonary macrophages in COVID-19, and positively regulates the expression of biomarkers for COVID-19 severity. Thus, macrophage re-programming through modulation of GSK3 -MAFB axis has potential therapeutic strategy for COVID-19 and other inflammatory diseases.

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TLR7 activation in M-CSF-dependent monocyte-derived human macrophages potentiates inflammatory responses and prompts neutrophil recruitment

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Toll-like receptor 7 (TLR7) is an endosomal Pathogen-Associated Molecular Pattern (PAMP) receptor that senses single-stranded RNA (ssRNA) and whose engagement results in the production of type I IFN and pro-inflammatory cytokines upon viral exposure. Recent genetic studies have established that a dysfunctional TLR7-initiated signaling is directly linked to the development of SARS-CoV-2-induced severe COVID-19. We previously showed that TLR7 is preferentially expressed by macrophages generated in the presence of M-CSF (M-MØ), whose MAFB-dependent transcriptome resembles pathogenic pulmonary monocyte-derived macrophage subsets in severe COVID-19. We now report that TLR7 activation in M-MØ triggers a weak MAPK, NFκB and STAT1 activation and leads to defective production of type I IFN. Nonetheless, TLR7 engagement re-programs MAFB+ M-MØ towards a distinctive transcriptional profile. Specifically, TLR7-activated M-MØ acquired the expression of genes that characterize inflammatory macrophage subsets in COVID-19 and other inflammatory diseases, including genes encoding neutrophil-attracting chemokines (CXCL1-3, CXCL5, CXCL8) reported as biomarkers for severe COVID-19. Functionally, TLR7-activated M-MØ displayed enhanced pro-inflammatory responses towards secondary stimulation and a robust production of neutrophil-attracting chemokines (CXCL1, CXCL5, CXCL8), which was dependent on the transcription factors MAFB and AhR. Interestingly, CXCL1 and CXCL5 release from M-MØ was also promoted by SARS-CoV-2 but not by Virus-like particles. As defective TLR7 signaling and enhanced pulmonary neutrophil/lymphocyte ratio associate with severe COVID-19, these results suggest that targeting macrophage TLR7 might be a therapeutic strategy for viral infections where monocyte-derived macrophages exhibit a pathogenic role.

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IRE1 α -XBP1 Activation Elicited by Viral Singled Stranded RNA via TLR8 May Modulate Lung Cytokine Induction in SARS-CoV-2 Pneumonia

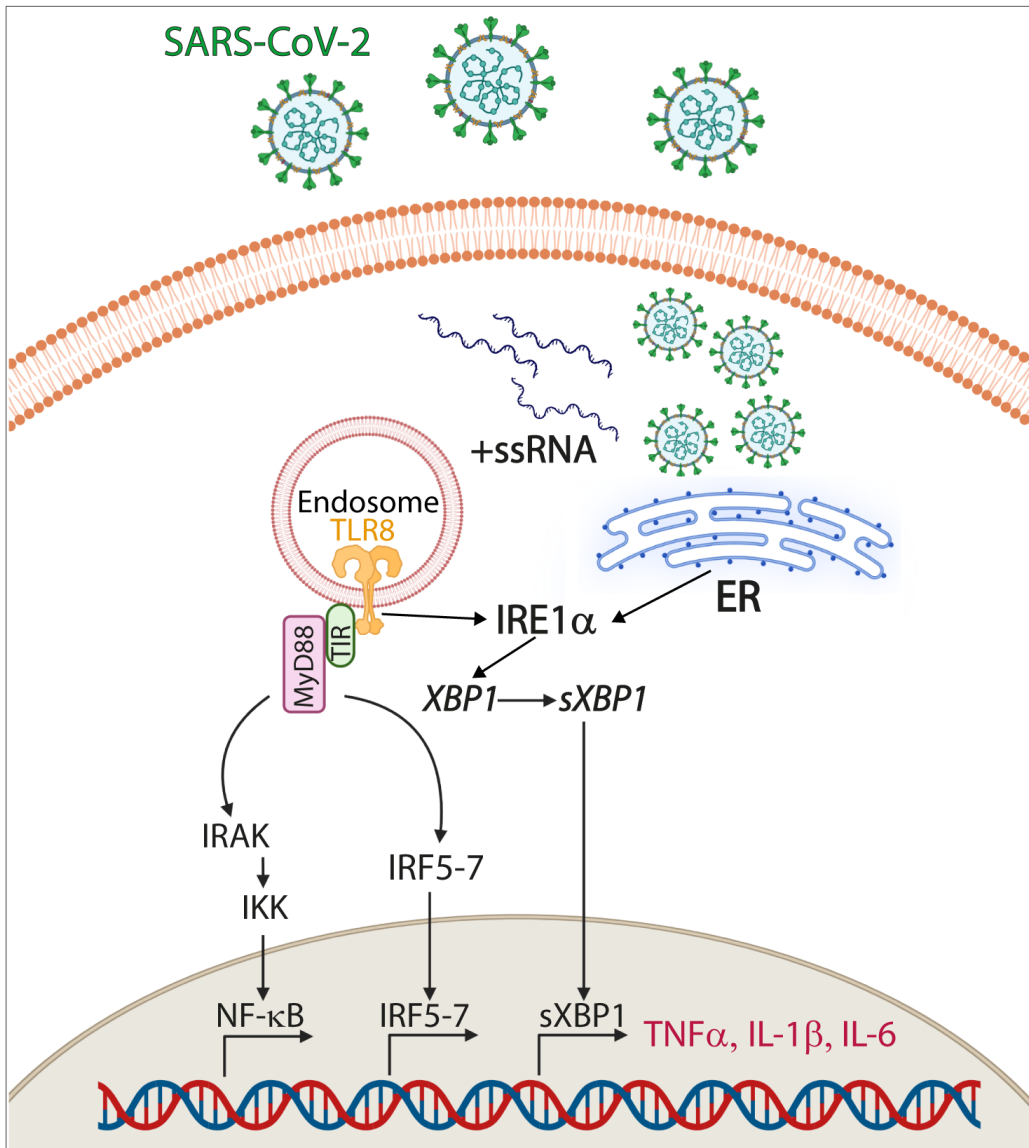
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Background: Infection by SARS-CoV-2 causes hyperinflammation and pneumonia in several patients. This differs from common viral respiratory infections and therefore urges a pathophysiological analysis. Viruses use host cell organelles to produce their proteins and thus overwhelm the protein-folding capacity of the endoplasmic reticulum (ER). This process evokes ER stress and activation of the unfolded protein response (UPR), an adaptive signaling pathway directed to preserve ER homeostasis. Of note, the IRE1 α -XBP1 branch of the UPR is activated in many viral diseases, and viral RNA might trigger the IRE1 α -XBP1 branch via Toll-like receptors. This is not unexpected, since activation of TLR2 and TLR4 by their cognate ligands underpins cytokine expression through IRE1 α -driven XBP1 splicing (sXBP1).

Methods: Nasopharyngeal swabs of SARS-CoV-2 infected patients and samples of bronchoalveolar aspirates (BAAs) of patients under mechanical ventilation because of severe pneumonia were analysed, as well as in vitro experiments using monocyte-derived dendritic cells (MDDCs) activated with ssRNA40, a positive-sense, single-stranded RNA (+ssRNA) like SARS-CoV-2 RNA.

Results: After observing a high incidence of sXBP1 in RNA obtained from nasopharyngeal swabs of SARS-CoV-2 infected patients, a systematic study was undertaken in samples of bronchoalveolar aspirates (BAAs). The presence of sXBP1 and a high expression of IL1B, IL6, and TNF mRNA were detected during active infection. Monocytic/macrophagic populations showed a reduction of markers associated with antigen presentation and survival, as well as the IFN stimulated gene MX1. In contrast, the expression of the mRNA of the serine protease TMPRSS2 involved in S protein priming showed a high expression. TLR8 mRNA showed an overwhelming expression as compared to TLR7 mRNA. MDDCs activated with ssRNA40, a selective TLR8 ligand induced sXBP1, and the expression of IL-1 β , IL-6, and TNF α at mRNA and protein levels. These responses were blunted by the IRE1 α ribonuclease inhibitor MKC8866 and enhanced by the non-toxic IRE1 α -XBP1 activator IXA4.

Conclusions: Experiments showed that activation of immune cell receptors by pathogen-associated molecular patterns mimicking viral RNA induced a pattern of cytokine expression like that observed in bronchoalveolar aspirates of patients with critical COVID-19 pneumonia. The analogies between the results observed in BAAs and the effects of +ssRNA suggest IRE1 α ribonuclease inhibition as a druggable target for severe COVID-19 disease. Overall, the study revealed that cell organelle overload and engagement of receptors targeted by viral RNA team up to produce the proinflammatory cytokines commonly associated with viral sepsis.



Graphical Abstract

IRE1 α -XBP1 Activation Elicited by Viral Singled Stranded RNA via TLR⁸ May Modulate Lung Cytokine Induction in SARS-CoV-2 Pneumonia

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Shared genetic mechanisms among stroke post-COVID-19 cases and ischemic stroke subtypes

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Background: SARS-CoV-2 infection causes inflammation and vascular dysfunction generating a pro-thrombotic environment associated with secondary thrombotic complications. Amongst them, COVID-19 patients have a higher risk of developing ischemic stroke. However, the biological mechanisms underlying ischemic stroke occurrence after SARS-CoV-2 infection remains unclear. Meta-analyses of GWAS, led by the MEGASTROKE consortium, have identified stroke risk genetic loci associated to subtypes of stroke of different etiology. This study aimed to identify genetic commonalities between stroke triggered by COVID-19 and known subtypes of spontaneous stroke.

Methods: A Genome-Wide Association Study (GWAS) from MEGASTROKE consortium was used to generate Polygenic risk scores (PRSs) across different p-value thresholds ($p=0.05$ - $p=5e-8$) using PRSice-2. For all ischemic stroke (AIS) we used 34217 cases and 406111 controls; large-artery atherosclerosis (LAA) 4373 cases and 297290 controls; cardioembolic stroke (CE) 7193 cases and 355468 controls; and small-vessel occlusion (SVO) 5386 cases and 343560 controls. For undetermined stroke etiology (UND) 984 cases and 5590 controls from a Spanish stroke cohort were used. PRSs were tested in a cohort of 43 European patients with an ischemic stroke that occurred after COVID-19 hospitalization (<8 days) (IS-COV) and 726 population controls.

Results: We found significant associations of IS-COV with PRSAIS, PRSLAA and PRSUND. We did not find any association with PRSCE and PRSSVO.

Conclusions: The results of this study suggest that ischemic stroke cases due to COVID-19 share genetic mechanisms with stroke caused by large-artery atherosclerosis and stroke of undetermined cause. The results support the notion that COVID-19 increases the risk of developing ischemic stroke in patients genetically predisposed to large-artery atherosclerosis.

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Effect of 2´5´-olygoadenylate synthetase genes on SARS-CoV-2 infection and the induction of innate immune responses

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Background: Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) infections cause different clinical symptoms ranging from asymptomatic patients to patients suffering severe respiratory disease leading to death in some of them. Genetic and functional studies have shown inborn-errors of interferon (IFN)-related genes in severe COVID-19 patients explaining why some young patients devoid of co-morbidities succumbed to infection. The objective of this study was to identify mutations in IFN-stimulated genes (ISGs) genes, focusing in 2´5´-olygoadenylate synthetase (OAS) genes. Upon double-stranded (ds)RNA binding, the OAS1, OAS2, and OAS3 proteins synthetize 2´-5´-polyadenylates which activate the endonuclease RNaseL. This endonuclease degrades cellular and viral RNAs, inhibiting viral replication.

Methods and results: We sequenced the whole exome of around 400 patients who required hospitalization after SARS-CoV-2 infection and found ultrarare mutations in OAS1 and OAS3 genes. We have analyzed in cell cultures the effect of the OAS1 and OAS3 mutations identified in our patients, and found that some of them impair the activation of RNaseL. In addition, by using OAS1 and OAS3 knock-out cells generated in our laboratory and performing overexpression experiments, we have shown that OAS3 negatively modulates pro-inflammatory responses induced by immune challenges, and that RNaseL activity seems necessary for this function. In addition, by using OAS3 knock-out mice intratracheally treated with poly(I:C), an analog of dsRNA produced during viral infections, and using OAS3 knock-out mice intranasally infected with SARS-CoV-2, our preliminary data showed that OAS3 counteracts the induction of innate immune responses in the mouse infected-lungs and curbs viral replication.

Conclusions: Our results show the antiviral activity of OAS1 and OAS3 against SARS-CoV-2 infection and the negative regulatory effect of OAS3 against the subsequent inflammatory responses. Given the contribution of exacerbated inflammatory responses to COVID-19 disease severity, our results suggest that OAS1 and OAS3 could play a role limiting the severity of the clinical symptoms after SARS-CoV-2 infection.

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Mapping the serum proteome of COVID-19 patients in a Spain population. A guidance for severity assessment.

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Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), whose outbreak in 2019 led to an ongoing pandemic with devastating consequences for the global economy and human health. According to World Health Organization, COVID-19 has affected more than 481 million people worldwide, with 6 million confirmed deaths. The joint efforts of the scientific community have undoubtedly increased the pace of production of COVID-19 vaccines³, but there is still so much uncharted ground to cover regarding the mechanisms of SARS-CoV-2 infection, replication and host response. These issues can be approached by proteomics with unprecedented capacity paving the way for the development of more efficient strategies for patient care. In this study, we present a deep proteome analysis that has been performed on a cohort of 72 COVID-19 patients aiming to identify serum proteins assessing the dynamics of the disease at different age ranges. A panel of 53 proteins that participate in several functions such as acute-phase response and inflammation, blood coagulation, cell adhesion, complement cascade, endocytosis, immune response, oxidative stress and tissue injury, have been correlated with patient severity, suggesting a molecular basis for their clinical stratification. Eighteen protein candidates were further validated by targeted proteomics in an independent cohort of 84 patients including a group of individuals that had satisfactorily resolved SARS-CoV-2 infection. Remarkably, all protein alterations were normalized 100 days after leaving the hospital, which further support the reliability of the selected proteins as hallmarks of COVID-19 progression and grading. The optimized protein panel may prove its value for optimal severity assessment as well as in the follow up of COVID-19 patients.

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SARS-CoV-2 Spike protein-promoted effects on human dendritic cells

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Background: Dendritic cells (DCs) are professional antigen presenting cells that constitute the bridge between innate and adaptive immunity. Tight regulation of the activity of these cells warrants configuration of an effective T cell response while avoiding exacerbating inflammation. Understanding DCs biology is key for counteracting pathogens and designing effective vaccine strategies.

DCs are predicted to play a crucial role in the clinical evolution of the infection by the Severe Acute Respiratory Syndrome (SARS) coronavirus (CoV)-2. In addition, DCs function is relevant in the development of long last immunity against this virus, either following infection or induced by vaccination protocols based on the Spike protein.

DCs maturation process upon Spike protein binding is expected to be a key event in the induction of immunity against this virus, nonetheless our knowledge regarding this issue remains limited. In this work we evaluated the response of human DCs to SARS-CoV-2 S protein, or to a fragment encompassing the receptor binding domain (RBD) challenge.

Methods: Monocyte-derived DCs were generated from different donors and challenged with either SARS-CoV-2 Spike protein, or with a fragment encompassing only the Receptor Binding Domain (RBD) region. DCs early response was evaluated by using western blot to determine the activation status of different signalling pathways known to play a crucial role in inflammation. Expression of maturation markers was determined using flow cytometry, whereas the expression of cytokines was evaluated by Enzyme-Linked Immunosorbent Assay (ELISA) and Quantitative Reverse Transcriptase Polymerase Chain Reaction (qPCR). Expression of ACE2 (angiotensin-converting enzyme 2) along the differentiation of human monocytes to mature DCs was also evaluated by different methods.

Results: Immature DCs challenge with either Spike or RBD proteins induced strong maturation and activation of the cells. Both proteins promoted significant increases in the expression of markers like the MHC molecules, as well as in that of costimulatory receptors like CD40, CD80, CD83, CD86. The effect of RBD was stronger than S in all the molecules analysed and was statistically comparable to those exerted by LPS.

DCs interaction with the SARS-CoV-2 S protein also promotes activation of MAPK, AKT, STAT1, and NFκB, which correlates with the expression and secretion of distinctive proinflammatory cytokines including IL-6 and TNF-κ.

Human DCs express ACE2 and differences in the expression of ACE2 along the differentiation of human monocytes to mature DCs and inter-donor were found.

Conclusion: Our results show that SARS-CoV-2 S protein promotes inflammatory response and provides molecular insights between individual variations and the degree of activation and dysregulation of the immune response.

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The SARS-CoV-2 E protein interacts with distinctive PDZ proteins in immune cells

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Background: PDZ proteins are central in the assembly of multiprotein complexes that regulate cell polarity. Viral pathogens target host PDZ proteins by expressing viral proteins containing a PDZ binding motif. Targeting of PDZ proteins promotes viral replication in part through disruption of epithelial cell polarity.

SARS-CoV-1 and -2 harbour the protein E, a viroporin with a conserved PDZ binding motif in their carboxyl-terminal region. In epithelia, SARS-CoV1 E interaction with PDZ proteins like PALS1 promote disruption of cell junctions, which contribute to the pathogenicity of this coronavirus.

SARS-CoV-2 infects different epithelium, like those from lung and intestinal tract, and also non-epithelial cells, including cellular components of the innate immune response, like dendritic cells or alveolar macrophages. Viral targeting of PDZ proteins in immune cells is expected to result in impaired immune responses and virus dissemination.

The targets of the SARS-CoV-2 E protein in immune cells are so far unknown, but their identification might offer valuable clues to understand how SARS-CoV-2 alters immune response. Identification of PDZ viral targets in immune cells is also necessary to highlight the function of PDZ proteins in immune fitness.

Methods: The ORF encoding for SARS-CoV-2 E protein was cloned in the pEZYEGFP vector using the Gateway system. The construct generated allow to express the E protein fused to a GFP-tag at the amino terminal. The viral fusion protein was expressed in THP-1 cells, a human monocyte cell line that can be differentiated to different macrophages and dendritic cells subsets. GFP tagged proteins were immunoprecipitated using the GFP-trap system, and associated proteins analyzed by liquid chromatography coupled to Triple-TOF Mass Spectrometry. The proteomic results were compared with 155 human proteins containing PDZ motifs in order to determine the SARS-CoV2 E protein PDZ-dependent interactome.

Results: Analysis of the interactome of GFP-E protein in THP-1 cells provided 372 proteins that fall into different functional groups. We found that only 8 of these proteins harbor PDZ domains, including the previous SARS-CoV-E protein partner Syntenin, a finding that validates our strategy. Three of the identified partners are cell polarity proteins, and other two are proteins with a role in cell homeostasis. Lastly, one of the interactors is a cell mediator with chemoattractant properties and with a role in inflammation. Variations in the expression along the differentiation process of monocytes to macrophages and dendritic cells was found in some of the SARS-CoV-E protein partners.

Conclusion: Here we identify SARS-CoV-2 E protein PDZ interactome in THP-1 cells. We found novel interactions of this viral protein with distinctive proteins whose function has been related with polarity and immune response. Our findings support the notion that viral targeting of PDZ proteins alters inflammatory response.

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Identification of the transcriptome and TCR repertoire against SARS-CoV-2 immunodominant peptides from long-term convalescent COVID-19 patients

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Background: V(D)J gene-segment recombination is responsible for the huge variability of different $\alpha\beta$ T-cell clonotypes. The high excess of possible TCR $\alpha\beta$ determines that different people do not share same receptors. However, shared (public) TCR $\alpha\beta$ have been found to be associated to specific infections and diseases. We propose to identify public TCR $\alpha\beta$ in CD8+ T cells from long-term convalescent COVID-19 patients (6-12 months after infection), focusing on people who are >60 years old and express the HLA-A02 allele (present in the 40% of people). Knowledge of immunodominant peptides and the public TCR $\alpha\beta$ specific for infectious diseases could serve to develop long-lasting vaccines based on T-cell responses and novel treatments based on TCR-engineered T cells.

Methods: Our cohort consists of 50 long-term convalescent patients and 20 healthy donors (unvaccinated and vaccinated). We analyzed the CD8 response to specific peptides (18 SARS-CoV-2 and 1 CMV) by measuring the intracellular expression of IFN γ and TNF α by flow-cytometry in HLA-A02+ CD8+ cells from convalescent patients. For this purpose, peripheral blood cells were expanded for two weeks in the presence of our pool of peptides, anti-CD28 antibody, and IL-2. Specific CD8+ clonotypes of each peptide were purified based on flow-cytometry using tetramers. 12 populations were purified populations based on labelling with an oligonucleotide-linked antibody to simultaneously analyze their transcriptome and TCR repertoire in single cells using the Rhapsody platform (Becton-Dickinson). We also characterized different antibody isotypes against viral proteins in plasma using the Multiplex Serological SARS-CoV-2 assay (Immunostep) and a sensitive method to measure the reactivity against native spike protein (S), as well as their neutralization capacity in infection assays in human ACE-2-expressing HEK-293T or Vero cells, using two S- and GFP-expressing pseudotyped viruses. Results: Our analyses indicated the presence of higher levels of IgA than IgG1 against S in plasma from long-term convalescent patients. IgG1 levels correlated with the neutralization index. Interestingly, neutralizing titers (IC50) and neutralizing IgG levels were extremely low in these samples compared to those from recently convalescent or vaccinated patients. Reactivity analyses of CD8+ HLA-A02:01+ cells present in convalescent patients confirmed the five most immunodominant peptides reported. These peptides come from S and Orf1ab proteins: S269-277, S269-277, Orf1ab139-147, Orf1ab3886-3894 and Orfab4094-4102, and no changes have been observed in the variants of SARS-CoV-2 virus identified in successive waves of infection. We are in the process of isolating 40 CD8+ tetramer+- specific populations for analysis of their transcriptome and TCR repertoire in single cells. The resulting bioinformatics analyses of the peptide-specific TCR sequences identified in single T CD8+ cells will be discussed.

Conclusions: Long-term convalescent patients have higher levels of IgA than IgG1 against S, and their neutralization indexes correlate with IgG1 levels. Interestingly, these patients have extremely low neutralizing capacity compared to their anti-S IgG/IgG1 levels. We confirmed the reported immunodominant HLA-A02:01 SARS-CoV2 peptides in our cohort. Analyses of transcriptome and TCR repertoire in response to these peptides in CD8+ cells will be presented. Future experiments will analyze a cohort of convalescent patients <60 years old with a higher number of peptides.

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What do microbial and immune fecal determinants tell us about the severity of COVID-19 in infants?

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The coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2 has spread rapidly worldwide, seriously endangering human health. Although SARS-CoV-2 had a lower impact on the pediatric population, children with COVID-19 have been reported to suffer gastrointestinal (GI) symptoms in a higher rate than adults. Additionally, in some case they suffer from the so-called multisystem inflammatory syndrome (MIS). The aim of this work was to evaluate the severity of COVID-19 disease in pediatric population, with emphasis on intestinal microbiota and fecal biomarkers; trying to identify possible dysbiosis and immune intestinal dysfunction associated with mayor risk of hospitalization.

This study involved 19 patients with COVID-19 under 36 months of age hospitalized during the pandemic in Spain at 6 different hospitals, and included a comparable age- and sex-matched healthy non-vaccinated control group (n=17). The patients and controls were ranged by age in two groups: newborns (from 0 to 3 months old) and infants (from 6 to 36 months old). To characterize the microbial intestinal communities, sequencing with Illumina technology of total 16S rDNA amplicons and intergenic sequences (ITS) of bifidobacteria was used. Calprotectin and a range of human cytokines, chemokines and growth factors were measured using ELISA and a multiplex system. Microbial indole profiling was analyzed by a LC-MS metabolomic platform.

A significant reduction in the abundance of sequences belonging to the phylum Actinobacteria was found in those infants with COVID-19, as well as in the Bifidobacteriaceae family. Also, we observed a different profile of bifidobacteria in patients, mainly represented by lower percentages of *Bifidobacterium breve* (a typical species that characterized early infancy), as compared with controls. In the group of hospitalized newborns, calprotectin was almost absent in comparison with the age- matched healthy controls. We also observed a general reduction in the excretion of key immune factors in infected patients, with less detection of IL-7 and IL-10, as compared with controls.

Hospitalized infants with COVID-19 were depleted in some gut bacteria, such as bifidobacteria, crucial for the proper establishment of a functional intestinal microbiota, and important for the development of a competent immune system. Our results point to a less active or immature immune system at intestine level in young babies infected by SARS-CoV2 that required hospitalization

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The gut microbiome as possible biomarker of the risk and evolution of the COVID-19 infection

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Background: The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is responsible for coronavirus disease 2019 (COVID-19), which was declared a pandemic in March 2020 by the World Health Organization (WHO). The COVID-19 presents with a wide range of symptoms. Although the lungs are the most affected organs, some studies suggest that the intestinal tract may be involved in the pathogenicity of COVID-19, as SARS-CoV-2 can replicate in intestinal enterocytes. The gut contains the largest number of immunocompetent cells in the body and, together with the microbiota, play a key role in the regulation of the local and systemic immune response.

Methods: This study aims at understanding if the intestinal microbiome features of the individual before and during the disease course is related to the risk and evolution of the infection by the SARS-CoV-2. To this end, a large population study has been conducted, including a total of 927 participants of 3-83 years of age, who were recruited since November 2021 and followed-up for one year. The inclusion criterion was not having suffered from COVID-19 infection, confirmed by the clinic history and specific serologic test. For this study, stool samples were collected at baseline from all participants and at different times of the infection from those becoming positive for COVID-19 during the follow-up. The composition of the faecal microbiota of forty participants who were infected with the virus and a control group with similar sociodemographic characteristics was analysed. For this purpose, genomic DNA was extracted from faeces and paired-end sequencing was performed on an Illumina MiSeq targeting the V3-V4 hypervariable region of the 16S rRNA gene. An average sequencing depth of 130.000 raw reads per sample was obtained. The quality of the raw FASTQ files was analysed using the software fastqc and multiqc. DADA2 package was used to trimming and filter samples, Phyloseq package to perform the diversity analysis and tidyverse to generate the figures and graphics. All bioinformatics analyses were done using the R software.

Results and conclusions: First, we compared the gut microbiome of individuals who become SARS-CoV-2 positive (cases) versus that of individuals who remained healthy (control) at baseline. Second, we assess the potential changes of the microbiome of infected individuals comparing before and at different times during the infection until their recovery. Our results will be presented and discussed in the light of the evidence published so far on the relationship of the intestinal microbiota and the COVID-19 infection.

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Exploring differences in the intestinal microbiota of COVID-19 positive patients and controls to find biomarkers that help disease management

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Background: COVID-19, caused by the SARS-CoV-2 infection, was declared a pandemic by the WHO in March 2020. The infection is characterized by a broad range of clinical manifestations, mainly affecting the respiratory system, although there is increasing evidence of gastrointestinal tract involvement. The aim of this work is to identify differences between the intestinal microbiota of positive COVID-19 patients and healthy people in order to identify possible alterations related to disease severity and prognosis, and which modulation could help in infection management.

Methods: Stool samples and data were collected from 63 COVID-19 positive subjects and from 49 age- and sex-matched COVID-19 negative controls. All samples were subjected to heat shock at 70°C for 10 minutes to inactivate the SARS-CoV-2 in patients' samples. DNA was extracted using QIAmp® Fast DNA Stool Mini Kit, and the V3-V4 region of the 16S rRNA gene was sequenced on an Illumina® MiSeq platform (2×300 bp paired-end reads).

Raw reads were filtered for quality assurance and clean pairs of reads were merged into contig sequences. An amplicon sequence variant (ASV) table was constructed and chimeric sequences were removed. Taxonomy was assigned by crossing sequences with SILVA v.138 database. All these steps were performed using DADA2 v.1.24 in R v.4.2. Taxa with a prevalence below 5% were removed from subsequent analyses.

Bacterial diversity within samples was computed through richness (or observed) and Simpson alpha diversity indexes using Phyloseq v.1.40. Pairwise comparisons between groups were performed by non-parametric Wilcoxon test after testing the normal distribution of the data with a Shapiro-Wilk test as implemented in R. Differences between microbial communities were analyzed by Bray-Curtis beta-diversity index using Phyloseq.

The microbiome differential abundance analysis between cases and controls was performed using DESeq2 v.1.36, whose implemented function normalized our data and performed hypothesis testing using Wald test. The resulting p-values were corrected using the BH FDR procedure.

Results: We obtained 907 ASV in the whole dataset, with 69 genera included in 10 phyla. We also identified 192 species, but at this level, we could identify 18% of the dataset only. Alpha and beta diversity indexes revealed differences between COVID-19 patients and controls. The controls showed less bacterial diversity than patients, although the bacterial diversity was generally low in both study groups probably due to the advanced age of some controls and the occurrence of some comorbidities.

Differential abundance analysis of bacterial taxa revealed differences between patients and controls. Individuals infected with SARS-CoV-2 presented increased genera and species related to opportunistic pathologies, while controls showed higher abundance of genera and species typical of the commensal microbiota of healthy people. The differences in microbiota considering differences in disease severity are also being investigated.

Conclusions: Subjects infected with SARS-CoV-2 showed an altered intestinal microbiota with increased abundance of opportunistic pathogens compared to the control group which could contribute to disease pathogenesis. This suggests that restoring the microbiota composition might help patient recovery. Further functional microbiome analysis would provide more insights into the possible mechanisms whereby the microbiota could contribute to the infection.

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Searching for new therapeutic targets to prevent lung microvascular endothelial barrier disruption and pulmonary edema caused by SARS-CoV2-induced cytokine storm

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Acute respiratory distress syndrome (ARDS) is a severe respiratory failure characterized by exacerbated inflammation of the lungs due to an acute rise in pro-inflammatory mediators such as IL-6, IL-1beta, IL-8, MCP-1 and TNF-alpha, among others, in response to infection. This phenomenon is known as a cytokine storm, which compromises the integrity of the entire pulmonary vasculature, causing increased infiltration of immune cells, subsequent edema and finally multi-organ failure and death.

This unstoppable inflammatory response turns into a life-threatening condition that has come under the spotlight upon the on-going pandemic coronavirus disease 2019 (COVID-19). COVID-19 and sepsis share the systemic inflammation pattern that lead to multiorgan failure. In fact, several COVID-19 patients died from a sepsis-derived cytokine storm. Pulmonary endothelial barrier function has emerged as a new therapeutic target to preserve lung integrity in severely ill patients of COVID19.

Our general aim is to identify the inflammatory signature inducing vascular hyperpermeability and pulmonary edema during COVID-19-derived cytokine storm. To do that, we study microvascular barrier function in response to pro-inflammatory cytokines by measuring transendothelial resistance (TEER). Taking advance of the collection of plasma samples of COVID-19 and sepsis patients available from IdiPAZ, we stimulated microvascular endothelial cells to study the molecular mechanisms underlying pulmonar endothelial barrier dysfunction. We finally search drugs approved for clinical use with potential therapeutic on cytokine-induced lung endothelial barrier dysfunction. We are focused on two strategies: 1) testing drugs that counteract cytokine- induce signaling derived from the cytokine storm and 2) testing drugs that strengthen the human lung endothelial architecture with a specific interest in targeting RhoA- and Rac1-mediated signalling to filamentous actin. Both strategies aim to reduce ARDS during the cytokine storm induced by SARS-CoV-2 infection.

Our findings suggest that the simultaneous treatment with anakinra, an antagonist of the IL1 receptor, and ruxolitinib, that inhibits JAK1/2, reduce the endothelial barrier disruption triggered by an in vitro cytokine storm and serum from patients with sepsis. Furthermore, the ROCK inhibitor ripasudil in combination with sphingosine-1-phosphate (S1P), also prevent the sepsis-derived lung endothelial hyperpermeability.

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Wastewater-based epidemiology of SARS-CoV-2 and its potential fecal-oral transmission

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Background: In the context of the COVID-19 pandemic, wastewater analysis has proven to be a very useful tool for epidemiological surveillance. Quantification of SARS-CoV-2 in wastewater provides real-time knowledge of the population incidence, and sequencing enables us to identify variants circulating at the population under study. Although SARS-CoV-2 can be excreted in feces, which allows analysis through sewage, the possible fecal-oral transmission pathway is not yet established and requires experimental validation.

Methods: From May 2020 to present, 4-hour composite samples were collected three times per week from the main wastewater treatment plants in the metropolitan area of Valencia (Spain). In addition, starting in March 2021, stool was collected from 62 COVID-19 patients admitted to the Hospital Universitari i Politècnic La Fe (Valencia). All samples were concentrated by high-speed centrifugation, RNA was extracted and SARS-CoV-2 was quantified by RT-qPCR in duplicate for two regions of the viral genome (N1 and N2) using standard curves. SARS-CoV-2 variant analysis was performed by RT-PCR and nested PCR targeting four relevant regions of the spike protein-coding gene by in-house primers and sequenced by the Sanger method. In addition, PCR products were cloned for minority variant analysis. The viability of SARS-CoV-2 was studied by inoculating Vero E6 cell cultures with the samples. Supernatants were collected at 0 hours post infection (hpi), 48 hpi and 6 days post infection, and cytopathic effects were observed. All supernatants were analyzed by RT-qPCR to determine viral infectivity.

Results: More than 300 composite wastewater samples were collected and SARS-CoV-2 RNA quantification was performed. SARS-CoV-2 RNA was amplified in 75% of the RT-qPCRs, with an estimated detection limit of 100 viral genome copies per liter (gc/L), and viral load remained between 10^2 and 10^5 gc/L. SARS-CoV-2 RNA concentration correlated strongly with disease reporting rates over 14-day periods (Pearson $r = 0.962$, $P < 0.001$). A concentration >1000 gc/L showed $>95\%$ sensitivity and specificity as an indicator of more than 25 new cases per 100,000 inhabitants. Time series were similar for wastewaters data and declared cases. In addition, Sanger sequencing showed the variants circulating in the population under study, which allowed estimating the proportion of variants during the transition periods between variants. A total of 62 stool samples were collected. SARS-CoV-2 RNA was detected in the 55% of the patients, and viral load ranged from 10^3 to 10^7 gc/g. Sanger sequencing provided the SARS-CoV-2 variants present in samples with a viral load of at least 10^3 gc/g. Positive stools and 8 wastewater samples were tested in cell culture for SARS-CoV-2 infectivity. However, no cytopathic effects were observed and RT-qPCR showed equal or lower concentrations in all cultures.

Conclusions: There is a strong correlation between SARS-CoV-2 load detected in sewage and cases reported by public health authorities, demonstrating the importance of waste water-based epidemiology for monitoring viral pathogens. In addition, sequencing of wastewater samples informs on variants circulating in the population under study. Finally, no evidence of infective particles in feces is observed, suggesting that fecal-oral transmission is not a primary route of SARS-CoV-2 transmission.

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Novel nanobiomaterial with antimicrobial activities

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The development of antimicrobial compounds among pathogenic bacteria and fungi is one of the most significant health issues of the 21st century. Recently, advances in nanotechnology have led to the development of nanomaterials, particularly metals that exhibit antimicrobial properties. These metal nanomaterials have emerged as promising alternatives to traditional antimicrobial therapies [1,2]. Recently, different nanomaterials, including for example metal and metal oxide nanoparticles (NPs) or carbon nanostructures have demonstrated antimicrobial activities and have been used as alternative biocides for example against microbiologically influenced corrosion (MIC) [3,4]. One of the key points is to find a particular strategy which being able to connect the materials with biocide properties with the materials to be protected. Here we described a novel technology [5,6] where a biomolecule, particularly a protein, is used to induce the formation of metal nanoparticles creating a novel type of materials with antimicrobial properties, where these proteins showed important role, mainly formation and control of material at nanoscale size but also act as agent for attaching to different surfaces. The efficiency of the strategy has been tested successfully in different materials and has been proved to show good antimicrobial activities [7].

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Capsid-integrity RT-qPCR to assess SARS-CoV-2 infectivity in environmental samples

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Background: The role of environment-to-human COVID-19 spread has been a matter of debate because mixed results have been reported concerning SARS-CoV-2 stability on surfaces and the presence of SARS-CoV-2 RNA in sewage. Up to now, no alternative and accessible procedures for cell culture have been described to evaluate SARS-CoV-2 infectivity on environmental samples. Several strategies based on virus integrity have been developed using viability markers to selectively remove false-positive qPCR signals resulting from free nucleic acids and damaged viruses. These have finally allowed an estimation of viral infectivity.

Methods: We screened monoazide dyes and platinum compounds as viability molecular markers on SARS-CoV-2 RNA targets. A platinum chloride-based RT-qPCR was then optimized using genomic RNA, and inactivated SARS-CoV-2 particles. Furthermore, initial assessment compared two different swabbing procedures to recover inactivated SARS-CoV-2 particles from fomites coupled with two RNA extraction methods. Procedures were validated with human (E229) and porcine (PEDV) coronavirus surrogates.

Results: The optimized capsid-integrity RT-qPCR efficiently removed the PCR amplification signals from heat and gamma-irradiated inactivated SARS-CoV-2 suspensions that had been collected from specified surfaces. Moreover, capsid-integrity RT-qPCR was further validated on wastewater samples naturally contaminated with SARS-CoV-2. The results showed that the optimized Capsid-integrity RT-qPCR completely prevented the amplification in all samples.

Conclusions: The Capsid-integrity RT-qPCR approach provides a more accurate ascertainment of the infectious virus detection and it may complement analyses to foster risk-based investigations. Additionally, its application in naturally contaminated wastewater samples supports the idea that SARS-CoV-2 present in sewage is not infectious.

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Toward biobased, biodegradable and transparent fibers for the mask manufacture by melt electro-writing

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Background: The main aim of this research is to obtain biobased, biodegradable and transparent fibers by melt electro-writing for the manufacture of facial masks against pollutants and microbes, in particular SARS COVID 19 at present time.

Methods: The material for fabrication of the fibers has been selected based on its biobased source and its capacity for biodegradation in the environment. For this reason, poly(lactic acid), grade named as PLA-LL602 and supplied by the company ErcrosBio, has been chosen in the present research as a polymeric matrix. Different plasticizers, also from biobased sources, have been added to this matrix to improve their mechanical behavior -Acetyl Tributyl Citrate, (ATBC), Dibutyl Adipate (DBA) and Epoxidized Soybean Oil (ESO)-. PLA compounds with plasticizer contents of 20 wt.% have been obtained by melt extrusion in a Haake Minilab extruder at 190°C, with a rotor speed of 70 rpm, using a mixing time of 3 min. Prior to extrusion, the PLA pellets have been dried in an oven at 80°C for 24 h.

The fibers have been obtained by melt electro-writing with a NovaSpider Pro (CIC nanoGUNE). The device consists of a tube with pressure that is able to be moved in the x-y-z axes, together with a collector, which can be a flat plate or a drum, connected to a negative voltage supply. The distinct materials have been loaded into the tube, whose needle has an inner diameter of 0.30 mm. The different parameters (voltage, pressure, height and collector speed) have been optimized to produce the fibers with the best characteristics. All samples have been characterized by Differential Scanning Calorimetry (DSC), Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR- FTIR), Scanning Electron Microscopy (SEM), and Optical Profilometer (OP).

Results: Several compounds based on PLA and different plasticizers (ATBC, DBA and ESO) have been successfully obtained by melt extrusion. The fibers manufactured from these materials and processed by melt electro-writing have a homogeneous thickness and do not present any defect on their surface as can be observed by the SEM and OP images. The best results are achieved in the PLA-DBA material, where a significant reduction in the fiber diameter is possible, this being of one order of magnitude compared to that found in the pristine polymer. In addition, these fibers do not show any evidence of degradation even having been processed at a temperature much higher than their melting temperature (T_m), as corroborated by the results of DSC and ATR-FTIR.

Conclusions: The use of melt electro-writing has been proved to be a satisfactory technique to produce oriented fibers with a controlled diameter without requiring the use of polluting solvents. Incorporation of plasticizers reduces the processing temperature of the original matrix down to 20 °C, and it allows decreasing the fiber diameter up to one order of magnitude. Comparatively, the use of flat collector allows drawing established patterns while the fiber diameter can be significantly reduced by using drum collector.

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Impact on Human Health and the Environment of the use of face masks for protection against COVID-19

Ethel Eljarrat¹ ; Sandra Callejas¹ ; Julio Fernández-Arribas¹ ; Rafael Bartrolí¹ ; Teresa Moreno¹

From the COVID-19 pandemic, the consume of single-use or reusable facemasks has widely increased by the general public, since they have been recommended or even enforced as personal protection against the SARS-COV-2. A great number of these facemasks are produced from synthetic polymers, such as polypropylene, polyurethane, or polyacrylonitrile, among others. This fact could establish facemasks as a source of human exposure to the chemical compounds incorporated into these polymers during their production, as plasticizers. Even though, very few studies have evaluated the potential risk to humans that would result from the prolonged use of facemask.

In this study, organophosphate esters (OPEs), an emerging group of pollutants with reported toxic effects, as well as heavy metals were determined in 39 different facemasks corresponding to four types: surgical, KN-95, FFP2 and reusable. Subsequently, an inhalation study was carried out to evaluate human exposure to these toxic chemicals, as a result of the use of the previously analyzed facemask. For this experiment, dummy heads equipped with personal environmental monitors were used. An air volume equivalent to 8 hours of human breathing was passed through the mask and collected in PM2.5 glass fiber filters, where the chemicals given off the mask were attached. The inhalation experiments were performed under three different conditions (initial conditions (22°C and 60% humidity); high temperature (35°C and 60% humidity) and high humidity (22°C and 80% humidity)). For both, the facemask and the fiber filters analysis, ultrasonic solvent extraction was performed. Sample purification and analysis were performed online with liquid chromatography coupled to a tandem mass spectrometer (LC-MS/MS).

OPEs were detected in all analyzed masks, with levels ranging between 24,7 to 20.427 ng/mask, being KN-95 the most contaminated ones. Results obtained from inhalation experiments showed that temperature positively affect the release of OPEs from the facemask. In addition, high temperature conditions are the most realistic, since the human breathing temperature is 33- 34°C. At these conditions, the mean release percentage is 6%, being the reusable masks the ones that showed a lower degree of release. Once the degree of inhalation has been calculated, the daily intake of OPES due to the use of facemasks has been evaluated, showing always levels safe for the human health. Regarding heavy metals, the highest levels (up to 150 µg/mask) were for Zn and Cu, since these metals are applied as viricides. However, the release percentage for metals is lower than for OPEs, being in most cases < 1%. This leads to levels in the air breathed through the mask below regulated levels in air.

Finally, an environmental impact assessment was carried out, calculating the waste generated globally by the use of facemasks (from 0,2 to 6,3 million tons per year), as well as the amount of OPEs released into the environment (from 12 to 2.360 Kg per year), being the reusable masks the most sustainable option. Thus, considering health recommendations, we can conclude that the best option would be the use of reusable facemasks in outdoor environments, and of FFP2 in indoor environments.

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Polymer coatings with excellent virucidal properties. Preparation, characterization and rapid inactivation of coronaviruses: human HCoV-229E and SARS-CoV-2.

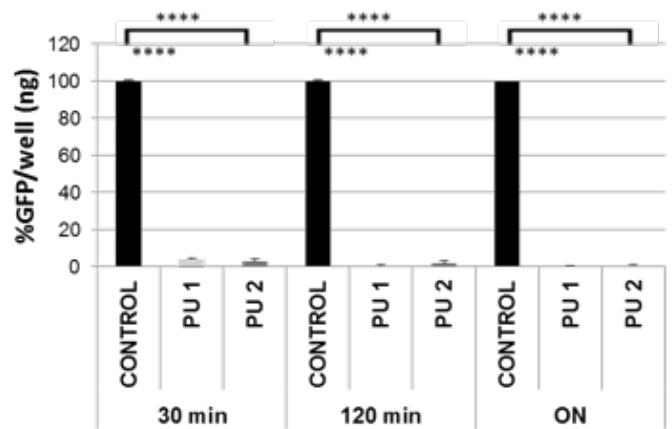
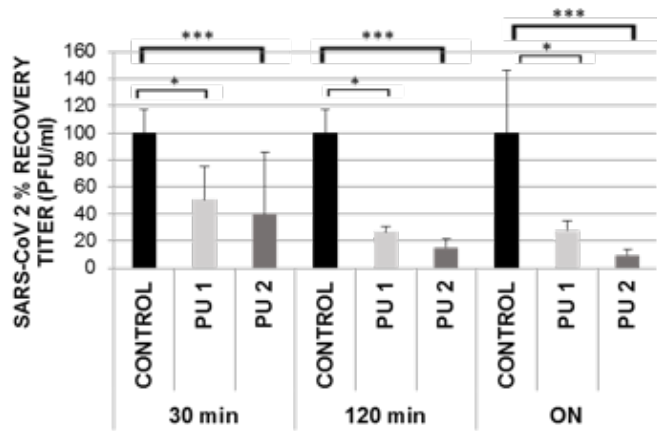
Cástor Salgado Soneira¹; **Raquel Cue López**¹; Vanesa Yuste Sánchez¹; Enrique Martínez Campos¹; Laura Montalvillo Jiménez¹; Aurora Fraile²; Fernando García-Arenal²; Ángela Vázquez Calvo³; Antonio Alcamí Pertejo³; Carolina García Sánchez¹; Paula Bosch Sarobe¹

Background: The COVID19 pandemic caused one of the most dramatic sanitary impacts on the society in the last 100 years, and a massive economical damage. From the transmission routes that contributed to the spread of respiratory viruses, the infection via contaminated objects is relevant. A virus deposited on a surface can infect individuals through self-inoculation with their hands. This mechanism of transmission is especially important in indoor public spaces where there is a large influx of people, through railings, handles and other frequently touched surfaces. The objective of this work we present is the incorporation of N-halamine functional groups to commercial coating formulation, for the development of virucidal polymer surfaces with high antipathogenic efficiency and a broad-spectrum of antiviral activity.

Methods: Materials: Commercial polyurethane and epoxy coatings (GAIRESA) have been functionalized through a procedure compatible with the curing reaction recommended by the supplier, to obtain clear coatings with N-halamine groups grafted onto their surface. Complete surface characterization has been performed by conventional techniques. The extent of functionalization was measured by titration. Stability and rechargeability of the functional group were studied under wet (immersion) and dry (storage in dark) conditions. Virucidal activity was studied against HCoV-229E-GFP, SARS-CoV2 (strain MAD6) and tobacco mosaic tobamovirus (TMGMV P00/10). SARS-CoV2 infectivity after contact with the polymers was evaluated in Vero-E6 cells by lysis plate counting, while the total content of GFP protein was measured in HUH-7 cells in the HCoV-229E GFP inactivation assay. Tobacco plants used for the study of TMGMV were kept in a greenhouse at 20 to 25°C and the necrotic lesions per leaf were counted after the infectivity assays.

Results: The materials were functionalized with the active group on the surface without altering the physical properties of the coatings (degree of crosslinking, gloss). The regenerability and stability of the functional group were studied. The virucidal efficacy of the coatings have been checked against SARS-CoV-2 and HCoV-229E and one very resistant enveloped vegetal virus (TMGMV). Inactivation is obtained against both coronaviruses after less than 30 minutes of contact with the modified coatings, and after 2 hrs against TMGMV. The virucidal activity is maintained after at least three cycles of successive contact with the virus, without the need for a chlorine reloading process.

Conclusions: The virucidal materials are easy to prepare, non-toxic and the functional group is fully regenerable for at least five deactivation/reactivation cycles. The coating surface exhibits a non-cytotoxic and stable behavior both under wet and dry conditions. All coatings show high virucidal activity after short contact times (30 minutes) against human coronaviruses (HCoV-229E and SARS-CoV-2) and also against a vegetal virus. The coating surfaces remain active after at least three reinfection cycles, without the need for a chlorine reloading process. Both capabilities (efficacy and reusage) enhance the applicability and usefulness of these PUR coatings. All these features make this process very promising to be used for modification of commercial epoxy and polyurethane coating formulations to acquire high virucidal activity against viruses of socioeconomic or agricultural impact.



Graphical Abstract

POLYMER COATINGS WITH EXCELLENT VIRUCIDAL PROPERTIES. PREPARATION, CHARACTERIZATION AND RAPID INACTIVATION OF CORONAVIRUSES: HUMAN HCoV-229E AND SARS-CoV-2.

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A new Aerosol Chamber: a tool to assess the stability of viruses in aerosols.

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Although airborne infectious disease transmission events are documented in the veterinary and agricultural biosafety, clinical medicine and public health, the COVID-19 pandemic has highlighted the dangers of airborne pathogen transmission. Viruses such as measles virus, influenza virus, respiratory syncytial virus, human rhinovirus (hRV), severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 are known to be transmitted through aerosols, but little is known about the dynamics of these aerosols. To better understand the stability of viruses in bioaerosols, an aerosol chamber has been designed and constructed.

This aerosol chamber uses dynamic fog cones constituted of liquid nanometric droplets (Counterfog® technology) to generate bioaerosols. In addition, the aerosol chamber has a suspended particle sensor (SPS30, Sensirion) that allows monitoring of particles of different sizes over time. After the generation of virus aerosols, a filtration system through 1 µm polytetrafluorethylene (PTFE) filters connected to the aerosol chamber and a vacuum pump allows bioaerosol recovering. From the PTFE filters, the presence of the virus can be evaluated by viral titration and PCR. In this study, we evaluated the effect of different pressures (11 or 5 bar) to generate the bioaerosol over viral viability. The stability of the bioaerosol generated inside the aerosol chamber and the viability of the recovered virus over time was also evaluated. The viruses employed in this report were bacteriophage φ29, human coronavirus 229E (hCoV-229E) and hRV-14. This aerosol chamber is a useful tool for studying the viability of airborne viruses in BSL2 and BSL3 facilities. Moreover, it could be used to evaluate the efficiency of different filters for capturing biological samples in air and air purification systems.

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Analysis of characteristics exhibited by different commercial masks in order to develop new bio-based, biodegradable and transparent masks

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Background: The main aim of this research consists of comparison of different commercial masks in order to solve some drawbacks that they present and design new biobased, biodegradable and transparent masks against pollutants and microbes, in particular against SARS COVID 19 at present time, taking the best characteristics of each of them.

Methods: The types of masks studied were mainly surgical and FFP2 ones and the brands selected were chosen randomly. Thus, the samples used were: a surgical mask, three non-biodegradable FFP2 masks, a biodegradable FFP2 mask and a partial transparent mask.

The masks were structurally characterized by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) and Scanning Electron Microscope (SEM). The mechanical properties were analyzed by strain-stress tests under tension.

Results: All the masks studied have been manufactured by electrospinning, i.e., using solvents during their production, fact that implies subsequent stages for their removal. Depending on the type, masks is composed by more or less number of layers, which favors different degrees of protection against SARS COVID 19 (or other pollutants or microbes). The fibers from which the masks are made exhibit nanometric size in diameter and, as expected, they show homogeneity in their sizes since they were produced from electrospinning. In addition, the common defects of using this methodology for obtaining fibers are observed in the SEM micrographs.

Mechanical properties prove that masks have higher values of Young’s modulus in the transverse direction than in the longitudinal one. This parameter is not greater than 100 MPa in the former and around 50 MPa in the longitudinal direction.

Conclusions: The use of melt electrospinning could be a satisfactory alternative to produce the fibers required in the mask manufacturing, avoiding the use of polluting solvents and turning out more eco-friendly. Moreover, working with solvents increases production costs and processing time along with feasible health damage due to the solvents trace. Keeping in mind the environment preservation as trending topic, the manufacture of biodegradable masks is the future. For this reason, knowing the products that are currently on the market, how they are morphologically, what materials they are made of and what their production process is, is a fundamental stage in the development of new compounds and in this case of new masks with improved performances.

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How to monitor sub-lineages of different SARS-CoV-2 variants? A case study of Omicron sub-lineages in Murcia (Spain)

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Background: The SARS-CoV-2 variant Omicron (B.1.1.529) emerged in South Africa in November 2021 but since its detection, many different sublineages have emerged and spread worldwide. Currently, Omicron is made up of several sublineages, and they have to be monitored to determine their relative relevance and the global circulation.

Methods: A combination of RT-qPCR, duplex RT-qPCR, digital PCR and whole genome sequencing approaches have been used to determine the levels of SARS-CoV-2 as well as the emergence of different Omicron sublineages, particularly BA.1, BA.2, BA.4 and BA.5. From January to June 2022, up to 200 wastewater samples were weekly collected from 12 wastewater treatment plants (WWTPs) to determine the prevalence and concentration of Omicron sublineages.

Results: SARS-CoV-2 loads in wastewater ranged from 3.2 to over 6.6 log₁₀ genomic copies/L. In the majority of the samples, the Omicron variant of concern (VOC) was present. Changes in sublineages were observed among samples obtained in different time periods. Omicron BA.1 was dominant between January to March, when Omicron BA.2 started to become relevant. In fact, sublineage BA.2 was dominant from April to June. Sublineages BA.4 and BA.5 have been only detected in very low percentages. Metagenomic data of deep-sequencing of the complete S gene revealed that the majorities of samples contain the N679K, Q954H, and N969K Spike mutations of interest associated with omicron BA.1 and BA.2. Additionally, the mutations of interest, R493Q also associated to BA.4 and BA.5 was observed in four WWTPs. However, the presence of others VOC were not detected in this area throughout the study period. These findings highlight the effectiveness of wastewater monitoring as a powerful surveillance tool of SARS-CoV-2 variants that can complement clinical testing.

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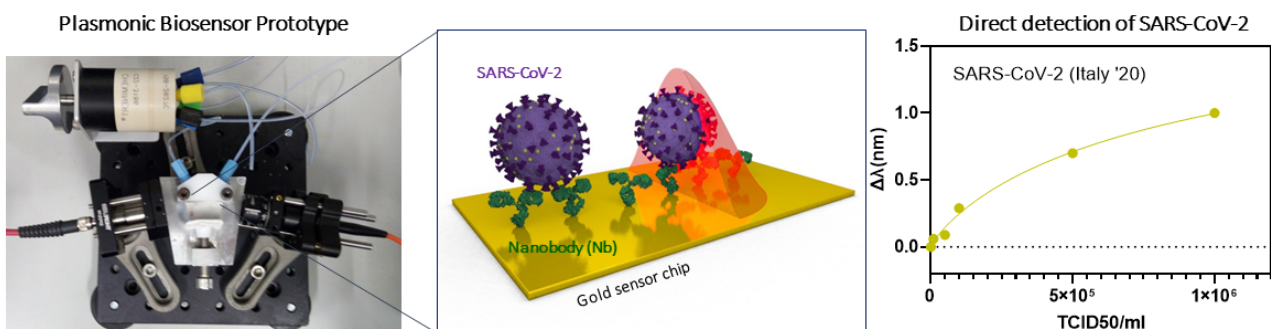
² Entidad Regional de Saneamiento y Depuraci n de Murcia (ESAMUR)

³ Instituto de Agroqu mica y Tecnolog a de Alimentos (IATA-CSIC)

A portable plasmonic biosensor for the direct and fast detection and quantification of SARS-CoV-2

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COVID-19 pandemic has revealed the growing need for efficient, reliable, and affordable diagnostic tools that allow the fast and sensitive detection, identification, and quantification of SARS-CoV-2 while ideally circumventing the sample transport to centralized laboratories to perform the analysis. Implementing such advanced technologies would contribute to ameliorating the inherent limitations of molecular analysis such as PCR, constricted to analytical laboratories and bulky instrumentation. Rapid diagnostic tests have significantly improved the diagnostics during critical infection waves. They are very practical and low-cost but might still lack sufficient sensitivity in some cases and cannot provide quantitative information. We propose the implementation of an optical biosensor based on plasmonics technology, which has been previously developed by our research group, and can provide sensitive, direct, real-time detection in just a few minutes. The biosensor is versatile enough to be applied to a wide variety of target analytes, just by selecting the appropriate specific bioreceptor that provides the necessary specificity. Herein, we have implemented a direct assay that requires the use of specific nanobodies (Nb), which have been specifically produced for SARS-CoV-2, and recognize the RBD region in the Spike (S) protein. The nanobodies were covalently immobilized on the surface of the sensor chip. The experimental conditions for the direct capture of the whole virus (previously UV-inactivated) have been optimized in standard buffer conditions and limits of detection of approximately 5×10^3 TCID₅₀/mL have been achieved in a direct, label-free strategy employing the original variant spread in Italy in March 2020. Several SARS-CoV-2 variants have also been tested to assess the specificity of our assay. In order to evaluate real samples collected during the pandemics, we have evaluated the effect of viral transport media (VTM) employed to conserve the nasal swaps from nasopharyngeal samples on the assay performance and we have reoptimized the assay conditions to minimize the nonspecific adsorptions coming from this fluid. We have also approached the evaluation of saliva, as a potential alternative sample that is easy to collect, and less invasive. Our strategy has demonstrated the potential of this biosensor for the direct detection of coronavirus with high reproducibility. The features of the biosensor prototype, which can be integrated into a user-friendly compact point-of-care device could contribute to a more efficient, decentralized analysis and diagnostics of COVID-19, providing quantitative information on the viral load in a fast and sensitive manner.



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Lung ultrasound aberration correction with singular value decomposition beamforming and pulse-coded excitation

Josep Rodríguez Sendra^{1,2}; Noé Jiménez González²

Background: The poor propagation of acoustic waves in air-filled tissues is a challenge for ultrasound imaging. The lung is composed of alveoli, which are tiny air-filled sacs at the ends of the bronchioles, responsible for the exchange of oxygen and carbon dioxide between the lung and the blood. The complex propagation phenomena in these tissues are translated into different image artefacts, and its mere appearance can be profited to diagnose different diseases. However, as information goes beyond anatomy and morphology, trained personnel is needed to interpret images. In this work, we propose advanced beamforming and coding methods for ultrasound imaging reconstruction, with the aim of providing quantitative and automatic methods to evaluate lung tissue.

We use singular value decomposition (SVD) to perform fast aberration correction during real-time ultrasound imaging. The method mitigates aberrations from second-order random scattering allowing for ultra-fast imaging in lung tissue. Importantly, as B-lines do not arise as a multiple random scattering process, the algorithm will keep the B-lines in the image, as this valuable artefact is commonly used to identify diseases. In addition, we incorporate pulse-coded excitations to improve the signal-to-noise ratio and mitigate attenuation in lung tissue. The combination of aberration-free ultrafast imaging with pulse-encoded excitation has the potential to penetrate deeper in lung tissue, allowing its quantitative characterization by ultrasound. In this work, the performance of the above techniques is evaluated in a lung phantom, and safety tests were performed according to international recommendations.

Methods: A lung phantom was constructed using agar-gelatine to simulate skin and muscle tissue, pig ribs to simulate human ribs, a layer of nitrile to simulate pleura and a triphasic porous structure (wet sponge) to simulate lung tissue. Steel wires were placed at different distances into the sponge to quantify the penetration of ultrasound. For the ultrasonic measurements, a research imaging system (Vantage 256) was used, with a 64-channel phased array imaging probe with a centre frequency of 2.7 MHz and a bandwidth between 1.7 and 3.7 MHz, and a 128-channel probe using a central frequency of 7.5 MHz. Two types of transmissions were used, a conventional short pulse and a synchronised sine sweep excitation. On these two emissions, 3 types of beamforming approaches have been applied: planewave compounding, multiple planewave compounding, and multiple planewave compounding using SVD, where a total of 100 transmissions are emitted in an angular scan (-60° to 60°), the IQ image is formed for each of the receptions and an SVD is applied, to obtain an image capable of correcting the aberrations.

Results and conclusions: We have performed a total of 12 tests with the same conditions as the phantom. By comparing the different beamforming and compounding strategies, we observe that by using synchronized sine sweep excitation penetration and the signal-to-noise ratio are increased, and by using SVD beamformer aberrations can be mitigated. The results of this work open the possibility of retrieving valuable information from the microporous structure of lung tissue, allowing the use of quantitative ultrasound and automatic methods to evaluate lung tissue.

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The occupational health and medicine surveillance unit as a support platform for the PTI Global Health of the CSIC

Marta Bermejo Bermejo¹ ; Juncal Sánchez Alonso¹ ; **Miriam Miranda García**^{1,2} ; Rebeca Jiménez Peral¹ ; Esther Velasco¹

Objectives:

1. Describe the competencies, functions and activities of a Health Surveillance-Unit Occupational Medicine and how to include among them the support to the PTI of Global Health.
2. Synthesize actions to support PTI during the pandemic period.
3. Proposals to support the Global Health PTIs in the present and future

Method: development and fit within the regulatory framework of Law 31/95 and RD 843/2011 of its field of competence. Material and human resources.

Support activities for the PTI Global Health:

- Epidemiology: collaboration in clinical and/or clinical-occupational epidemiological research. New challenges in agents and emerging risks.
- Advice for compliance with the requirements of the Bioethics Committee.
- Informed consents of patients-workers/healthy controls: advice, documentation and reporting.
- Cooperation for the development of laboratory techniques and/or health process.
- Collaboration with researchers in the collection of clinical samples.
- Advice for compliance with specific health surveillance measures, including the development and application of specific medical protocols, in tasks with potential biological, new agents/emerging risks, etc.

Conclusion:

In the last two years we have seen that intersectoral collaboration is essential if we want to make progress in the field of biomedical research. The close collaboration that has been maintained between the Health Surveillance Unit and the different research centers has made it possible to achieve important advances in health prevention and promotion.

¹ Unidad de Medicina del trabajo y vigilancia salud

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Artificial Intelligence algorithms for real-time lung ultrasound assisted-diagnosis in COVID-19

M. Muñoz¹, J.F. Cruza¹, G. Cosarinsky¹, J. Camacho¹ and J. L.-Herraiz²

Background, motivation and objectives: Due to the COVID-19 pandemic, lung ultrasound has emerged as one of the most promising medical techniques for the diagnosis and monitoring of pneumonia, the main critical complication of SARS- COV-2 infection. But the lack of trained personnel in this area has limited the expansion of its use. The learning curve of junior technicians could be reduced by computer aided diagnosis allowing to extend the use of lung ultrasound. This work presents the implementation of real-time algorithms based on Artificial Intelligence (AI) to guide the operator during the exploration and advise about possible diagnoses according to the lung artefacts found.

Methods: A real time algorithm has been developed to facilitate the lung exploration and to detect the presence of pneumonia combining AI models implemented with Keras and Tensorflow 2 and signal processing algorithms using python language. This flow helps the technicians to be guided through several coloured labels on the screen in order to obtain the best image conditions according to the region explored, the movement and probe orientation and the similarity with previously labelled pulmonary images. First, the acquired image is evaluated by the AI models in order to identify if it is suitable for processing or not. Once the image is marked as valid, another AI model is used to detect the pleura, which is the key to be able to detect the presence of typical lung patterns such as A-lines, pleura irregularity, B-lines and consolidations.

Due to the computational cost of AI algorithms, it was necessary to use multiple parallel processes using an i7 octacore CPU to obtain a continuous image refresh rate.

Results and conclusions: A rate of 16 frames per second was processed, without any delay in processing, being capable to detect in real-time the typical artefacts that allows differentiating between healthy lungs and pneumonia. This is an acceptable image rate to track with human eye fluently.

In conclusion, it was confirmed that it is possible to develop real-time algorithms for pneumonia detection based on AI models using CPU. Future work includes computational improvements to speed up processing such as the use of graphical processor units (GPUs) and novel Adaptive Compute Acceleration Platform (ACAP) devices.

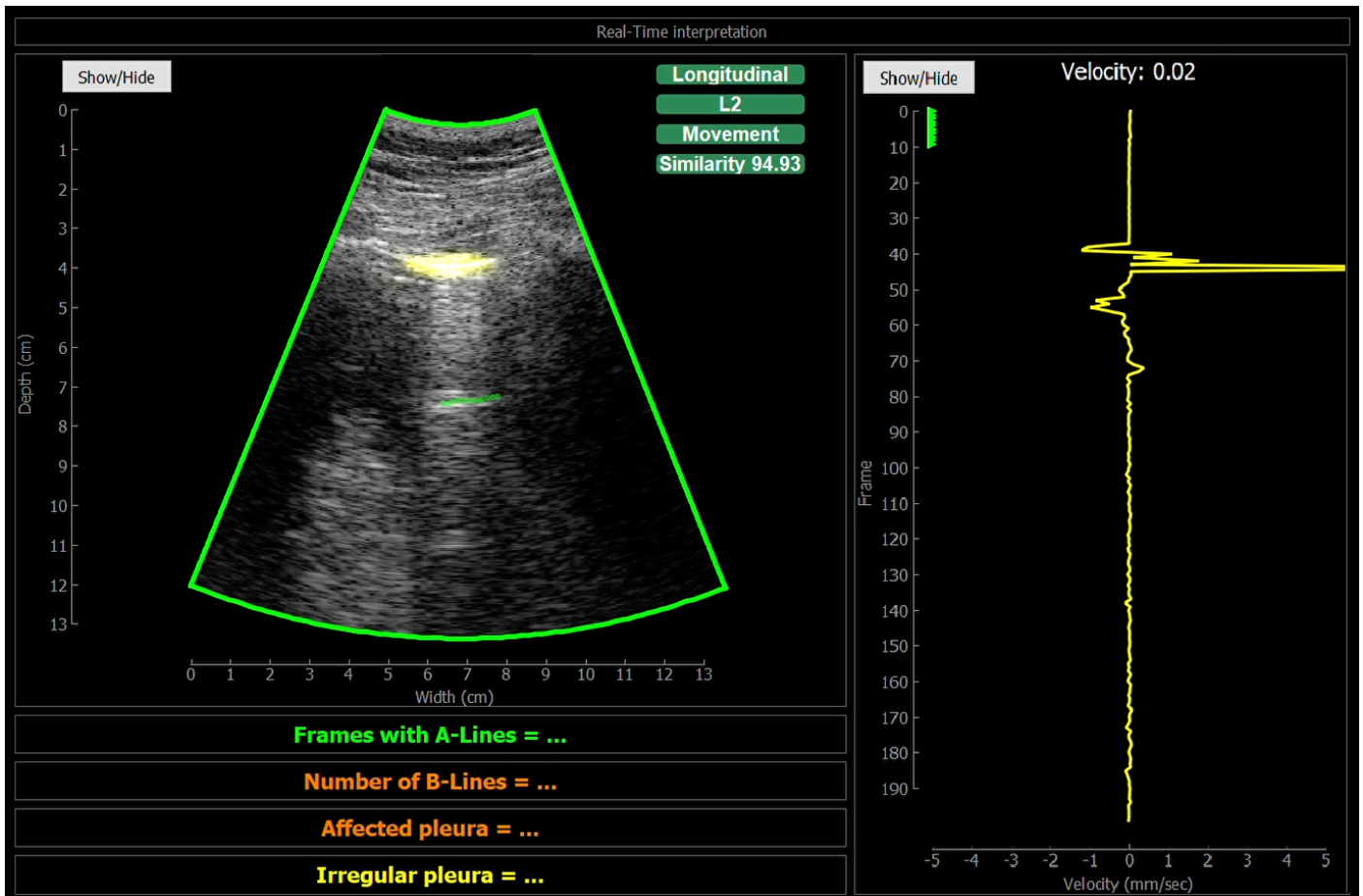


Figure 1. Real-time pleura and A-lines detection sample screen with probe movement graph

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FokI-driven signal amplification platform for enhanced detection of viral RNA species

Juan Ramón Tejedor¹ ; Annalisa Roberti¹ ; Mario F. Fraga¹

Background: Adequate signal quantification is a critical parameter for accurate pathogen diagnosis. The quantitative real time, reverse transcription polymerase chain reaction (RT-PCR) technique is a gold standard in microbiology laboratories. However, while this technology excels in terms of its specificity and sensitivity, it does require specialized equipment and alternative approaches that can be scaled to facilitate real-time surveillance of a particular disease have been proposed in recent years. We have recently developed a novel molecular detection methodology for the identification of SARS-CoV-2 RNA in COVID19 positive samples. This signal amplification approach, which is powered by the nickase activity of the FokI restriction endonuclease, enhanced the detection signal of its target molecules in a highly specific manner. In addition, we were able to detect other human B-coronaviruses in a multiplex setting, including various SARS-CoV-2 variants, highlighting the potential of this method for the detection of multiple viral targets of interest. Taking these advantages into account, and in order to reinforce the diagnostic potential of this technique, we have now extrapolated our observations to an additional set of respiratory viral pathogens of interest to the public health system.

Methods: We designed an extended set of dumbbell-like fluorescent sensing oligonucleotides against 11 human single-stranded RNA respiratory viruses, including Influenza A, Influenza B and Influenza C (family Orthomyxoviridae), Alphacoronavirus (229E and NL63) and Betacoronavirus (OC43, HKU1 and SARS-CoV2) (family Coronaviridae), Rhinovirus and Parechovirus (family Picornaviridae), Respirovirus (HPIV1 and HPIV3) and Rubulavirus (HPIV2 and HPIV4) (family Paramyxoviridae), Metapneumovirus (HMPV) and Orthopneumovirus (hRSV) (family Pneumoviridae). FokI-assisted digestion of such detection probes in the presence of their cognate target molecules was performed to test the signal amplification efficiency of this approach in other target scenarios. In addition, to improve the detection limit of this system, a molecular coupling has been implemented between the FokI-assisted reaction and the isothermal rolling circle amplification technique under the mentioned conditions.

Results: We are currently testing the signal amplification efficiency of such dumbbell-like fluorescent sensing oligonucleotides in the context of synthetic target nucleic acids, both at the level of the simple signal amplification approach (driven by FokI) and in the context of the molecular coupling with the rolling circle amplification technology. An update of such results will be presented and discussed on the site during the congress.

Conclusions: The implementation of the proposed signal amplification platform for diagnostic purposes highlights the possibility of considering this FokI-assisted reaction as a potential methodology for the identification of viral nucleic acids of particular relevance to human health.

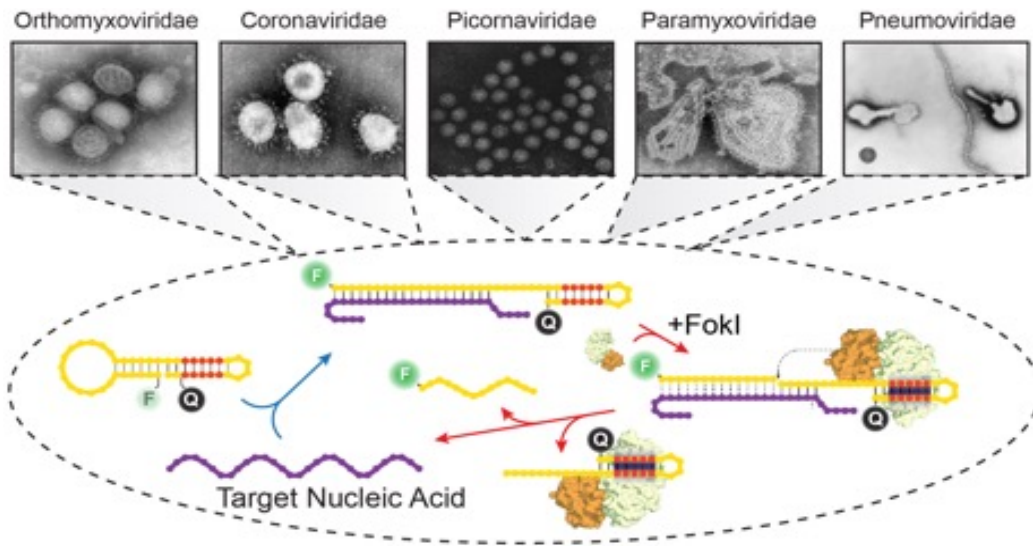


Figure 1.

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Point of Care (POC) system based on a calorimetric LFA (C-LFA) for the detection of genetic material.

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Laboratory diagnosis play an important role in many diseases' management. Early diagnostic is the key to successful treatment providing care at the initial stages. It is especially important in diseases such as viral or bacterial infections and cancer, where time is a crucial factor. The diagnosis often is made by clinical criteria but it is also necessary carry on specific diagnosis methodologies as medical imaging or laboratory testing. Several biomarkers, especially molecular biomarkers as DNA, RNA or proteins, have being found to supplement this important clinical diagnosis. Regarding this, accurate and fast laboratory tests are necessary. Reverse-transcriptase real-time polymerase chain reaction (RT-PCR) is the most sensitive and specific test for genetic material detection, however, this method takes several hours. Rapid diagnostics tests (RDTs) have being developed to provide quicker results but generally they suffer from lack of sensitivity. Point-of-care (POC) biosensors such as chip-based and paper-based biosensors are typical rapid, portable, cost-effective, and user-friendly, which can be used for genetic material detection. Combining conventional POC biosensor, how is the lateral flow methodology, with inorganic nanoparticles, as gold nanoprisms (AuNPrs), a novel ultrasensitive Calorimetric Lateral Flow Assay (CLFA) was developed. Gold nanoprisms (AuNPrs) biofunctionalized with a specific DNA oligonucleotide to recognize the desired genetic material, will be used as thermal transducers for the biosensor. Due to their optical properties, AuNPrs can convert light into heat. The desired genetic material, either RNA or DNA, is further recognized by the AuNPrs and the capture biomolecule deposited on the nitrocellulose strip, the subsequent no visible test line irradiation with a NIR laser will generate a visible spot in a thermosensitive paper than could be further quantified. With this novel nanobiosensor, we are able to detect genetic material with high specificity and sensitivity, overcoming the classical limitations of LFIA tests (low sensitivity and lack of quantification). Calorimetric Lateral flow assays have a wide array of applications and can test a great variety of samples, here the detection of two different biomolecules have been illustrated: detection of viral RNA from Covid-19 and Influenza viruses and the detection of miRNA from exosomes (EVs) derived from neurons.

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SARS-CoV-2 detection exploiting different class 2 CRISPR-Cas systems

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The pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has highlighted the challenges in diagnostics of viral infections, especially when a fast, massive, and reliable intervention is required to reduce the transmission. In recent years, together with suitable nucleic acid amplification methods, clustered regularly interspaced short palindromic repeats (CRISPR) systems have been repurposed for diagnostic applications[1], showing advantages in terms of broad applicability. First, we use the formation or destruction of canonical and non-canonical protospacer adjacent motifs in the nucleic acids of interest to detect specific mutations with the Cas12 nuclease[2]. For example, in collaboration with the groups of P. Domingo-Calap (I2SysBio) and J. Navas (UniCan), we can identify genomes harboring the E484K spike mutation or Omicron from patient samples without sequencing. Second, in collaboration with the groups of G. Gómez (I2SysBio), J.A. Daròs (IBMCP), and D. Navarro (INCLIVA), we unveil the small RNA landscape associated with the SARS-CoV-2 infection with the aim of extracting suitable biomarkers of infection and prognosis. We design a CRISPR-Cas13 system to detect without the need of pre-amplification a truncated tRNA that is greatly overexpressed upon infection, thereby sensing indirectly the virus.

Third, we develop a novel strategy to detect nucleic acids based on a Cas9 nuclease, whose mode of action relies on strand displacement rather than on collateral catalysis[3]. Given a pre-amplification process, a suitable molecular beacon interacts with the ternary CRISPR complex to produce a fluorescent signal. We show that a multiplexed detection of SARS-CoV-2 from patient samples is possible. Overall, all these results based on repurposed CRISPR-Cas systems would represent useful preparedness actions for future pandemics.

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Antibody production and immunoassay development for the detection of Nucleocapside and Spike 1 protein from SARS-CoV-2

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Background: In search for detecting early infection of SARS-CoV-2 causing coronavirus disease-2019, to help fight this disease. The development of a multiplexed immunoassay for the determination of antigens from SARS-CoV-2 would improve the sensitivity and specificity of the assay. The current commonly named “rapid test” just detect the presence or absence of the nucleocapsid (NC), however we believe that detecting also Spike 1 (S) protein will improve the clinical value of this assay. Thus, it is proposed the production of different antibodies with the final aim to select the most suitable combination with the highest detectability, robust against different transport media and robust also to the main variants of concern alfa, delta and omicron. Both multiplexed ELISA and rapid test (lateral flow immunoassay) have been developed.

Methods: Rats and mice aged 8 – 10 weeks were immunized with 100 µg/mice of NC and S protein in PBS. Spleen cells were fused with P3-X63/Ag8 murine myeloma cells and culture in DMEM supplemented. Stable anti NC and S proteins monoclonal hybridoma cell lines were selected by indirect ELISA and culture to obtained monoclonal antibodies that were purified from the culture supernatant. Polyclonal antibodies were produced by direct inoculation of both proteins of interest (NC and S1 proteins from Wuhan) separately. Three final sera were obtained. Most of the antibodies produced were characterized by Surface Plasmon Resonance in order to determine the affinity parameters.

The screening of the most suitable pair or antibodies for immunoassay development was performed by ELISA. Finally, the LFIA development was performed using standardized methods well established in our laboratories for antibody immobilization, antibody gold nanoparticle conjugation and construction of the final rapid test.

Results: After the screening of the mAbs production, it was possible to isolate a pair of mAbs for S1, and a pair for NC, with high sensitivity. Taking into consideration also pAbs produced, all available Abs were characterized by SPR, showing in all of them KD of 10⁻⁸-10⁻¹⁰ M⁻¹. The selectivity profile was determined against different regions of the S1 and NC. Specifically, for S1 antibodies, the main variants of concern were tested showing that few of them were robust against S1 independently of the variant tested. The limit of detection (LoD) obtained by ELISA was 0.1 ng/mL for NC and 0.5 ng/mL for S. Each pair were implemented in a LFIA test that could reach a of 0.9 ng/mL for NC protein and 5 ng/mL for S protein. The S protein variants were detected by its corresponding combination of monoclonal antibodies with similar LoD 5 ng/mL. A robustness study has been performed to validate different commercial transport media, revealing that most of them can be used in the immunoassay analysis.

Conclusions: A great collection of antibodies against the main proteins for concern from SARS-CoV-2 have been produced and characterized by SPR, ELISA and LFIA. Both ELISA and LFIA showed high sensitivity and specificity of the selected combinations of monoclonal antibodies specifically for NC and the different variants of concern (alpha, delta and omicron).

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CSIC Chemical Library (Quimioteca CSIC)

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Background: Rapid urbanization, increased mobility and global economic interdependence exacerbate the threat of emerging pathogens and add to the challenge of containment. Being adequately prepared to detect, manage, and respond to emerging infectious disease outbreaks has never been more imperative.

In this context, the outbreak of COVID-19 pandemic highlighted the necessity to access to a large collection of synthetic molecules as candidates for antiviral drug discovery. To meet this challenge, the CSIC promoted the constitution of “Quimioteca CSIC” [QCSIC, Institutional Chemical Library] by collecting, organising, classifying and storing chemical compounds produced by CSIC scientists.

The purpose of QCSIC is focused on:

- Enhance the value of the compounds obtained within the Institution.
- Establish contacts and promote collaborations between CSIC groups and/or with private Foundations or companies from different fields (chemists, biologists, biochemists, clinicians, etc.) interested in screening the chemical library in order to obtain hits and/or leads for their projects.
- Creation of new Intellectual Property Rights (IPR).

QCSIC includes an original collection of quality-controlled substances with a consistent coding system in a format compatible with high-throughput screening (HTS) techniques, and their inventory in a virtual database, Lg-Chimio. Currently, the QCSIC is implemented at three different CSIC centres (IIQ, IQM, and ICAQ), although the long-term goal is to include all CSIC organic and medicinal chemistry centres and groups.

As a permanent and transversal initiative, the QCSIC is not limited to find antiviral drug candidates; but applies to nearly all type of biological targets. As such, the QCSIC is of interest to academic and industrial chemists and biologists. For chemists, it is a way to enhance and value their molecules beyond their primary purpose, by supplementing them with new biological data. For biologists, the QCSIC offers a collection of substances with original and diversified structures with the possibility of providing analogues of hit compounds. For all partners, the QCSIC is a valuable tool for starting new interdisciplinary research projects, enabling them to accede to new proposal calls and new financing possibilities. These collaborations will accelerate the discovery of chemical probes and lead compounds, thus providing the researchers with new intellectual property rights (IPR) and patents. The QCSIC will thus be offered to all those scientists public or private who wish to screen it.

Ultimately, the QCSIC is a valuable way to preserve the heritage and know-how of the research groups, the research institutes and the Institution.

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Quality assurance of Machine Learning algorithms

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ML is gradually becoming a major large-scale enhancement of modern medicine. It is increasingly presented as a ubiquitous tool in a large number of clinical applications that range from clinical decision support such as diagnostic, follow-up and prognosis aid methods, medical image classification and pattern recognition findings, to health systems support such as prediction of clinical outcomes, risk stratification for mortality, readmissions or ICU admissions, and for self-care of patients as well.

Depending on the field of application, the training of an ML algorithm can be subject to a series of unintended biases that could lead to unreliable models. Essentially, potential biases in ML are related to the dataset and how it has been collected. Thus, incorporation biases, automation biases, sampling biases, convergence biases, overgeneralisation biases and confirmation biases, among other risks, may occur. These biases can be related to mislabelled data, the lack of relevant data with respect to the number of samples needed to train, the variability of the population, the lack of generality due to differences in clinical devices, as well as in medical procedures, or mixing data sources, among others. Hence, one must analyse the available data and its origin before using them to build models.

To ensure quality in the results of ML algorithms, a large number of tests related to data must be performed. Reproducibility, stability and traceability must be guaranteed. These tests may require a large number of additional actions with the data. This includes a great persistence of the training data, external validation for robust assessment of the predictive accuracy, quality collections to verify performance under changes in the underlying distributions of the data, and proper selection of performance indicators and methods to obtain new data for lossless model retraining should be provided from the original values to determine the stability of the results.

In addition, there are now methods and tools that can be used to assess and reduce the risk of bias in ML training datasets: PROBAST, TRIPOD or STARD present a number of elements that should be taken into account when conducting studies with predictive models, presenting their results and assessing the risk of bias. This helps to improve the quality of the results of these models, their reproducibility and the expectations of implementation in real production environments.

The aim of this communication is to present a set of good practices, including the use of these tools, to try to ensure the best results in AI models in order to facilitate their implementation in the health system and improve their impact on health.

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A single dose of an MVA vaccine expressing a prefusion-stabilized SARS-CoV-2 spike protein neutralizes variants of concern and protects mice from a lethal SARS-CoV-2 infection

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Background: Novel safe, immunogenic, and single-dose effective vaccines are needed to control the COVID-19 pandemic, caused by SARS-CoV-2.

Methods: We generated an optimized COVID-19 vaccine candidate based on the modified vaccinia virus Ankara (MVA) vector expressing a full-length prefusion-stabilized SARS-CoV-2 spike (S) protein, termed MVA-CoV2-S(3P), containing 3 mutations in the furin cleavage site (R682G, R683S, and R685S) and three proline substitutions in the S2 region (A942P, K986P, and V987P) for improved stability.

Results: The S(3P) protein was expressed at higher levels than the non-stabilized S in cells infected with the corresponding recombinant MVA viruses. One single dose of MVA-CoV2-S(3P) induced higher IgG and neutralizing antibody titers against parental SARS-CoV-2 and variants of concern than MVA-CoV2-S in wild-type C57BL/6 and in transgenic K18-hACE2 mice. In immunized C57BL/6 mice, two doses of MVA-CoV2-S or MVA-CoV2-S(3P) induced similar levels of SARS-CoV-2-specific B- and T-cell immune responses. Remarkably, a single administration of MVA-CoV2-S(3P) protected all K18-hACE2 mice from morbidity and mortality caused by SARS-CoV-2 infection, reducing SARS-CoV-2 viral loads, histopathological lesions, and levels of pro-inflammatory cytokines in the lungs.

Conclusions: These results demonstrated that expression of a novel full-length prefusion-stabilized SARS-CoV-2 S protein by the MVA poxvirus vector administered as a single dose enhanced immunogenicity and efficacy against SARS-CoV-2 in animal models, further supporting MVA-CoV2-S(3P) as an optimized vaccine candidate for clinical trials.

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The non-replicative antibiotic resistance gene-free plasmid vector pPAL for the development of DNA vaccines.

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Background: The pPAL vaccination platform consists of the pPAL plasmid, whose production has been fine-tuned under good manufacturing practice (GMP). The main objective is to use this plasmid, which doesn't contain antibiotic resistance genes, for the development of DNA vaccines and thus its worldwide distribution. We have generated the Neoleish vaccine against natural infection of canine leishmaniasis, which elicits protection in 60% of vaccinated dogs. Leishmaniasis is a vector-borne parasitic disease that, causes more than 50,000 deaths per year in its visceral form.

Methods: The generation of the pPAL plasmid was carried out from the pCIneo expression mammalian cells plasmid. The procedure consisted of replacing the antibiotic resistance genes npt and bla by the fab I gene from a modified Escherichia coli strain (SURE) with a reduced of genetic material, and its cor responding promoter. A PCR cloning procedure was applied to obtain the recombinant pPAL-LACK vaccine using XbaI and EcoRI target sequences. In vitro expression of the LACK gene in the human HEK293 cell line was verified by Western blot. Evaluation of the humoral immune response of the NeoleishR vaccine in Beagle dogs against canine leishmaniasis was carried out by detecting the level of total IgG and IgG1 and IgG2 subclasses by ELISA. The evaluation of the parasite load in the main target organs (bone marrow, spleen and liver) was performed by qPCR. In addition, the percentage of CD4+ cells and the level of cytokines IFN γ and IL-10 were determined by lymphoblastic transformation test (LTT) in PBMC.

Results: We have developed an antibiotic resistance gene-free plasmid, which requires the use of triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol) instead of an antibiotic as a selection agent (Alcolea et al., 2019). Selection of transformants that carry our plasmid of interest is based on the tolerance to this molecule, which toxic to the bacteria.

The fab I gene, encodes the enoyl-ACP reductase (ACP being the acyl carrier protein), an enzyme essential for fatty acid synthesis in E. coli and is sensitive to triclosan.

This plasmid has been used in the development of a naked DNA vaccine using a homologous prime-boost regimen via intranasal route (NeoleishR), against canine leishmaniasis, achieving 60% protection. This vaccine is based on the LACK gene (activated protein kinase C receptor analog) of Leishmania infantum, which causes zoonotic visceral leishmaniasis. The expression of the LACK gene in HEK293 cells was confirmed by Western blotting. Protection by this vaccine is characterized by a reduction 60 of parasite load and symptomatology, including a robust cellular immune response with a high production of IFN γ in protected dogs, which is an essential factor in the development of vaccines against an intracellular parasite.

Conclusions: The pPAL vaccination platform could be used in the development of DNA vaccines against other infectious diseases. The non-replicative pPAL plasmid which doesn't contain antibiotic resistance genes, therefore allowing its worldwide distribution.

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Lyophilized homodimers of the RBD domain of spike protein as vaccine against SARS-CoV-2

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The unprecedented research carried out to counteract COVID-19 pandemics, has resulted in the development of efficacious vaccines, most of them based on the full-length spike (S) protein (420 kDa) of SARS-CoV-2 as antigen. However, few approaches have resorted to using the S protein's receptor binding domain (RBD) as a vaccine candidate. The RBD is an essential target to eliciting protective immune responses against SARS-CoV-2. Additionally, its reduced low molecular mass bestows RBD (29 kDa) relevant potential advantages, i.e. reduced large-scale production costs and improved long-term stability for the development of second generation of COVID19 subunit vaccines. Accordingly, we have expressed two RBD versions (RBD1 and RBD2) in insect cells. Both recombinant RBD polypeptides are secreted to the extracellular medium, from where they are efficiently purified applying two subsequent chromatographic steps, i.e. affinity chromatography followed by size exclusion chromatography. Expression of both recombinant proteins results in the production of a combination of dimer/monomer RBD molecular species.

Interestingly, the relative abundance of the dimeric species is significantly higher following RBD1 expression. Immunogenicity analyses carried out in mice showed that anti-RBD antibody titer induced by RBD1 is considerably higher than those recorded after immunization with RBD2. This indicates the existence of a positive correlation between RBD homodimerization and immunogenicity. The consistent induction of high SARS-CoV-2 neutralizing antibodies titers in RBD1-immunized mice led us to further explore the potential of this polypeptide as a vaccine candidate. We have developed a pre-industrial process leading to the production of stable, lyophilized, pure RBD1 protein lacking recombinant tags. To analyze RBD-1 protection potential, groups of transgenic K18-hACE2 mice were immunized intramuscularly at 21 days intervals, with three doses of lyophilized RBD1 (50 µg each). 18 days after the last boost, mice were challenged with a lethal dose (2×10^4) of SARS-CoV-2 (Wuham strain). Immunization with RBD1 induced a complete protection (100%) of challenged animals. Additionally, RBD1-immunized mice did not show clinical signs of disease nor body weight reduction after virus challenge. This work is supported by PTI Salud Global. grant: SGL2103058.

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A fast and reliable vaccine platform based on Vaccinia MVA: COVID-19 vaccine candidates confer efficient protection in the mouse and hamster disease models.

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Its past use as Smallpox vaccine, together with improved genetic modification techniques, have expanded the use of Vaccinia virus as a vaccine not only for humans but also for farm animals or even wildlife. As a vaccine vector, it offers many advantages, low virulence, good immune response, good genetic stability and temperature resistance (it is not necessary to maintain the cold chain). In addition, it allows the introduction of large portions of exogenous DNA, making it possible to introduce multiple genes in a single vaccine vector. We developed a novel system for the isolation of recombinant vaccinia virus, starting from a double mutant virus ($\Delta A27L-\Delta F13L$), which is unable to be transmitted from cell to cell. Reintroduction of the A27L and F13L genes acts as a powerful selection to mediate dual insertions into the viral genome.

This selection system facilitates the combined expression of multiple foreign proteins from a single recombinant virus. We have applied this system to the MVA strain of Vaccinia virus and generated a panel of vaccine candidates against SARS-CoV-2 that express protein S separately or in combination with other SARS-CoV-2 proteins, in order to broaden the immune response to vaccination. We have characterized the viruses with the different combinations and tested the response in a mouse and hamster model in order to select vaccine candidates. Prefusion-stabilized S protein of different SARS-CoV-2 variants were expressed as complete proteins that were correctly transported to the cell membrane. Mice vaccinated with the recombinants induced anti-S neutralizing antibodies and were able to induce protection against a lethal dose of SARS-CoV-2. Similarly, Syrian hamsters were protected against SARS-CoV-2 infection by vaccination, which prevented lung pathology, a variety of clinical signs and, importantly, virus dissemination in the brain. As a whole, those results point to these vaccine candidates as inducers of a robust immunity. Notably, this vaccine platform facilitates the fast isolation of new vaccine candidates. Results obtained by co-expression of additional SARS-CoV-2 genes will be presented.

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Discovery of novel pikfyve inhibitors as potential antiviral agents

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Background: PIKfyve is a kinase involved in endosomal maturation. It is responsible for the synthesis of phosphatidylinositol-3,5-biphosphate (PI(3,5)P₂) from phosphatidylinositol-3-phosphate, and is implicated in various trafficking events associated with the endocytic pathway, which are essential for endosomal maturation. Given that most of the circulating viruses infect cells through the endocytic pathway, PIKfyve inhibition could be an interesting target to avoid Ebola virus, SARS-CoV-2 or African Swine Fever virus entry (1). However, despite the potential of PIKfyve as a therapeutic target, the number of reported inhibitors as well as their chemical diversity is still lacking.

Methods: We have performed structure-based virtual screening of our in-house chemical library, named MBC library (2) on the crystal structure of PIKfyve (PDB: 7K2V) to search for new inhibitors. To increase the chemical diversity, a two-step virtual screening, consisting on a shape screening based on the structure of known PIKfyve inhibitors, such as apilimod or YM201636, followed by another structure-based virtual screening of the EU-OPENSOURCE chemical library have been performed. In parallel, we have also implemented in our laboratory the NanoBRET™ Target Engagement (TE) assay with the aim to evaluate intracellular kinase activity.

Results: New small hits with suitable intracellular PIKfyve kinase inhibitory activity have been found and their antiviral effect demonstrated.

Conclusions: The in-house implementation of NanoBRET™ TE Assay combined with virtual screening has allowed the identification of a novel family of PIKfyve inhibitors with antiviral activity.

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New thiophene derivates as potential drugs against Ebola virus

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Background: Ebola virus (EBOV) enters the cell through a set of complex interaction processes. Mainly it is internalized via micropinocytosis, although a clathrin-dependent endocytosis is used as an alternative entry route (1). The clathrin-dependent endocytosis is a recruitment process that allows the internalization of external agents into the cells. Cyclin G-associated kinase (GAK) is a kinase belonging to the Numb-Associated Kinases (NAKs) family and it is essential for clathrin trafficking (2). It has been reported that some GAK inhibitors protect against morbidity and mortality in murine models of Ebola infection (3).

Methods: Novel GAK inhibitors were identified through virtual screening in the ATP binding site of GAK (PDB: 5Y7Z) and subsequent antiviral evaluation in a lentiviral EBOV-glycoprotein-pseudotyped infection assay. The so identified hits were further optimized.

Results: A new family of GAK inhibitors, bearing a thiophene ring, was found to be active in pseudotyped EBOV-viruses. After exploring different chemical modifications around the selected hit, some structure-activity relationships were established and the binding mode at the active site of GAK proposed.

Conclusions: This novel family of antiviral compounds demonstrated to have a great potential to be developed as anti-EBOV drugs, highlighting GAK as a promising new target for antiviral agents development.

Acknowledgments: The project leading to these results has received funding from “la Caixa” Banking Foundation under the project code LCF/PR/HR19/52160012 and from the European Commission – NextGenerationEU (Regulation EU 2020/2094), through CSIC’s Global Health Platform (PTI Salud Global). M. M. holds a predoctoral FPU grant (FPU18/03493) from MICINN.

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CryoEM structures of the SARS-CoV-2 spike bound to antivirals

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Background: Single-particle cryoelectron microscopy (cryoEM) has played a key role in the fight against COVID-19. The molecular mechanisms for the action of some of the currently approved drugs targeting the SARS-CoV-2 RNA-dependent RNA polymerase, the fast developments of the current available vaccines and antibody therapies are examples of the impact of the knowledge gained from the cryoEM structures of SARS-CoV-2 proteins in complex with proteins (ACE2 or antibodies/nanobodies) or small compounds. Our aim is to use this technology to understand structurally how certain antiviral compounds and proteins targeting the spike may inhibit viral entry.

Methods: 1) Production of wild-type and mutated spike and ACE2 proteins using baculovirus/insect cells. 2) Spike binding kinetics: protein-protein and protein-small compound interactions measured by BLI Biolayer interferometry (BLI) and/or microscale Thermophoresis (MST). 3) Buffer optimization for cryoEM grid preparation of spike variants by thermal shift assays and negative-staining electron microscopy (NSEM). These techniques are also used to adjust the molar ratio of spike:ACE2 and spike:small-compound complexes. 4) Structural characterization by cryoEM.

Results: At IBV-CSIC we have created a pipeline for the production and characterization of several spike variants and ACE2 decoys. While this pipeline is described in detail in other oral/poster communications, this communication is centered around one of the pillars within this pipeline; the structural characterization of possible drug candidates bound to the SARS-CoV-2 spike by cryoEM. In this way, we have successfully solved structures of the spike bound to: A) protein inhibitors as ACE2 decoys; B) a small inhibitory compound; C) mixtures of proteins and small-compound (nanobody-heparan derivative) working cooperatively as inhibitors. These protein/drug candidates were previously selected based on the results obtained in our interactomics platform, whereas their concentration and the buffer conditions for cryoEM grids preparation were established based on thermal shift assays and NSEM.

Conclusion: CryoEM is a powerful tool to directly visualize the effect caused by a potential drug on a protein target. In a short period of time we have developed this technique in our institute to be applied to the SARS-CoV-2 spike protein, not only to obtain high-resolution structures of SARS-CoV-2 spike variants of concern (see WP4) but also to obtain the structures of complexes of the spike with various inhibitory compounds of very different nature.

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Proteolytic chimeras targeting purine biosynthesis: at the crossroads of antiproliferative and antiviral effects

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Background: Targeted protein degradation has emerged as an innovative and versatile approach for the development of novel and more efficient therapies. In a conventional approach, proteolytic targeting chimeras (Protacs) are heterobifunctional molecules that simultaneously bind a target and an E3 ubiquitin ligase, thus inducing ubiquitination and proteasome-dependent degradation of the target. In alternative designs, chimeras recruit not E3s but other factors with the capacity of inducing degradation of the target.

Purine metabolism exerts a pivotal regulation of multiple cellular functions. Among them, synthesis of nucleotides as building blocks of nucleic acids represents a limiting step in processes demanding high levels of DNA or RNA production. Therefore, blocking key steps in purine synthesis exerts a control in either cell proliferative or viral infection events.

Methods: In the present work we have produced and characterized a series of 11 degrading chimeras targeting a key factor in purine biosynthesis (defined herein as “Target P”). We have compared the efficiency of chimeras based on recruitment of E3s, chaperones, 26S proteasome and autophagosome, in the degradation of Target P. Additionally, we have tested different linker sizes in chimera synthesis.

Activity of compounds were tested in human cell cultures. Target P degradation was assessed by immunoblotting. Effect in cell viability was analyzed by MTT. Association of chimeras with their interactors has been assessed by surface plasmon resonance and by specific cellular ligand-receptor recognition assays. Cell permeability was estimated by quantitation of compounds in medium and cell extracts by UPLC-HRMS. Effect of compounds in SARS-CoV-2 infectivity was analyzed in the Virus Biotechnology Platform, at the CNB (CSIC).

Results: We have observed degradation of Target P by chimeras based on 26S and chaperone recruitment, but not when using E3- and autophagy-based chimeras. Furthermore, linker length has appeared to strongly influence chimera activity. Best behaved compounds exhibit KD constants around 15 μ M. We have established correlations between depletion of the target and decrease of cell viability. Testing the antiviral effect of compounds has not revealed so far a neat antiviral effect due to a strong decrease of cell viability.

Conclusions: At the present stage, a series of Target P-degrading chimeras has been developed. Target P depletion has appeared to strongly correlate with decrease of cell viability but not with SARS-CoV-2 infectivity decrease. Further studies are required to determine the potential of these novel compounds as therapeutic tools.

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Scipion-Chem: a traversal tool for the development of antiviral drugs

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Background: The field of Virtual Drug Screening (VDS) studies and develops computational techniques directed to finding new drugs (ligands) for known biological targets (receptors). VDS is mainly used as an initial step prior to the typical drug screening workflow, allowing only the most promising ligands to move to experimental testing and thus reducing the considerable cost of this experimental workflow. In this communication, we present Scipion-chem, a computational platform for VDS that combines several widely used software suites and biochemical tools and offers them to the user with a friendly Graphic User Interface (GUI).

Methods: Scipion-chem is developed over Scipion, a workflow engine [1] particularly well-suited for structural studies of biological macromolecules. Previously, Scipion-em was developed as a platform oriented to Electron Microscopy (EM) and protein structure determination. Now, in Scipion-chem, we have included workflows for Structural Based and Ligand Based Screenings including some of the most common programs used for VDS (such as Schrödinger, AutoDock, RDKit or Gromacs). This platform, under ongoing development, also offers consensus tools in order to compare and combine results coming from these different programs.

Results:

- 1) Scipion-chem offers complete, flexible and functional workflows for VDS along with an adaptable and user-friendly interface.
- 2) Scipion-chem offers consensus tools which are able to effectively combine the information obtained from different programs.

Conclusions: Scipion-chem provides a user-friendly and useful tool for working on VDS projects due to its flexibility, its intuitive GUI and the incorporated workflow control, which allow the user to easily design and analyze automated and customizable workflows.

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Small-Molecule Inhibitors against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

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The current coronavirus disease 19 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is having devastating consequences, with more than 629M infected people and over 6.2M deaths worldwide (<https://covid19.who.int/>, accessed on 24th May 2022).

Although vaccination advances at good pace, effectiveness against emerging variants is unpredictable. The virus has displayed a remarkable resistance to treatments and no drugs have been proved fully effective against COVID-19. Thus, despite the international efforts, there is still an urgent need for new potent and safe antivirals against SARS-CoV-2, especially for non-hospitalized patients, to prevent hospitalization and death.

A Peptidomimetic's group library, belonging to IQM-CSIC Library, was screened at a fixed initial dose (10 μ M) in the CNB Antiviral Platform. This assay is a phenotypic cell-based assay that recapitulates every aspect of the virus replication cycle in Vero E6 cell monolayers infected with the SARS-CoV-2 NL2020 strain. Several hits, belonging to different compound classes, shown protective effect against virus-induced cytopathic effect.

In this communication, we will present the hit-to-lead process of one of the identified families that has allowed us to select leads for further toxicity and efficacy studies in vivo.

The selected candidates have shown EC₉₀ values of 2 M against SARS-CoV-2 with CC₅₀ 50 M in different human cell lines. Furthermore, they have shown a selectivity profile against other viruses, showing no antiviral activity against recombinant human West Nile virus and vesicular stomatitis virus. Additionally, the selected candidates are effective in preventing propagation of SARS-CoV-2 showing viral RNA load reductions of 10⁵, values comparable to those of remdesivir.

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Discovery and characterization of broad-spectrum antivirals against RNA viruses with dual mode of action against SARS-CoV-2

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COVID-19 pandemic has evoked the need for increasing our preparedness towards emerging viral infections. While pandemic contention is achieved through vaccination of the general population, effective antiviral therapies may be essential to improve clinical outcome of the infection in infected individuals. To increase the antiviral armamentarium against SARS-CoV-2, we screened a large collection of small molecules with the aim of identifying novel compounds with antiviral potential through an unbiased phenotypic screening approach. A family of compounds with antiviral activity in the low micromolar range was identified. These compounds were effective at interfering with SARS-CoV-2 infection after the infection had been initiated and reduced viral spread by several orders of magnitude. These compounds displayed comparable antiviral activity not only against other coronaviruses but also against distant RNA viruses, such as vesicular stomatitis or West Nile viruses, suggesting broad-spectrum antiviral activity.

Structure-activity relationship carried out with dozens of analogs, led to identification of lead compounds that shared structural features with a drug with clinical potential and well characterized mode of action on a cellular biosynthetic route. The clinical candidate showed antiviral activity which was rescued with a metabolite downstream of the inhibition point. Interestingly, infection could only be rescued with the same metabolite in some of the newly discovered compounds, arguing for a different mode of action in some of the new antivirals. Next, we took advantage of chemical proteomics approaches to identify host cell pathways alteration of which may result in reduced viral spread. As comparison, we studied in parallel the impact of the clinical candidate to determine whether the newly discovered family shared not only structural similarities but also similar proteomic alterations. The results of this analysis suggest that both the clinical candidate and the newly discovered family modulated similar cellular pathways. However, cells treated with the newly discovered compounds show an additional cluster of genes altered by the treatment in a host pathway often exploited by RNA viruses, suggesting a potential dual mode of action against SARS-CoV-2.

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Photopolymerizable Glyconanomicelles as Inhibitors of SARS-CoV-2 Entry

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Background: Rapid urbanization, increased mobility and global economic interdependence exacerbate the threat of emerging pathogens and add to the challenge of containment. Being adequately prepared to detect, manage and respond to emerging infectious disease outbreaks has never been more imperative. Among the different strategies that are being developed to achieve this objective, those based on multidisciplinary approaches, such as nanomedicine, are considered the most promising. Nanoparticles (NPs) and viruses operate at the same length scale, and this is what makes nanotechnology approaches in treatment, vaccine development and immune engineering so appealing. Indeed, some formulations are already approved for clinical use against influenza, HIV, and HBV. In the case of COVID-19 pandemic, Moderna' and BioNTec/Pfizer's mRNA-based vaccines, which are among the first to reach the market, are, indeed, based on lipid nanoparticle platforms. In this work, we present our results on the use of organic nanoparticles designed to block the adhesion of the SARS-CoV-2 virus, thanks to a multivalent exposition of Spike protein's specific ligands. The synthesized nanosystems have also an internal cavity capable of housing small molecules/active ingredients with therapeutic potential against COVID-19, making them very appealing for combination therapies applications.

Methods: Various micelles have been synthesized and characterized from self-associating and photopolymerizable diacetylene amphiphiles. The incorporation and release studies of different antiviral agents have been carried out by UV-Vis spectroscopy and by HPLC.

The ability of the micelles to inhibit SARS-CoV-2 virus entry has been evaluated using a surrogate system based on retroviral pseudoparticles bearing a luciferase reporter and decorated with SARS-CoV-2 protein S (Spp) or vesicular stomatitis virus protein G (VSVpp), as control. As a positive specificity control, a neutralizing antibody was used that inhibits the entry of SARS-CoV-2 by binding to protein S. In parallel, direct cytotoxicity (in the absence of viral pseudoparticles) of the micelles has been evaluated by MTT assays in Vero-E6 cells.

Results: The photopolymerizable micelles have been shown to be endowed with important ability of incorporating large amounts of antivirals, and to improve their solubility in water more than 10.000 times. A slight reduction (2-3 times) has been observed in the luciferase activity derived from both Spp and VSVpp at a concentration of 50µM with four micelles. On the other hand, metabolic activity experiments have shown an absence of toxicity of the micelles.

Conclusions: The results obtained suggest that, although the micelles tested do not have the capacity to interfere selectively with the entry of SARS-CoV-2, at least effectively, the epitope exposed on their surface does play a key role in their activity. By optimizing this epitope, the micelles may be able to present an antiviral effect by inhibiting the entry of SARS-CoV-2. Indeed, the results recently obtained with multivalent systems of sialic acid derivatives (Nature. Comm. 2022, 13,2564), support this conclusion.

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Characterization of the in vitro polymerization activities of different SARS-CoV-2 nsp12 variants from patients' Isolates

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Background: In a recent study we have characterized the SARS-CoV-2 mutant spectra present in diagnostic samples from COVID-19 patients in terms of complexity, mutation frequency, and the association of these parameters with infection severity [1]. 41 amino acid substitutions in nsp12 (the RNA-dependent RNA polymerase, RdRp) have been recorded in the 30 nasopharyngeal swab samples analyzed. These substitutions were positioned in the 3D structure of the nsp12-nsp7-nsp8 replication complex and 6 of them (P323L, V373L, V560A, D499G, D618N, and M668V) were selected for new in vitro biochemical studies, based on their potential functional effects on the RdRp activity.

Methods: The nsp12 genomic mutations were introduced in the vector encoding the SARS-CoV-2 RdRp complex pRSFDuet-1(14-his-nsp8/nsp7)(nsp12) [2] by site-directed mutagenesis. The nsp12 active site mutant (D760A, D761A), which lacks polymerase activity was also prepared as a negative control. WT and mutant replication complexes were produced in E.coli BL21 Star (DE3) cells, and purified in three steps: nickel affinity, anion exchange, and size exclusion chromatographies. Polymerase activities were measured using a nonradioactive coupled-enzyme assay with a fluorescently labeled RNA primer 5'-5'-AGAACCUGUUGAACAAAAGC3' annealed to the unlabeled RNA template 5'AUAUUCGGCUUUUGUUCAACAGGUUCU3' as previously described [3]. The primer extension studies were performed in a 20 l reaction mixture in buffer (20 mM HEPES, pH 8, 20 mM DTT, 5 mM MgCl₂, 0.01 U RiboLock), 10 nM RNA and 1.5 M RdRp. The RdRp-RNA complex was previously incubated 3' at 37°C. The reaction was initiated by the addition of rNTPs (125 M). Polymerization was stopped at different time points by addition of the quenching buffer (8 M urea, 90 mM Tris base, 10 mM EDTA, 0.02% SDS, 0.1% bromophenol blue). Polymerization products were analyzed by Urea-PAGE (18%), in Tris base-boric acid-EDTA buffer. Reaction products were visualized using an Odyssey infrared imaging system and analyzed using the Fiji software.

Results: Comparative kinetic data show that the WT and mutant, P323L, D560A and D499A, polymerases exhibit similar polymerization rates, with P323L being slightly faster. In addition, the V373A variant, whose mutation is located at the interface between the polymerase fingers and the NiRAN domain, initiates a fast nucleotide incorporation in a similar way to the rest of the mutants, but after a few minutes, RNA synthesis slows down and stops prematurely. Finally, the RdRp complex that contains the D618N mutation, involving the catalytic aspartate of motif A, is inactive as expected. We will also present the data of the new biochemical experiments of nucleotide incorporation in presence of the nucleoside-triphosphate forms of the mutagenic analogues favipiravir, ribavirin and β -D-N⁴-hydroxycytidine that are currently underway.

Conclusions: We expect to understand at the biochemical level how the different RdRp variants interact with standard nucleotides and nucleotide analogues in the context of the SARS-CoV-2 replication complex, to distort its copying fidelity to either impede completion of the replication cycle, or to generate non-viable RNA progeny.

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Drug combination studies of cell-targeted antivirals against SARS- CoV-2

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Background: The coronavirus disease pandemic (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has triggered the search for multiple antiviral compounds. An alternative strategy to conventional antiviral drugs that interfere with viral components is the identification of compounds targeting cellular functions. Following this approach, we have focused our studies on lauryl gallate (LG), an ester derivative of gallic acid widely used as an antioxidant, and valproic acid (VPA), a branched short-chain fatty acid with a known therapeutic role against neurological diseases. Our previous results, which demonstrated the efficacy of LG and VPA in inhibiting different viruses, led us to test their effect on human coronaviruses, including SARS- CoV-2.

Methods: MTT cytotoxicity (CC50 values determination) and virotoxicity (possible antiviral toxicity on the viral particle) studies were performed for all de antivirals, antiviral combinations and coronavirus-susceptible cell lines. The inhibitory effect of LG or VPA was assayed on Vero E6, Caco-2 or Calu-3 2B4 cells infected with SARS-CoV-2. Antiviral combination studies were conducted following Chou-Talalay method, where synergy was quantified according to CI (Combination Index) values, determined by CompuSyn software.

Results: A consistent decrease of 2 to 4 logarithmic units in virus yields in all susceptible SARS- CoV-2-infected cell lines tested was observed in the presence of non-toxic concentrations of each antiviral, with an average IC50 value of 2.1 M for LG and 8.6 mM for VPA. By evaluating the effect of time of addition of the antiviral during infection, similar levels of inhibition were obtained when adding the drug before (-3 hpi), at the time (0hpi) or after (2hpi) infection, supporting a post-virus entry mechanism of action. Specificity of LG among other related compounds, such as gallic acid and epicatechin gallate, predicted to be better inhibitors according to molecular docking studies, was also demonstrated. The combined effect of these compounds was also evaluated, together with remdesivir, a virus-targeted drug with a proven effect against coronaviruses multiplication, searching for possible synergistic effects, at non-toxic concentrations of combined drugs. Synergy was found between LG and VPA and in the combination of the three drugs, according to Chou-Talalay method CI values. The combinations of remdesivir with LG or VPA also showed synergy, although to a lesser degree.

Conclusions: Our results show that cell-targeted antiviral drugs can reduce virus production upon coronavirus infection, suggesting a robust synergistic effect between LG and VPA, and to a lesser extent between the other drug combinations. These findings reinforce the interest of testing the effect of these cell-targeted compounds as a first line of disease defense or as a vaccine complement to minimize the gap in antibody-mediated protection evoked by vaccines, either in the case of SARS-CoV-2 or for other possible emerging viruses.

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Broad-range antiviral activity against coronaviruses exerted by non-coding RNAs derived from a viral genome

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Background: The ncRNAs are small and non-infectious synthetic RNA transcripts mimicking in sequence and structure different domains in the non-coding regions (NCRs) flanking the genome of foot-and-mouth disease virus (FMDV). These molecules elicit a robust and broad-spectrum antiviral effect based on type I interferon (IFN) induction and have been successfully assayed both in cultured cells and in vivo against a variety of viral pathogens, including zoonotic viruses causing severe disease in humans. In the context of the COVID-19 pandemic, the identification of novel and effective therapeutic solutions for new and emerging viruses is urgently needed. Here, the antiviral activity of the ncRNAs against human and swine coronaviruses has been tested in cultured cells and also in a mice model against infection with SARS-CoV-2.

Methods: The antiviral activity induced by transfection with the ncRNAs was measured by autocrine and paracrine assays against infection with SARS-CoV-2, HCoV-229E-GFP or porcine transmissible gastroenteritis virus (TGEV). For that, Caco-2, Calu-3 2B4, Huh-7, Vero E6 or ST cells were used. The activation of the innate immune response in transfected cells was analyzed by RT-PCR and immunoblot. For in vivo antiviral assays, K18-hACE2 transgenic mice were inoculated intraperitoneally with the ncRNAs 24h before intranasal infection with SARS-CoV-2. Animals were euthanized 3 days later and viral titers and cytokines levels in lung were determined by RT-qPCR.

Results: The FMDV ncRNAs are potent antivirals against SARS-CoV-2 in human colon and lung epithelial cells. The production of infectious SARS-CoV-2 was reduced in a dose dependent manner in ncRNA-transfected cells, mostly blocking infection with 10-20µg/ml of RNA at the highest MOI assayed (MOI: 0.5). Transfection with RNA doses as low as 1-0.1 µg/ml still induced a >100-fold reduction in viral titers at a MOI of 0.5. A potent activation of the innate immune response in human cells susceptible to SARS-CoV-2 infection after transfection with the ncRNAs was detected, in agreement with the inhibitory effect observed. The ncRNAs were also highly efficacious against common human HCoV-229E and TGEV in hepatocyte-derived Huh7 and swine testis ST cells, respectively. Delivery of naked ncRNA in K18-hACE2 transgenic mice 24 h prior to infection with SARS-CoV-2 reduced viral load and inflammation in lungs. A statistically significant decrease was measured in the ncRNA-inoculated group for viral RNA, IL-6, CXCL10 and CXCL11, compared with the control group.

Conclusions: In this study, we show the strong protective effect of the ncRNAs against SARS-CoV-2 in human intestinal and lung epithelium cells. Transfection of the ncRNAs triggered the induction of type-I IFN and IFN-stimulated genes, linking the activation of innate immune response with the measurable antiviral activity exerted. The efficacy of the ncRNAs was also confirmed against infection by the common cold human coronavirus HCoV-229E and the swine coronavirus TGEV, both members of the genus Alphacoronavirus. Remarkably, the FMDV ncRNAs showed anti-SARS-CoV-2 activity in mice reducing viral load and inflammation in lungs. Our results provide valuable insights for developing new anti-coronavirus strategies based on the broad-spectrum antiviral activity of the FMDV ncRNAs against both current circulating and future emerging coronaviruses.

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In vivo experimental platform for the evaluation of the efficacy of novel therapies against SARS-CoV-2

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Background: The current SARS-CoV-2 pandemic has highlighted the need for the rapid development of novel tools to combat viral threats. Whereas vaccines have played an important role in prevention, there is still a need for effective therapies to combat this disease. In this context, the last step of preclinical characterization of drug candidates is the efficacy testing in animal models.

Methods: We have established, characterized and validated a novel transgenic mouse model expressing the human angiotensin converting enzyme 2 (hACE2) for the evaluation of therapeutic candidates against SARS-CoV-2.

Results: Transgenic mice expressed the hACE2 receptor in the airways and showed a high susceptibility to SARS-CoV-2 infection using a virus isolate from the first epidemic wave. The virus replicated reaching high titers in lungs and induced lethality in this model. Histopathological analyses demonstrated the presence of lesions similar to those characteristics of COVID-19 in humans. The induction of proinflammatory cytokines was also confirmed in the lungs of infected mice. Through multiple collaborations with other groups of the PTI+ Salud Global, we have used this mice model to perform efficacy analyses of a wide variety of drug candidates.

Conclusions: The mice model developed has proven its suitability for the evaluation of the antiviral activity of multiple candidates against SARS-CoV-2, thus contributing to the preclinical development of novel drug candidates.

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SARS-CoV-2 pathogenesis mediated by E protein PBM was prevented by modulators of CFTR function

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Coronaviruses (CoVs) of genera α , β and δ encode proteins that have a PDZ-binding motif (PBM) consisting of the last four residues of the E protein (PBM core), which may bind over 400 cellular proteins containing PDZ domains, making them relevant for the control of cell function. Three highly pathogenic human CoVs have been identified to date: SARS-CoV, MERS-CoV and SARS-CoV-2. The relevance of their E protein PBMs in virulence has been studied. First, recombinant variants of these viruses missing each of E protein PBMs were generated and their pathogenicity was analyzed in mice. The PBMs of these three CoVs were virulence factors. A collection of SARS-CoV mutants in which the E protein PBM core was replaced by the E protein PBM core from virulent or attenuated CoVs was constructed and their virulence analyzed. A gradient of virulence, depending on whether the alternative PBM core introduced was derived from a virulent or an attenuated CoV was observed.

The gene expression patterns associated with the different PBM motifs in lungs of mice infected with SARS-CoV was analyzed by deep sequencing of the mRNAs expressed in lungs of infected mice, and it was observed that the E protein PBM motif of SARS-CoV and SARS-CoV-2 dysregulated the expression of genes related to ion transport and cell homeostasis. Specifically, a decrease of the mRNA expression of the cystic fibrosis transmembrane conductance regulator (CFTR), which is essential for edema resolution, was observed. The reduction of CFTR mRNA levels was associated with edema accumulation in lungs of mice infected with SARS-CoV. The effect of compounds that modulate the expression and activity of CFTR on the replication of SARS-CoV-2 was studied in cell cultures and it was observed that these compounds drastically reduced the production of SARS-CoV-2 and protected against its infection in mice model. These results showed the high relevance of the PBM motif in the replication and virulence of CoVs, and have allowed the identification of cellular targets for the selection of antivirals.

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Genomics and NGS (GENGS) Core Facility at the CBM Severo Ochoa, and its role with the PTI+ Global Health

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The Genomics and Next-Generation Sequencing (GENGS) Facility at the Centro de Biología Molecular (CBM) Severo Ochoa has over 16 years of experience in the implementation and development of cutting-edge technologies in molecular biology and genomics, and over 12 years in NGS. During this time, we have worked with a great diversity of samples and from any kind of organisms, and helped internal and external researchers (academic, clinical and industrial) with technical and scientific support in the experimental design, undertaking, data analysis, etc, of nucleic acid extraction, quality control, real-time (RT)-PCR, digital PCR and NGS related projects. In addition, our service mediates between sequencing platforms and users. We actively explore, validate, optimize, and implement new genomics and NGS technologies, using different methodologies and computational analysis of big data. We also perform statistical analysis, and custom software. We generate back top-quality data and information and reports along the project, with rapid turnaround times at a competitive cost. The facility organizes seminars and on-demand courses for users.

Regarding the PTI+ Global Health, we have been involved in different projects and helped a diversity of researches, from general advice to design, performance and/or data analysis. In this respect, for example, we have:

- Developed four COVID RT-qPCR assays, protected by CSIC as trade secret.
- Performed around 3000 diagnostic COVID RT-qPCR, with their corresponding personal reports, from clinical samples obtained by the CSIC Occupational Health and Medicine Surveillance Unit (Marta Bermejo, UVSyMT) at the Comunidad de Madrid (CM) through the Centinela project (Margarita del Val, CBM).
 - The ISCIII authorised and the CM recognised the GENGs Facility as a lab to perform COVID RT-qPCRs and issue European Digital COVID Certificate (CCD). The GENGs officially reports to the CM the COVID PCR results.
- Created bio-banks with samples and RNAs obtained in the Centinela Project. These bio-banks are available for the PTI researches, being already used by some of them.
- Compared RT-qPCR COVID results of nasopharyngeal, oral and saliva samples from 100 individuals.
- Set up a digital PCR equipment and an assay to detect COVID from different samples.
- Sequenced the RT-qPCR positive samples, in collaboration with the Biomedicine Institute in Valencia (Iñaki Comas, IBV) and Antonio Alcamí's lab (CBM), to deeply analyse time-course mutations of samples obtained from the same individual, from different individuals of the same family/lab, evolution of lineages through time, etc.
- Generated personal reports informing about SARS-CoV2 lineage (from nasopharyngeal samples obtained for diagnostic COVID RT-qPCR).
- Performed data analytics from clinical, RT-qPCR and sequencing information, and combined this processed data for different studies.
- Interacted with different companies in relation to COVID RT-qPCR assays.
- Participated in the HISOPOC (printed 3D swabs) project (Juan Rodriguez, ICTP).
- Developed four new “combo RT-qPCR” assays to detect respiratory virus from human samples (Antonio Alcamí, CBM). (In progress)
- Extracted 500 RNAs from COVID nasopharyngeal clinical samples from Barcelona Hospitals (Iñaki Comas, IBV).
- Helped with projects design. Example: Genotyping of COVID patients at elderly homes. Diego Ramiro (CCHS).

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Micro Electron Diffraction CSIC Facility: New tools for structure determination

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Micro-electron diffraction is a technique for atomic structure determination of crystallized molecules using an electron microscope. Data sets of crystals that are too small for X-ray diffraction experiments can be collected and processed for structure resolution in a matter of a few hours. It has been a revolution not only in the field of structural biology but also in the field of chemistry since it allows to work with both macromolecules and small molecule compounds.

The CryoEM facility at the National Centre for Biotechnology has implemented MicroED in a Talos Arctica and made the technique available for the scientific community. Initial experiments with samples from several users have proved the robustness of the workflow for small molecule and macromolecule structure resolution. New additions are being included for the handling of Vlarger crystals not suitable for MicroED using a Zeiss CrossBeam 550 cryo-FIB-SEM microscope offering the possibility to mill lamellae from crystals that are too large for microdiffraction but too small for X-rays. We believe that this service will be of great help to many research groups by providing them with a fast and effective method for the resolution of their molecular structures as well as resuming projects stopped due to the limitation of the size of their crystals.

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Tryptophan trimers efficiently inhibit SARS-CoV-2 entry into the host cells

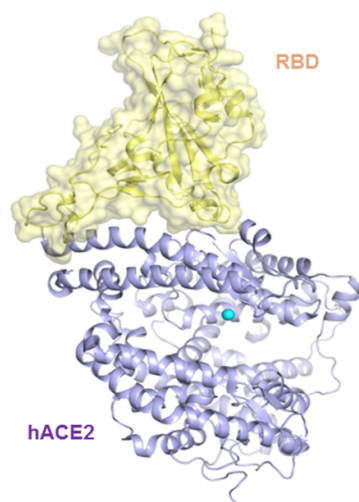
Marta Gargantilla López¹; Clara Francés²; Anmol Adhav³; Belén Martínez-Gualda¹; Alberto Marina³; Vicente Rubio³; Ron Geller²; Ana San-Félix¹; María-Jesús Pérez-Pérez¹

Background: The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a new betacoronavirus responsible for the largest pandemic the world has suffered in recent years, COVID-19. Coronaviruses are characterized by spike proteins that are crucial for the entry process into the host cells. Particularly, the spike protein of SARS-CoV-2 binds to the human angiotensin converting enzyme 2 (hACE2) through its receptor binding domain (RBD) (Figure 1), leading to viral entry and subsequent infection of the host cell. Thus, inhibition of SARS-CoV-2 by blocking the interaction of the spike protein with hACE2 represents an appealing antiviral strategy complementary to the treatments already approved. Our research group has been extensively involved in the search for entry inhibitors of different virus, as recently reported for potent HIV and EV-A71 antivirals. In the current project, 200 compounds were evaluated as entry inhibitors in a preliminary HTS assay, resulting in the identification of tryptophan trimers as promising hits that have been further studied.

Methods: Compounds from our own collection were evaluated in a HTS assay using VSV pseudoparticles expressing the SARS-CoV-2 spike protein in Vero cells. Novel tryptophan trimers, based on the identified hits, have been synthesized through a new protocol that involved optimization of already described chemistry procedures. The antiviral activity has been confirmed against SARS-CoV-2 in Vero E6 and A549-hACE2 cells in BSL3 labs. Microscale thermophoresis studies have been carried out to study the interaction of the antiviral compounds with the RBD and spike of SARS-CoV-2, and how this affects the KD of the interaction of the RBD/spike with hACE2.

Results: Several structural analogues of the hits based on a tryptophan scaffold have been synthesized providing a battery of trimers with different substituents in the periphery and at the focal point. This has allowed the establishment of SAR and the improvement of solubility in a saline solution. Some compounds were able to block viral entry in the pseudoparticles assay with IC50 values in the low micromolar or submicromolar range and in the absence of toxicity to host cells (CC50 >100 µM). The antiviral activity was confirmed against SARS-CoV-2 in Vero E6 and A549-ACE2 cells. The bio-physical studies evidenced that these compounds were able to interact with the RBD and spike of SARS-CoV-2 (Wuhan) and indeed they significantly affected the KD of the interaction of the RBD with hACE2.

Conclusions: These tryptophan trimers are able to block SARS-CoV-2 entry by interfering with the hACE2-viral spike interaction, thus representing an interesting alternative for the development of novel therapies and/or prevention of the viral infection.



Graphical Abstract

Tryptophan trimers efficiently inhibit SARS-CoV-2 entry into the host cells

Fig 1: X-Ray of the complex of RBD of the spike of SARS-CoV-2 with hACE2

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Synergistic inhibitory effect of remdesivir and ribavirin against SARS-CoV-2 and human coronavirus 229E

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Background: The current COVID-19 pandemic caused by the emerging severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is significantly impacting public healthcare systems. Until this moment, more than 530 million cases and more than 6.2 million deaths have been reported. Antiviral treatments against SARS-CoV-2 are currently under development. We previously described that favipiravir and ribavirin exert a synergistic lethal mutagenesis on hepatitis C virus (HCV) during replication on human hepatoma Huh-7.5 cells, and synergy was required to extinguish high fitness HCV. Therefore, we are interested in identifying synergistic combinations of lethal mutagens which are effective against SARS-CoV-2.

Methods: Vero E6 cells and human hepatoma Huh-7 cells were infected with SARS-CoV-2 (iso late WA1/2020, obtained from BeiResources) and HCoV-229E, respectively. Synergy has been documented using the following concentration ranges: for HCoV-229E, remdesivir (0-50 nM) and ribavirin (0-50 µM); for SARS-CoV-2, remdesivir (0-7.5 µM) and ribavirin (0-100 µM). Cells were preincubated for 16 hours with the inhibitors prior to infection at a MOI of 0.01 PFU/cell with virus adsorption time of 2 hours. The infection was continued four days in the absence or the presence of the drugs. Infectivity was titrated by plaque assay in triplicate. Drug concentrations and inhibitory activities were entered in the CompuSyn software to apply the Chou-Talalay method to identify a possible synergistic activity.

Results: The results document a synergistic activity by the combination of remdesivir and ribavirin acting on SARS-CoV-2 and HCoV-229E. The quantifications show average dose reduction indices (DRI) above 1, and average combination indices (CI) below 1, for both viruses. Furthermore, ultra- deep sequencing of SARS-CoV-2 in the presence of ribavirin revealed a four-fold increase in the number of mutations in viral RNA.

Conclusions. The combination of remdesivir and ribavirin shows synergistic antiviral activity against SARS-CoV-2. This may help to reduce the effective concentration of compounds to achieve a beneficial clinical effect. Synergy among nucleoside analogues may be a potential strategy to confront emerging viral infections.

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Natural products as antivirals. High-throughput screening of the MEDINA collection of microbial natural products.

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Coronaviruses (CoVs) have the ability to propagate and generate new species that cause epidemic diseases. The impact of the SARS-CoV-2 pandemic has revealed the urgent need for broad-spectrum antivirals to prevent future viral pandemics of unknown origin. Human coronavirus OC43 (HCoV- OC43), is a well-matched surrogate for SARS-CoV-2 because it is also a betacoronavirus, it targets the human respiratory system and is transmitted via respiratory aerosols and it exhibits several common features with SARS-CoV-2 with regard to its structure and biology. In order to provide capacities in the identification of novel antivirals, methodology for high-throughput screening against the human coronavirus HCoV-OC43 has been established. The growth parameters of HCoV-OC43 were analysed in several cell types for assay optimisation. Optimised and ongoing assays include inhibition of the cytopathic effect (CPE), estimation of antiviral activity by immunofluorescence and determination of viral load by quantitative RT-PCR.

The assays are available in 96 and 384 formats. Using these methodologies, we have performed a pilot screen of the MEDINA collection of microbial natural products and other available natural product compound collections. A primary screening for the detection of CPE inhibition in MRC-5 cells after 5 days of infection with HCoV-OC43 was performed with 1280 microbial extracts selecting as hits those that produced an inhibition higher than 40%. Cherry picking and dose response analysis together with low resolution HPLC-MS deconvolution identified a total of 14 extracts of high interest for further analysis. Additionally, the information obtained in the analysis identified the presence of multiple previously described natural product components and several novel compounds with potential antiviral properties. The methodology was also used to evaluate the activity of a series of pure natural products. Highly selective and potent hits were identified. Further analysis of extract composition using high-resolution technologies and identification of active components may lead to the discovery of novel natural products with therapeutic potential in the treatment of viral infections.

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Pharmacological modulation of the interaction between tubulin and a structural protein of SARS-CoV-2

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The cytoskeleton is the main communication/transport route within cells and many viruses abuse on this cellular machine to fulfil their cycle. We initially identified the interaction of a SARS-CoV-2 protein with tubulin using a proteomic analysis. We next confirmed the interaction and identified the viral protein domain involved through in vitro co-immunoprecipitation assays and analytical ultracentrifugation experiments. Then, we focused on unveiling the molecular mechanism of the interaction to determine if the SARS-CoV-2 protein promote a stable microtubule assembly (as roads for motors) or induce microtubule dynamics (as main force generation for transport).

We have combined biochemical, biophysical and structural studies to determine the ratio of protein-protein interaction and the resulting effect on tubulin assembly. We have found that this protein domain is able to promote microtubule depolymerization into rings and tubulin assembly into non-functional filaments likely because a preference for tubulin curved-conformation. Importantly, this effect is not dependent on nucleotide or nucleotide hydrolysis. Finally, tubulin is a well-known target in cancer diseases and there are four of the seven tubulin druggable sites exploited on chemotherapy. Hence, we have analyzed the ability of microtubule stabilizing (MSA) and destabilizing agents (MDA) on disrupting the interaction of SARS-CoV-2 protein with tubulin. We have found that MSAs keep microtubule structures even in the presence of the viral protein, while the effect of MDAs varies depending on their mechanism of action.

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Use of an interactomics pipeline to assess the potential of new antivirals against SARS-CoV-2.

Alberto Marina Moreno¹; Alicia Forcada-Nadal¹; **Anmol Adhav**¹; Antonio Rubio del Campo¹; Carla Sanz- Frassetto¹; Clara Marco-Marín¹; Jerónimo Bravo Sicilia¹; José Luis Llacer Guerri¹; María Jesús Pérez Pérez²; María Luisa López-Redondo¹; María Pilar Hernández-Sierra¹; Marta Gargantilla López²; Rafa Ruiz Partida¹; Roberto Gozalbo-Rovira²; Santiago Ramón-Maiques^{1,3}; Sara Zamora Caballero¹; Sonsoles Martín Santamaría⁴; Vicente Rubio¹

Background: In late 2019 SARS-CoV-2 infection appeared in China, becoming a pandemic in 2020. The scientific community reacted rapidly, characterizing the viral genome and its encoded proteins, aiming at interfering with viral spreading with vaccines and antivirals. The receptor binding domain (RBD) of the viral spike (S) protein plays a key role in cell entry of the virus. It interacts with the cellular receptor for SARS-CoV-2, the membrane-bound human Angiotensin Converting Ectoenzyme 2 (ACE2). With the goal of monitoring interference with this interaction by potential antiviral drugs, we have set up at the Institute for Biomedicine of Valencia (IBV-CSIC) an interactomics pipeline targeting the initial step of viral entry.

Methods: For the production part of the pipeline (pure RBD/Spike variants and soluble ACE2), see parallel poster. These proteins allowed monitoring of the RBD/Spike-ACE2 interaction in presence or absence of potential inhibitors. Thermal shift assays (thermofluor) were used for initial detection of compound binding at different ligand/protein ratios and media conditions (pH, buffers, chaotropic agents). Next, binding affinity and on/off kinetics were characterized using Biolayer interferometry (BLI), Surface plasmon resonance (SPR), Microscale Thermophoresis (MST) and/or Isothermal titration calorimetry (ITC). For protein-protein interactions, we mostly used BLI or SPR, whereas for protein-small compound analysis MST was generally best. Protein aggregation-dissociation was monitored by size exclusion chromatography with multiangle light scattering (SEC-MALS).

Results: Candidates proven by thermal shift assays to bind to RBD/spike protein without affecting the integrity of these proteins were subjected to quantitative affinity measurements. We successfully demonstrated that BLI, SPR and MST can be used to follow the interactions between SARS-CoV-2 proteins and the putative drug candidates, as well as to monitor the interference with Spike-Ace2 binding of potential drug candidates. While BLI and SPR displayed reproducible results in the measurement of protein-protein interaction (applied to soluble ACE2 used as a decoy), they were less suitable for measuring the binding of small molecules. The fact that most small compounds were only soluble in organic solvents made difficult to obtain a low signal/noise while using BLI, necessary for the assessment of the binding. We overcame that problem by using MST. After dilution of the compounds to the final experimental concentrations, the technique could detect a significant binding signal enough to calculate binding parameters. MST also allowed to measure the degree of interference that each compound was having on RBD/Spike-ACE2 interaction. The pipeline has been customized and validated with compounds of very different nature provided by different groups belonging to the PTI and other external laboratories, as well as with different Ace2 decoys designed at the IBV.

Conclusions: The interactomics platform at the IBV has been used to successfully develop two different antiviral approaches in order to fight COVID-19. It has allowed technical specialization of the staff as well as the development, in a very short period of time, of two ambitious projects. We have demonstrated that we can perform interactomic characterization for challenging projects as well as provide information about binding of antivirals to potential new SARS-CoV-2 variants of concern.

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ACE2-derived peptides for the inhibition of SARS-CoV-2 infection

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Keywords: SARS-CoV-2 spike inhibitors, ACE2- based peptides, macrocyclic peptides.

The severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) originated the current world- wide pandemic situation (COVID-19). The structural and biochemical basis of the SARS-CoV-2 infection mechanism has been widely investigated, showing that the receptor binding domain (RBD) of the virus surface spike protein interacts with the peptidase domain (PD) of angiotensin-converting enzyme 2 (ACE2) [1-3]. Structural studies of the RBD of SARS-CoV-2 with the full-length human ACE2 receptor exposed the amino acid residues that play a key role at the contact interface of the two proteins, which are mainly located on the helix 1 (residues 22-57) and -sheet 5 (residues 351-357) of ACE2 [1,2]. Therefore, peptides focused on ACE helix 1 containing these essential interface residues have been designed, synthesized and evaluated to identify potential inhibitors of the ACE2-RBD binding. [4,5] These studies reported that α -helix secondary structure is essential to obtain SARS- CoV-2 antiviral activity. Accordingly, we report the development of a long-range macrocycle ACE2 derived peptides with the aim to stabilise the helical structure of the peptides and consequently, increase the potential ability to block SARS-CoV-2 attachment to the host cell. Furthermore, more soluble versions of a discontinuous peptide based on a combinations of hotspot interactions with strong binding affinity to RBD domain described by Sadremomtaz et al. were synthesized [6]. All these peptides have been evaluated by microscale thermophoresis and some of the candidates have showed nanomolar binding affinities towards the RBD domain and the Spike protein. These results are the starting point for the design of novel SARS-CoV-2 peptide inhibitors.

This work has been supported by PTI+ Salud global, CSIC (SGL2103050).

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New amino acid derivatives for targeting SARS-CoV-2

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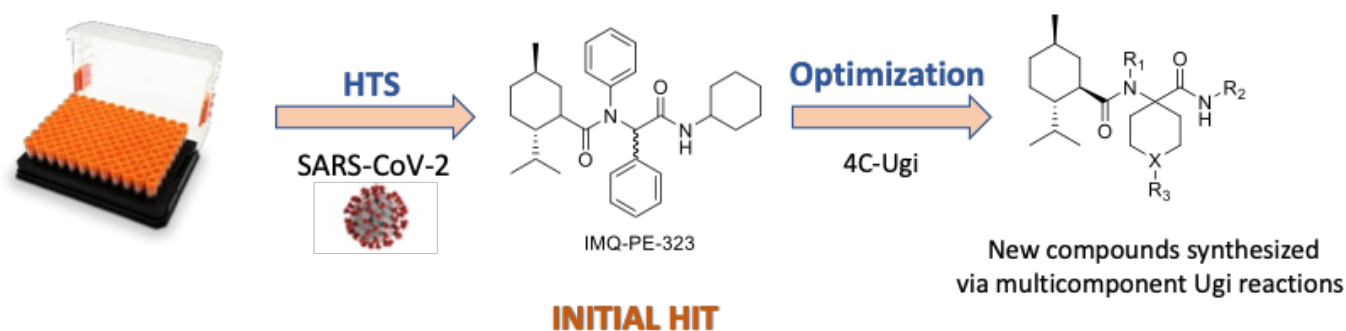
The Coronavirus disease-19 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 virus (SARS-CoV-2), represents a pandemic situation worldwide [1]. Despite of the development of vaccines, and the approval of few antivirals [2], new efforts are still needed to fight COVID-19 [3]. In this context, our research groups are working on the design, identification and optimization of small molecules as novel therapeutic strategies against SARS-CoV-2.

In a high-throughput phenotypic screening assay of our in-house chemical library we identified IQM-PE-323 as an interesting hit for the development of novel antivirals against SARS-CoV-2. To increase the activity on SARS-CoV-2 we carried out a medicinal chemistry program. The new compounds have been synthesized through a straightforward and quick procedure using multicomponent Ugi reactions. Some of them showed micromolar anti-SARS-CoV-2 activity in A549 –ACE2 cell cultures, but with a narrow therapeutic window. Due to the similarity with ML-188, a viral main protease (Mpro) inhibitor [4], we evaluated their activity on a Mpro enzymatic fluorometric in vitro assay. However, this family of compounds did not inhibit viral Mpro (IC₅₀ > 100 µM). Therefore, the observed antiviral activity must be due to a different mechanism of action. Further work is ongoing for a better characterization of this family of compounds.

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Graphical Abstract

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New antivirals targeting the energy metabolism of the virus-infected cell

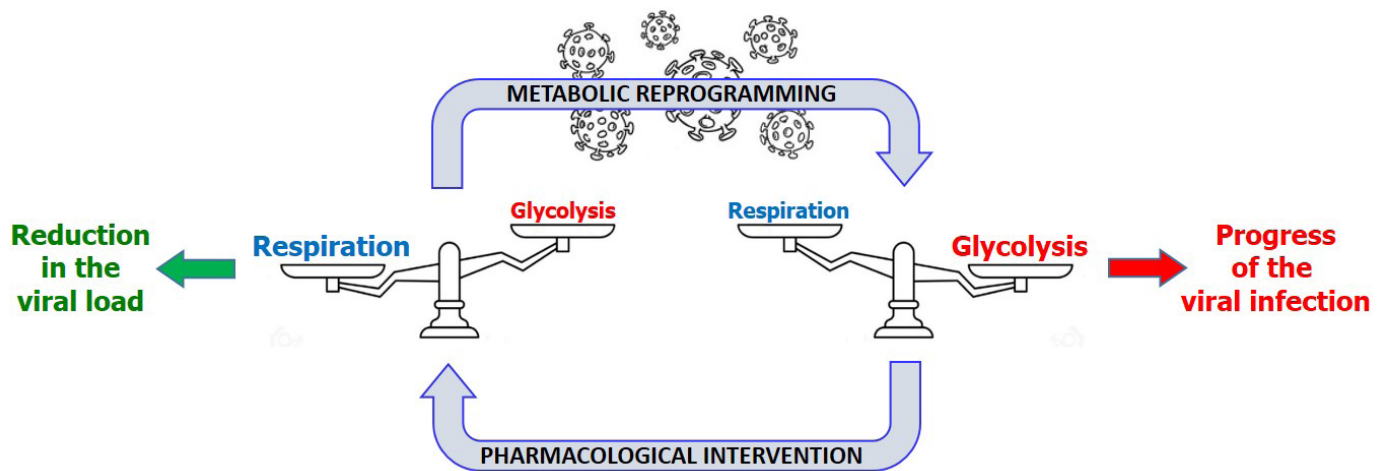
Paula Martínez de Iturrate¹; Bruno Hernández²; Patricia de los Santos¹; José María Sánchez-Puelles¹; Antonio Alcamí²; Luis Rivas¹; Eduardo Rial¹

Background: Viruses are obligate intracellular pathogens that require a host cell to multiply and expand the infection. Thus, they have evolved to use a variety of strategies to reprogram key signaling pathways in the host cell to obtain the metabolic intermediates (nucleotides, fatty acids, amino acids, etc.) required for the biosynthesis of viral macromolecules. The metabolic change resembles the one that occur in cancer cells, the Warburg effect. The project aims to reposition of existing drugs to revert the viral-induced metabolic reprogramming in the host cell.

Methods: The biological models used to test the antiviral action of known anti-tumour drugs include the cancer cell line Huh7, the lung fibroblast cell line MRC5 and differentiated alveolar epithelia cultured in transwell under air-liquid interface conditions. The model viruses used are the alfa-coronavirus strain 229E-GFP, the vesicular stomatitis virus VSV-GFP and the coronavirus SARS- CoV-2. The characterization of the energy metabolism of the cell lines is performed using an extracellular flux analyser XFe24 (Agilent-Seahorse).

Results: We have tested a set of inhibitors that target different components of the signalling pathways that are known to be involved in the metabolic reprogramming of tumour cells. The antiviral activity of these inhibitors is generally modest when tested on the hepatocarcinoma cell line Huh7 infected with the coronavirus 229E-GFP. However, some of these inhibitors do show a potent antiviral action when infection involves non-transformed cells.

Conclusions: The components of the signalling pathways involved in the viral-induced metabolic changes of the infected cell are suitable drug targets for the identification of new antivirals.



Graphical Abstract

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Residential environment perception by older adults in nursing homes during Covid-19

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Introduction: Nursing homes were the most vulnerable residential settings during COVID-19 pandemic, which experienced high rates of incidence and death from this cause. This paper aims to know: i) the assessment of the residential environment made by institutionalized older people and to examine the differences according to personal and contextual characteristics; and ii) which variables determine that a person is classified in different residential assessment categories.

Material and methods: The survey on COVID-19 Residential Settings (1), carried out between May- October 2021 in nursing homes in the Community of Madrid-Spain, was used. A multistage cluster sampling was designed with stratification of residences (ownership: public/private, location: municipality of Madrid/rest of the community, size: <60 beds and more) and residents (sex, 60 or more years old, with no cognitive impairment), with a sampling error= ±4.8% and a confidence level of 95% (p=q=0.5). Informed consent was signed by the participants and the study was approved by the CSIC Bioethics Committee. For this study, information on assessment of the residential environment (10 items) and personal and contextual characteristics (32 variables) was selected. Factor analysis (FA) and Cronbach's Alpha were applied to the construction of the residential assessment subscales. Through Cluster Analysis three groups of residents based on the residential assessment were obtained. One-factor ANOVA, test of means with Bonferroni correction and χ^2 test were run to examine the differences in the residential assessment groups. Logistic Regression Analysis (LR) with each of the three groups was used to address the second objective of the study.

Results: The sample consisted of 447 people (mean age=83.8; 63.1%=women; 50.8%=widowers/widows; 40%=less than primary education level). The results of the AF (4 principal components) and Cronbach's Alpha (≈ 0.8 in the variables of each principal component) determined the construction of 4 residential assessment subscales (relationships; mobility; residential aspects; and intimacy space). Three clusters of subjects were obtained according to residential assessment (medium-high assessment with all residential aspects=71.5% of cases; low assessment with mobility=15.4%; low assessment with all residential aspects=13.1%). In relation to the 3 clusters, statistically significant mean differences were observed with all the variables used, with the exception of educational level, concern about the COVID-19 pandemic, number of diagnosed diseases, and number of medications taken. The significant variables were used in 3 LR models according to the clusters, obtaining a high explanation of variance in each one. The results indicated that better personal and contextual conditions determine a better residential assessment.

Conclusions: If a better residential assessment is influenced by more favorable personal and contextual conditions, it is concluded that promoting age-friendly residential and social environments would help minimize the effects of pandemic outbreaks, in addition to maximizing the quality of life of vulnerable population, such as the older adults in long-term care facilities.

Keywords: COVID-19, institutionalized old people, residential assessment, Comunidad de Madrid, Spain.

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Living in a nursing home during COVID-19 in Madrid: a glimpse into an unexplored world

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Background. Nursing homes for the older people have been particularly vulnerable to COVID-19, as shown by high rates of infections and deaths. More than 50,000 people lived in nursing homes in the Region of Madrid (Abellán et al., 2021), among whom there were more than 6,000 deaths in these homes, including confirmed and suspected cases of COVID-19, according to the Report of the Covid-19 and Residence Working Group (IMSERSO, 2020). This paper aims at describing the protocol of a nursing homes research carried out during the COVID-19 pandemic in Madrid (Spain), providing information on the design, measures used and residents’ characteristics, as well as some salient findings.

Data. A specific questionnaire was designed with the intention of understanding the impact of the Sars-Cov-2 virus on the older population living in nursing homes in Madrid. A total of 447 people over 60 years of age without cognitive impairment, 220 in private residences and 227 in public residences, participated by answering questions related to issues such as personal situation regarding the pandemic, feelings and ways of coping, residential assessment, health and quality of life.

Results: The population analysed reported a perceived good or very good health status (58.6%), but with more than 5 illnesses, accompanied by polypharmacy. While more than half related having passed the disease, only 37% claimed to have been diagnosed. Overall, only 1 in 10 reported having sequelae after their infection. Among these, they did not include depression, albeit the majority (75%) informed about mild depression. Residents’ concern and/or fear of COVID-19 were widespread feelings, which conditioned the extensive follow-up of infection control measures taken. Positive feelings about coping and resilience may have contributed to this. Perceived loneliness is not a widespread feeling among residents, although it was exacerbated during the pandemic.

Living in a nursing home represents a fact of paramount importance in the older people’ lives. The residential assessment expressed was generally good, especially their safety and relations with their relatives, not so much the mobility in and out of the facility. This may have been influenced by the fact that a high percentage had a single room and did not leave it during the critical phase of the pandemic. The change in the pattern of activities has meant a maintenance of passive activities (watching TV, exercising), which are the most common, and a reduction in those involving mobility. In short, living in the residence during the pandemic provided them with a high quality of life, as did their satisfaction with living there, although this was reduced during COVID-19.

Conclusions: This work provides very useful materials for understanding the consequences of the pandemic on the resident population itself, and assessing strategies for improving their situation in critical periods. These results will be corroborated with a more in-depth qualitative approach.

Keywords: COVID-19, institutionalized old people, design study, Comunidad de Madrid, Spain

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Elderly Care Homes Survey 2020 and advance 2022: an overview of the characteristics of Elderly Care Homes in Spain

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This poster will present the results of the 2020 Survey carried out across all the Elderly Care Home Centers in Spain, c5600 and will offer as well a brief estimate of the survey which be carried out in October-November to this kind of Centers in Spain. From previous surveys, we estimated that the c5,600 centers can host up to 392,016 residents. 31.6% are concerted spaces between public and private institutions. Before launching the 2022 survey, our survey data is compared periodically with the data offered by the Autonomous Communities on their websites, so our estimation is that the number of Elderly Care Homes and the number of spaces that can host will increase, in line with the evolution of recent years. In the previous survey carried out, in 2020, we reached a 69% response rate, a figure that we hope to exceed this time.

All the georeferenced information will be displayed in maps and via the Spanish National Research Council Health Portal Spatial Data Infrastructure, an open platform which offer complete information about health and elderly care resources in Spain. The Elderly Care Home Centers Survey is the only survey carried out in Spain to offer information about these resources for the elderly at national level and has been widely used during the pandemic.

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Adult Day Care Centers Survey 2022, a map and characteristics of Day Care Centers in Spain

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This poster presents the results of the postal survey sent to 1,442 Adult Day Care Centers for the elderly, independent of the elderly care homes for the elderly, that exist in Spain. This postal survey was carried out in a first round in April 2022 to all centers, and in a second phase in July 2022, aimed exclusively at centers that did not respond our first survey and adding to this survey other newly created ones during this period of time. Therefore, the second round was sent to 671 centers in total. So far, the response rate is 53% and we hope to increase this response substantially during our second round.

Adult Day Care Centers attend daily 50,168 elderly and 41% of these spaces are provided by publicly owned centers. All the georeferenced information will be displayed in maps and via the Spanish National Research Council Health Portal Spatial Data Infrastructure, an open platform which offer complete information about health and elderly care resources in Spain. The Adult Day Care Centers Survey is the only survey carried out in Spain to offer information about these resources for the elderly at national level.

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The gut microbiota, a hallmark of human aging, could implicate risk and effect of COVID19 in the elderly

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Background: Branyas’ project aims to enrich and refine the predictive risk profiles of Covid-19, focusing on genetic, comorbidity, lifestyle, immunological and microbiome variables for which innovative bioinformatic and big data tools as well as powerful next generation sequencing technologies have been used. CIAL-CSIC researchers in this project attempted to deepen the compositional and functional profiles of the gut microbial communities of elderly nursing homes and search for potential microbial biomarkers capable of individually predicting COVID19 risk and prognosis in this community, its relationship with other factors and expand them to the entire population. A further objective is to study the trajectory of the human microbiome and prolonged symptoms over time and their association with cognitive and motor impairment in this vulnerable population.

Methods: Demographic, clinical, dietary, comorbidities and medication data, and biological samples (blood and stool) from 216 participants from different nursing homes in Catalunya were recruited during 2021. Forty-one participants dropped out for different reasons, and finally the study enrolled 175 participants, 73 had followed-up engagement for 3 months to observe possible changes in their gut microbiomes. Fecal samples (n=280) were obtained and stored under anaerobic conditions at -80°C until use. Fecal total DNA was extracted using an optimized protocol and shotgun sequencing analysis approach was performed to deepen the structural, compositional and functional microbial profiles present. Fecal short-chain fatty acids (SCFA) profiles were also assessed using Gas Chromatography with Flame-ionization Detection (GC/FID) as an indicator of the functionality of the microbiome.

Results: First comparisons with fecal SCFAs have suggested differences depending on participants’ grouping. The results showed slightly higher levels of acetic and propionic acids and total SCFAs in participants who did not suffer COVID19, suggesting a possible impairment in the production of these beneficial metabolites in those after COVID19 infection. Furthermore, by focusing on the severity of the symptoms of infected participants higher levels of acetic acid and total SCFAs in participants with mild/moderate symptoms compared to participants with severe symptoms were observed. However, analysis of SCFAs by grouping participants that suffer from Alzheimer’s disease or not did not show appreciable changes in SCFAs levels, but more variability in non-diseased participants was observed.

The taxonomic and functional profiles of the microbiome derived from shotgun sequencing analysis allowed assessment of the preliminary trends observed with SCFAs and find responsible microbial communities and functions. Furthermore, the compositional and functional characteristics will provide a global snapshot of the microbiome and possible changes depending on the conditions of the individuals, possibly showing biomarkers of COVID19 risk as well as determining which populations and functions could be involved in these relationships.

Conclusions: Analysis of the gut microbiome in the Branyas project allows deeper investigation into potential microbial biomarkers of COVID19 risk and their relationship with a range of other immunological, lifestyle and genetic variables. In addition, the integrated study of microbiome functionality through metabolomics and lipidomics will identify requirements of the elderly vulnerable population giving more effective strategies in the prevention and treatment of pathologies associated with immune and cognitive function.

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A snapshot of the older population in Spain 2022

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This document is the latest issue in an ongoing report series by Envejecimiento en red that aims to provide an overview of the characteristics and living conditions of the population age 65 and older in Spain.

Drawing on reliable national and international data provided by trustworthy organizations, this report presents summary statistics and trends of select demographic, health, economic and social characteristics of the elderly population and shows how these have changed over time. Findings are communicated with a broad audience in mind, avoiding technical vocabulary. The indicators chosen are those which lend themselves to ongoing estimation via comparable and consistent data and statistical sources and those most representative of the entire elderly population; they are not presented in order of importance or interpreted as to their repercussions or functional implications.

How many elderly people reside in Spain and what does it mean to be a part of this increasingly larger segment of society? What is the life expectancy and healthy life expectancy at age 65? What are the leading causes of death and the most common diseases? Does the elderly have economic security or are they more likely to live in poverty? This report uses interactive data visualization to answer these and more questions and to explore time trends and geographic variations in the characteristics of the older Spanish population.

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MADCOVID-CSIC: Research and design of scientific dissemination activities on COVID-19 aimed at the Spanish youth

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MADCOVID-CSIC is an outreach project aimed at young people concentrating on the most interesting aspects about the pandemic. These ranged from contagion to emotional impact. It is financed by the Recovery Fund and belongs to the Social Forum of the PTI+ Global Health.

Objectives:

- Disseminate, inform and promote knowledge about the pandemic among young people.
- Promote greater awareness of young people about the risks of COVID-19.
- Encourage young people to be the link of information and dissemination with their immediate family and friends.
- Help young people emotionally manage the pandemic.

Methods: First we sent a questionnaire to the teacher in order to determine the topics that interest the most: disease, contagion, vaccine, duration of the pandemic, misinformation, fake news and emotional management.

The content of the talks is adapted to priorities. Then, an assessment of the experience is made between speakers and teachers to detect changes in behavior and perception of the pandemic among the students.

Results and conclusions:

To date, more than 25 talks have been given in educational centers, 11 of them in collaboration with secondary schools associated with the CSIC’s Science dissemination Project “Ciencia en el Barrio”, orientated to socially and vulnerable neighborhood in Madrid and the surrounded metropolitan area. In the talks, emotional management is treated preferentially, through collaboration with a cabinet of psychologists that is receiving great interest from teachers and students. They learn to recognize and name emotions, normalizing them and discarding emotional garbage, favoring more emotionally proactive, tolerant and healthy attitudes.

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Uncertainty, trust and responsibility: keys to counteracting disinformation, infodemic and conspiracy mania

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The project RESPONTRUST explores the epistemic and ethical dimension of trust and responsibility in face of the profound uncertainty that COVID19 health emergency has caused in our society. Infodemic, disinformation and post-truth discourse changed our rationality patterns and hindered the successful implementation of measures to contain the virus, provoking instead an increase of conspiranoia, denialism, anti-science and anti-vaccine movements, as well as anti-democratic attitudes, consequence of the destabilizing ideological and affective polarization of society.

Given the key role of “critical, informed trust” during the health crisis, it is crucial to explore those factors that undermine or reinforce confidence and weaken or stimulate citizens’ sense of responsibility. Our project aims to analyze the correlation and flow of information between science, politics and society in order to better understand the phenomenon of disinformation amplified in the digital sphere.

The project team explores the main factors that, by disturbing the equilibrium of uncertainty, trust, and responsibility, override autoregulatory mechanisms of our epistemic practices. These are intertwined dynamics that are mutually reinforcing but can be heuristically distinguished as follows:

The public image of science and the mechanisms of access to scientific information by the general public.

The relationship between common sense and scientific knowledge, and the expectations placed on the average citizen with regard to expert knowledge.

The impact of disinformation, identifying technological (e.g. algorithmic design) and cognitive factors that contribute to its credibility, as well as ways to eliminate its harmful effects without affecting freedom of expression.

Arguments from “anti-science” movements that lead to skeptical conclusions.

New social phenomena such as post-truth relativism or the commodification of information as well as reinvigorated ones such as political polarization.

The dimensions of human vulnerability (particularly biases and cognitive vulnerability) as well as the psychological drivers of anxiety, denial, paranoia and political disaffection, in order to connect science with public concerns.

The conception of an ethical-epistemological equilibrium of the levels of uncertainty, trust and responsibility (crucial for cooperation, communication and knowledge transfer) serves as a methodological framework, a dynamic model which helps to scrutinize different mechanisms and factors implied in the disinformation processes. The research draws on an interdisciplinary perspective, guaranteed by the composition of the research team, that counts with specialists from areas such as philosophy, biology, psychology, anthropology, sociology and journalism, and applying a methodology based on the epistemological and ethical analysis of scientific literature, news and opinions posted in social networks.

Based on the results of these analyses, we formulate a series of structural proposals in terms of educational content, regulation of digital platforms, institutional and media communication strategies, and ethics in the digital sphere. We propose a multidimensional concept of structural responsibility that combines moral and legal responsibility with an epistemic and political one, a concept capable of encompassing the individual, institutional and corporate dimensions.

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Containment of COVID-19: Policy design and compliance

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Objectives: The aim of this Project is twofold: (i) to analyse the measures that regional and local authorities adopted to contain the spread of the Pandemic and the motivations behind. (ii) to study the drivers of citizens’ compliance with these measures.

Methods: We have applied two different research designs for each of these two objectives. For the first objective, we carried out semi-structured interviews with decision-makers in charge of enforcing the COVID-19 related measures at the regional and local levels in a sample of four regions (Asturias, Galicia, Madrid, and Valencia). We have coded the transcripts of the interviews. For the second objective, we administered an online survey in February 2022 with a representative sample of the population aged 18 and over living in Spain (i.e., N=3291) and we oversampled the four selected regions. The survey, apart from asking questions regarding compliance with COVID-19 related measures during the first and the second state of emergency, also included a conjoint to test the relative importance of different attributes that inform citizens’ preferences.

Results: We have produced a preliminary report describing citizens’ attitudes and opinions. The study confirms, with few exceptions, the strong orientation of Spaniards towards complying with norms with few variations at the regional level. Concerning policymaking, we have identified a relative significant convergence among regions (with a possible exception) in prioritizing health over other (i.e. economic) concerns. We have also identified a shared trust on citizens’ predisposition to comply as the basic belief inspiring the design of norms.

Conclusions: The project is not completed yet. Provisional conclusions point in the direction of strong willingness to comply with restrictive norms because of health considerations by citizens and a concomitant trust on compliance by public authorities. Because of this, policymakers, in general, did not consider strong enforcement mechanism as an essential instrument for extracting compliance.

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Determination of pathogenic potential of Spanish lineage 1 and 2 WNV strains in a mouse model

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West Nile virus (WNV) is the most widespread emerging arbovirus in the world. Its transmission cycle is complex and is driven by factors associated to the virus, the vectors, the hosts and the environment. The incidence and spread of WNV have increased in Europe in the last 2 decades with two genetic lineages (L1 and L2) currently circulating. L1 was first identified and isolated in Spain in 2007. Since then it is spreading throughout South-Western Spain, with cases in horses and humans reported in the area since 2010, including an unprecedented outbreak of WNV meningoencephalitis in Andalusia, with 77 human cases and 8 deaths in 2020. Meanwhile in Catalonia (North-Eastern Spain), L2 has been detected repeatedly in goshawks (*Accipiter gentilis*) and mosquitoes since 2017, with no human cases reported yet.

In this study, the pathogenic potential in mammals of recent Spanish isolates (year 2020) from L1 (Andalusia) and L2 (Catalonia), was evaluated in a mouse model and compared with previous Spanish isolates.

Groups of twelve four-week-old female Swiss outbred mice were intraperitoneally inoculated with 1000 pfu of each viral isolate and monitored daily for symptoms and death, up to 3 weeks after inoculation.

First clinical signs appeared at 6-7 days-post-infection (dpi) and mortality started between 7 and 9 dpi. Overall, Catalonian L2 isolates showed lower virulence profiles as compared to Andalusian L1 isolates in this animal model. Statistically significant differences in mortality and median survival times were found between these two groups. Pathogenicity profiles of lineage 2 isolates from Catalonia were similar to the observed for B956 strain considered of moderate virulence, whereas Andalusian L1 isolates of 2020 were similar to highly virulent isolates obtained previously from the same area.

The results obtained using this mouse model point out that the recent WNV L2 strains circulating in Catalonia could represent a lower risk for humans and horses to cause severe illness in case of infection as compared to recent WNV L1 isolates from Southern Spain. Remarkably, the latter come from the same area and year where severe human outbreaks occurred in 2020.

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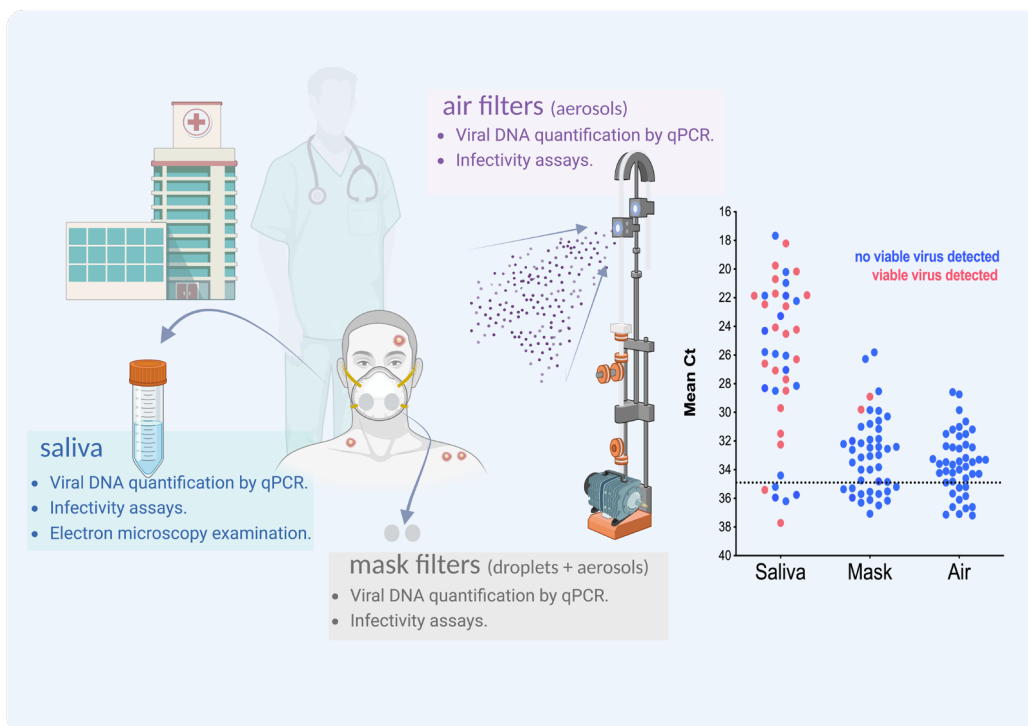
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Monitoring monkeypox virus in saliva and air samples

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Transmission of monkeypox virus (MPXV) occurs through direct contact, but transmission through saliva or exhaled droplets and aerosols has not yet been investigated. We evaluated in 45 samples from MPXV-infected patients the presence of virus in saliva, exhaled droplets within a mask and aerosols captured by air filtration through newly developed nanofiber filters. Viral DNA was detected by quantitative polymerase-chain-reaction (qPCR) and infectious virus was isolated in cell cultures. We identified high loads of MPXV DNA by qPCR in 85.3% (35/41) of saliva samples. Infectious MPXV was recovered from 66.7% (22/33) of saliva samples positive for MPXV DNA. We also found a significant association between the number of affected cutaneous territories or general symptoms and the viral load in saliva. Droplets exhaled from infected patients, detected inside a mask, contained MPXV DNA in 71.1% (32/45) of the samples and 2/32 of the positive samples showed infectious virus. MPXV DNA in aerosols, collected from the medical consultation room, were detected in 64.2% (27/42) of the cases, in spite of patients wearing an FFP2 mask. Infectious virus was not recovered from aerosol samples. High levels of MPXV DNA were identified in aerosols collected from a hospital isolation room housing a MPXV-infected patient.



Graphical Abstract

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Surface Modification of metal surfaces to provide antimicrobial properties

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Background: While aerosols are the main vector for microbial spreading, the survival of microorganisms on inanimate surfaces means that they play an important role in the transmission of infections. It is well reported literature, that viruses can remain active on surfaces for periods ranging from hours to days. Metallic materials are part of our daily lives in a wide range of applications. Among them, the 304 stainless steel is particularly important for transportation and architectural applications. So, the present research is focused on the functionalization of austenitic stainless steels with antimicrobial properties by developing continuous, nanostructured anodic layers containing fluoride ions.

Methods: The layers were prepared by anodization of mirror polished samples in an electrolyte composed of ethylene glycol, ammonium fluoride and water under a constant voltage.

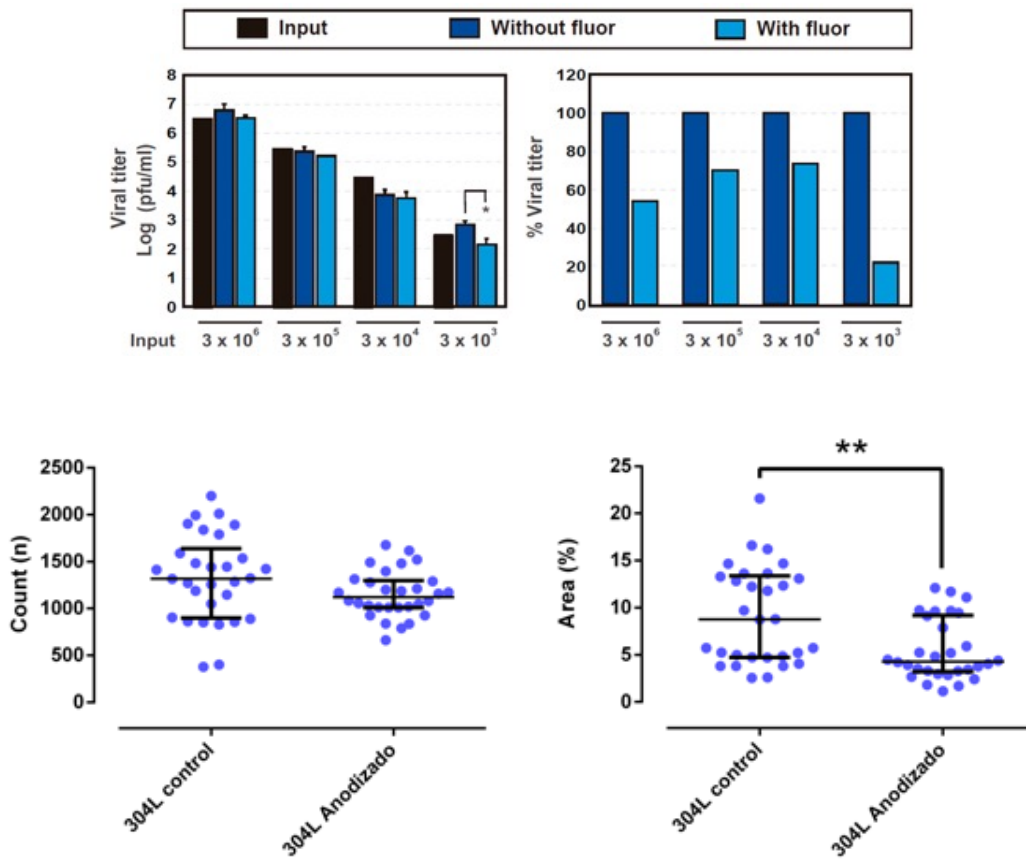
The first study was made with HCoV-229E-GFP virus. Three different viral loads were used [3×10^6 ; 3×10^5 ; 3×10^4 and 3×10^3 plaque forming units (PFU)] and were incubated on the layers with a final volume of 100 μ l. The contact time of the virus on the surface was approximately 1 hour. The virus was resuspended in 1 ml DMEM using vortex. Following a standardized titration protocol, the supernatants were serially diluted and inoculated in Huh-7 cells. Viral titer are expressed as PFU/ml. To control for the absence of contamination, layers with DMEM in the absence of virus were maintained in parallel.

Bacterial Adherence was evaluated using a *P. aeruginosa* ATCC27853 following the methodology previously described (Aguilera-Correa et al., Appl Environ Microbiol. 2019 Jan 9;85(2):e02271-18) at room temperature for 90 min incubation. The experiments were performed in triplicate. The statistical data were analyzed by the nonparametric unilateral Wilcoxon test with a level of statistical significance of $p < 0.05$. The values are cited as medians and interquartile range.

Results: Figure 1(up) showed that incubation in anodized surfaces with F reduces the viral titer of the virus. A new set of experiments have been recently done with SARS-CoV-2 following exactly the same protocol that was used with 229E. However, it was not found a reduction in the viral titers in anodized surfaces with F as compared to control samples (i.e. in absence of F).

Figure 1 (down) shows the results of the bacterial adherence study. Both the count and the % area covered by bacteria reveal that the anodized surface with F reduces the bacterial adherence of *P. aeruginosa* by up to 50% compared to the non-anodized surface (control samples).

Conclusion: Despite the good results obtained for the HCoV-229E-GFP, the results done with SARS-CoV-2 did not show a reduction in the viral titers in the presence of F as compared to the absence of F. It is interesting to know why two different coronaviruses behave differently. A new study is being carried out with new SARS-CoV-2 variants (omicron). Also, new studies are being carried out to test antibacterial properties with additional commercial and clinical strains.



Graphical Abstract

Figure 1. Up) HCoV-229E-GFP virus; down) *P. aeruginosa* ATCC27853

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Accumulation dynamics of DVGs during experimental evolution of betacoronaviruses

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Replication of RNA genomes by virus-encoded RdRPs often results in hypermutated genomes or in truncated versions of the genome (DVG), an especial case of the latter being the defective interfering particles (DIPs). DVGs are incompetent to complete the infection cycle by themselves, but coinfection with a helper virus that provides the missing protein(s) in trans may result in their amplification and transmission. DIPs are well known to modulate viral replication, to affect disease progression, to elicit immune responses, to promote viral persistence after infection, and in some cases also to accelerate viral evolution, thus they are an important part of the multicomponent viral system.

In this project, we focused our effort to characterize the dynamics of formation and evolution of DVGs in evolution experiments with betacoronaviruses. The long-term goal of the study was to select a library of DIPs with optimized interfering activity. As model virus for the evolution experiments, we have used the human coronavirus OC43 (HCoV-OC43). We performed more than 30 serial passages in two susceptible cell lines, BHK-21 and HCT-8. Experiments were carried out at low and high multiplicity of infection (MOI). High-throughput sequencing was performed at several evolutionary passages to identify DVGs.

Preliminary results show that the variability of DVGs in BHK-21 at high MOI increased first to significantly decrease after passage 23, while at low MOI no significant changes in the number of DVGs were detected. Some DVGs were persistently observed at high MOI, while others had a transitory existence and were lost. In HCT-8, the tendency was similar, though less drastic.

Regarding the abundance of DVGs, significant MOI-dependent changes were only found in the first-intermediate stages of the evolution in both experimental hosts. Comparing the differences among passages, at high MOI the virus maintained the trend to increase the abundance of DVGs in both cell lines, but in BHK-21 this effect was more noticeable. At low MOI the number of DVG counts was not increasing in HCT-8 and even slightly decreasing in BHK-21.

Next, we looked at deletions as they may be of special interest for positive strand RNA viruses. In fact, the most abundant DVGs were deletions (51.4 - 72.3%). Their size distribution at low MOI was bimodal, with short and long DVGs being common, and showed almost no changes during the evolution, except for intermediate passage in HCT-8, where shorter DVGs were more prevalent. In samples at high MOI, the size distribution was multimodal and shifted over time to longer DVGs. Hot-spot regions for generation of DVGs were found. They were similar to the ancestral strain, but density increased along with evolutionary passages (except for subgenomics), specially at high MOI.

In conclusion, we showed the pervasive formation of DVGs during HCoV-OC43 evolution, with the dynamics being MOI- and host-dependent.

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Exploring the accessibility of adaptive pathways of the SARS-CoV-2 spike protein by in vitro experimental evolution

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High mutation rates, short generation times and large population sizes fuel up the ability of RNA viruses to explore adaptive landscapes, resulting in fitness increases. Such evolvability is particularly relevant for zoonotic viruses, as it is the case of SARS-CoV-2, because they are still engaged in adaptation to their new hosts. During the ongoing COVID19 pandemic, a large number of SARS-CoV-2 variants have been identified. Some of these variants have been categorized by the WHO as VOC or VOI (variants of concern or of interest, respectively), due to the presence of mutations in the spike glycoprotein (S) that could be potentially important for its function. However, not all of them have been equally relevant according to their incidence in the population or to the severity of symptoms. One could expect that this relevance might only reflect differences in biological fitness of the variants, yet other factors could affect the epidemiological fate of these variants (e.g., demographic, economical, sanitary, social, political...). Our aim is to explore and describe the topography of the adaptive fitness landscape of the SARS-CoV-2 S protein in susceptible human cells, describing the number of potential fitness peaks, and evaluating the number of accessible adaptive walks. To achieve this goal, we have started focusing in the most prevalent mutations (present in >75% of the sequences) in the VOC and VOI identified by September 2021. Using maximum likelihood methods, we have inferred the ancestral amino acid sequences at each node of the diversification tree of the S protein. These sequences depict a set of potentially accessible evolutionary pathways followed by SARS-CoV-2 S protein along the pandemic. Fitness for all these S variants was assayed using pseudo-typed vesicular stomatitis virus (VSV), with the S protein in their envelope, by head-to-head competition assays in human (HEK293) or monkey (VeroE6) cells expressing the ACE2 receptor with their respective ancestors and descendants. To further evaluate the evolvability of all these variants and the likelihood of selection to find different evolutionary solutions, serial passages of the pseudoviruses carrying different S forms were performed.

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