Long Covid Coalition Conference

April 30, 2022 Dr. Carlo Brogna M.D., Chief director of Craniomed group research facility, Bresso (Mi), Italy

Toxins-like peptide in SARS-CoV-2 infection. Who produced them?

Topics presented:

1. Definitions: Long Covid; Symptoms; coagulation and neurological disorder; Present and past analogies, acetylcholine signalling; neuronal receptors.

2. Toxin-like peptides: What they are; where they come from; why they are important; how to recognize them, consideration in the acute phase, and Long Covid

3. Future scientific and industrial implications.

4. **What about in terms of public health safety?**

Toxin-like peptide in SARS-CoV-2 infection. Who produced them?

Non Topics presented:

- Vaccines
- Hydroxychloroquine
- Invermectin
- Other products

THIS PRESENTATION is presented **ONLY what we have observed in the lab**, published and reviewed, published and not yet reviewed, and some industrial data. We will **discuss how we have reported observations from our research into daily practice** in the care of **COVID-19 and Long Covid patients.**

1 .Definitions: Long Covid 1/3

Long Covid

- ***The definition of the Long Covid condition,*** described World Health Organitation (WHO) is as follows : *"Post COVID-19 condition occurs in individuals with a history of probable or confirmed SARS CoV-2 infection, usually 3 months from the onset of COVID-19 with symptoms and that last for at least 2 months and cannot be explained by an alternative diagnosis.*

Common symptoms

1. *fatigue*
2. *dyspnea*
3. *myalgia*
4. *cough*
5. *headache*
6. *cognitive dysfunction*
7. *joint pain*
8. *chest pain*
9. *altered smell*
10. *diarrhoea*
11. *altered taste*
12. *others*

Aiyegbusi Ol. et al, (2021) said :"*Symptoms may be new onset following initial recovery from an acute COVID-19 episode or persist from the initial illness.* ***Symptoms may also fluctuate or relapse over time****"(2).*

Aiyegbusi OL, Hughes SE, Turner G, Rivera SC, McMullan C, Chandan JS, Haroon S, Price G, Davies EH, Nirantharakumar K, Sapey E, Calvert MJ; TLC Study Group. Symptoms, complications and management of long COVID: a review. J R Soc Med. 2021 Sep;114(9):428-442. doi: 10.1177/01410768211032850. Epub 2021 Jul 15. PMID: 34265229; PMCID: PMC8450986.

1 .Definitions: Long Covid 2/3

In a prospective study (Chicago, Illinois, USA) (3) of ***100 non hospitalized*** patients,

- 50 nasopharyngeal swab and/or SARS-CoV-2 antibody positive
- 50 SARS-Cov-2 negative patients but recruited in accordance to ISDA Covid-19 symptom guidelines (4)
- **“brain fog” (81%), headache (68%),** numbness/tingling (60%), dysgeusia (59%), anosmia (55%), and myalgias (55%), 85% fatigue
- They concluded the results: “ ..both groups exhibited **impaired quality of life in cognitive and fatigue domains.** SARS-CoV-2+ patients performed **worse in attention and working memory cognitive tasks** compared to a demographic-matched US population”.

3.Graham EL, Clark JR, Orban ZS, Lim PH, Szymanski AL, Taylor C, DiBiase RM, Jia DT, Balabanov R, Ho SU, Batra A, Liotta EM, Koralnik IJ. Persistent neurologic symptoms and cognitive dysfunction in non-hospitalized Covid-19 "long haulers". Ann Clin Transl Neurol. 2021 May;8(5):1073-1085. doi: 10.1002/acn3.51350. Epub 2021 Mar 30. PMID: 33755344; PMCID: PMC8108421.

4. Hanson KE, Caliendo AM, Arias CA, Hayden MK, Englund JA, Lee MJ, Loeb M, Patel R, El Alayli A, Altayar O, Patel P, Falck-Ytter Y, Lavergne V, Morgan RL, Murad MH, Sultan S, Bhimraj A, Mustafa RA. The Infectious Diseases Society of America Guidelines on the Diagnosis of COVID-19: Molecular Diagnostic Testing. Clin Infect Dis. 2021 Jan 22:ciab048. doi: 10.1093/cid/ciab048. Epub ahead of print. PMID: 33480973; PMCID: PMC7929045

In an Italian study (5) of ***238 patients hospitalized*** for COVID-19 and followed up to month 12, a considerable presence of post-COVID symptoms is underlined.

- **A decrease in diffusing lung capacity for carbon monoxide** (DCLO ≤ of 80%) in 39.5% of subjects,
- **motor impairment** in 25.8% of patients who also had posttraumatic stress syndrome (PTS).

5. Bellan M, Baricich A, Patrucco F, Zeppegno P, Gramaglia C, Balbo PE, Carriero A, Amico CS, Avanzi GC, Barini M, Battaglia M, Bor S, Cantaluppi V, Cappellano G, Ceruti F, Chiocchetti A, Clivati E, Giordano M, Cuneo D, Gambaro E, Gattoni E, Loro A, Manfredi M, Morosini U, Murano F, Paracchini E, Patti G, Pinato DJ, Raineri D, Rolla R, Sainaghi PP, Tricca S, Pirisi M. Long-term sequelae are highly prevalent one year after hospitalization for severe COVID-19. Sci Rep. 2021 Nov 22;11(1):22666. doi: 10.1038/s41598-021-01215-4. PMID: 34811387; PMCID: PMC8608998.

1 .Definitions: Long Covid 3/3

The **need for oxygen therapy, preexisting hypertension**, and **chronic pulmonary conditions** have been highlighted as **major determinants** of long-term symptoms (6).

6. Galal I, Hussein AARM, Amin MT, Saad MM, Zayan HEE, Abdelsayed MZ, Moustafa MM, Ezzat AR, Helmy RED, Abd_Elaal HK, Al Massry NA, Soliman MA, Ismail AM, Kholief KMS, Fathy E, Hashem MK. Determinants of persistent post-COVID-19 symptoms: value of a novel COVID-19 symptom score. Egypt J Bronchol. 2021;15(1):10. doi: 10.1186/s43168-020-00049-4. Epub 2021 Feb 5. PMCID: PMC7863043

It would appear that there is
an increased risk for hospitalized patients to develop a Long Covid Syndrome

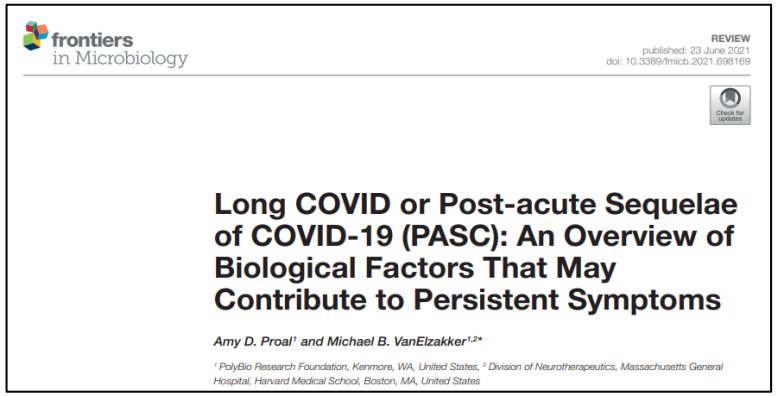
1 .Definitions: Coagulation and Neurological disorders

The **alteration of coagulation parameters** is related to **severe events** of COVID-19 (7). Some authors (8) have emphasized the importance of the **prothrombotic state in COVID-19 patients** for activation of coagulation mechanisms as they occur in **other viruses** such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), influenza virus, Ebola, and Dengue virus (DV) (9-12).

Guillain Barré syndrome (GBS) could be listed as a manifestation of Long Covid condition. The GBS is usually **rapidly progressive but self-limiting inflammatory polyneuropathy** characterized by muscle weakness and mild loss of distal sensation, which usually arises after an infectious process on an autoimmune basis. Approximately **one-quarter** of patients with Guillain-Barré syndrome had a recent **Campylobacter jejuni** infection (13). **Molecular mimicry** between microbial and nerve antigens is clearly a major driving force behind the development of the disorder, at least in the case of Campylobacter jejuni infection (14). **Double infection of campylobacter and coronavirus was also found in marmosets** (15).

From the beginning of the pandemic until November 2021, **approximately 90 cases of GBS** have been reported (16-19). A correlation with GBS cases was **observed during the Zika virus pandemic** (20). As early as **1941**, cases of **GBS associated with probable viral genesis** were shown (21) to develop in children who had had inflammatory upper airway disease days earlier, including **poliovirus infection.**

1 .Definitions: Coagulation and Neurological disorders -PACS


frontiers in Microbiology

REVIEW
published: 23 June 2021
doi: 10.3389/fmicb.2021.698169

Long COVID or Post-acute Sequelae of COVID-19 (PASC): An Overview of Biological Factors That May Contribute to Persistent Symptoms

Amy D. Proal[1] and Michael B. VanElzakker[1,2]*

[1] PolyBio Research Foundation, Kenmore, WA, United States, [2] Division of Neurotherapeutics, Massachusetts General Hospital, Harvard Medical School, Boston, MA, United States

Post-acute sequelae of COVID-19 (PASC)

Myalgic encephalomyelitis/ chronic fatigue syndrome (ME/CFS)

*«Some PASC patients **meet the diagnostic criteria** for myalgic encephalomyelitis/chronic fatigue syndrome **(ME/CFS)** – a neuroinflammation-linked condition characterized by a **range of debilitating chronic symptoms** including severe fatigue, musculoskeletal pain, and post-exertional malaise (worsening of symptoms following exertion)»*

*…"most cases of ME/CFS **begin with a viral infection**"……*

***"Immune dysregulation** driven by SARS-CoV-2 might also promote the collective **imbalance of the human body's microbial and viral ecosystems** in a manner that could result in PASC symptoms."*

*"Even **human blood** has been shown to **harbor communities of organisms**, especially in immunocompromised individuals"*

*"Under conditions of health, these host microbiome/virome communities are kept "in check" by a robust host immune response, and persist in a state of balance or homeostasis. However, **dozens of chronic conditions are now tied to dysbiosis: a collective imbalance of microbiome/virome** ecosystem composition and dynamics"*

1 .Definitions: Hyposmia/anosmia 1/2

- An **alteration in the neuro-modulatory transmission of acetylcholine** and its dependent receptors (nicotinic and muscarinic).
- Smell as an important **predictor of neurodegenerative diseases**.
- Both **nicotinic and muscarinic ACh receptor** (nAChR and mAChR, respectively) subtypes are expressed in the **glomerular layer of the olfactory pathway** and have various consequences on processing by providing distinct mechanisms by which ACh can modulate olfactory information
- Also, **coronaviruses in mice** resulted in decreased odor perception

1 .Definitions: Hyposmia/Anosmia 2/2

Parkinson's disease (PD)	Lewy body disease
Dementia	Schizophrenia: reduced expression of α7 acetylcholine receptors in the hippocampus
α-synuclein pathology	Common Cold- influenza viruses occasionally
Myasthenia gravis	Snake bite
Accidental toxin exposure	Coronavirus 229E
Poor industrial hygiene	Common Coronavirus
Low airborne toxins over long periods	Drugs
Alzheimer's disease (AD)	Others

Conflict

The regeneration of this epithelium occurs from the basal cells of the olfactory bulb (OB) in a period between 60 and 90 days

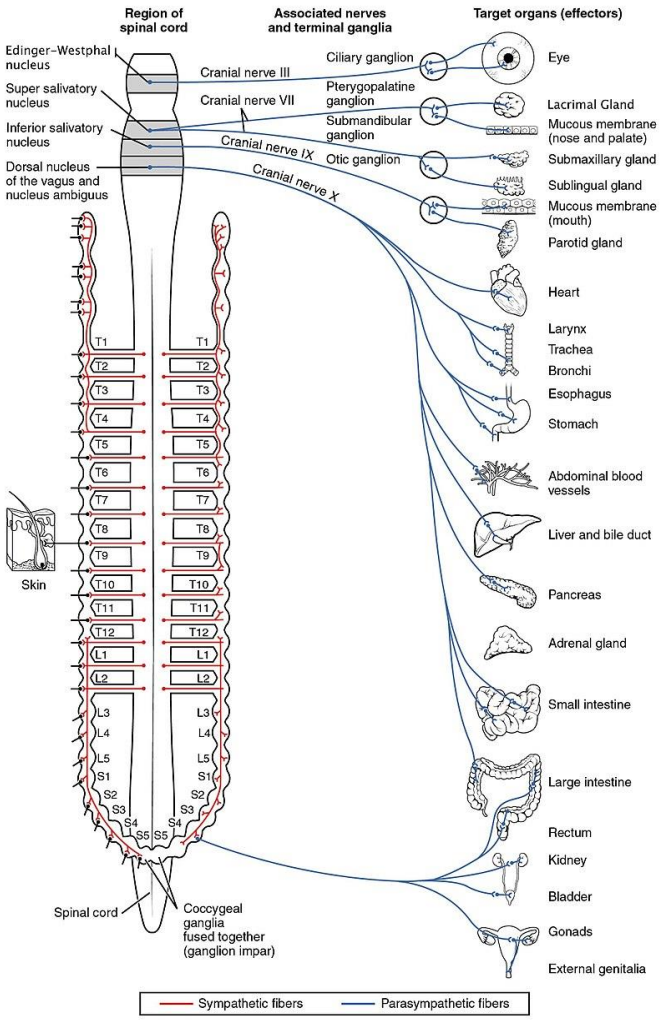
Hyposmia in COVID -19 patients don't have the same duration (40).
Recovery from loss of sense of smell could vary from 7 up to 28 days in those affected.

And more time in LONG COVID patients.

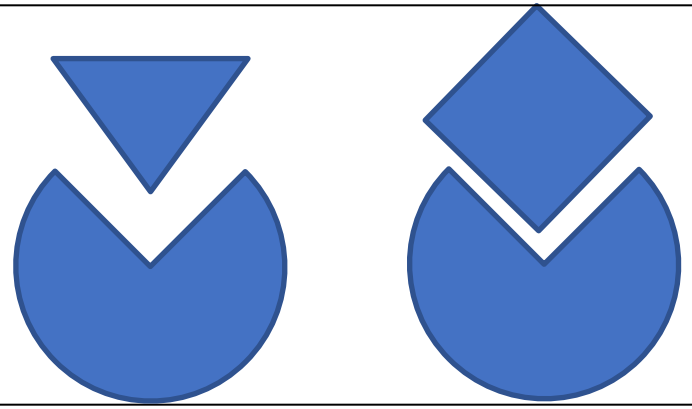
1 .Definitions: Autonomic nervous system (ANS) and receptors

The autonomic nervous system (ANS), formerly referred to as **the vegetative nervous system**, is a division of the peripheral nervous system that supplies smooth muscle and glands, and thus influences the **function of internal organs**.The autonomic nervous system is a **control system** that acts largely unconsciously and **regulates bodily functions, such as the heart rate, digestion, respiratory rate**, pupillary response, urination, and sexual arousal. This system is **the primary mechanism in control of the fight-or-flight response.**

The autonomic nervous system has three branches: **the sympathetic nervous system, the parasympathetic nervous system and the enteric nervous system**



Different molecules or drugs can bind to the same receptor and activate or block it



Receptors are macromolecules involved in the chemical **transmission of signals** within the cell or between cells; they may be located on the surface of the cell membrane or within the cytoplasm

The **pharmacological effect** is also determined by the length of time the drug-receptor complex persists (**residence time**)

For some receptors, transient occupancy of the drug produces the desired pharmacological effect, whereas **prolonged occupancy causes toxicity.**

1 .Definitions: Cholinergic Medications (CM)

C.M. pharmaceutical agents **acting on the neurotransmitter ACh** – Primary neurotransmitter of parasympathetic nervous system (PNS)

2 types of drugs: **direct-acting and indirect** –acting on receptors

Drugs Direct Acting: Choline esters (acetylcholine, methacholine, carbachol, bethanechol), alkaloids (muscarine, pilocarpine, cevimeline)

Drugs Indirect acting (**increase the level of ACh**): reversible agents (physostigmine, neostigmine, pyridostigmine, edrophonium, rivastigmine, donepezil, galantamine) and irreversible agents (echothiophate, parathion, malathion, diazinon, sarin, soman, nerve gas)

However also **venom animal toxins are cholinergic acting Drugs.**

1 .Definitions: ANS and receptors

Drugs Indirect acting increases the Ach level **inhibiting the level of acetylcholinesterase** and **butyrylcholinesterase (aChE and BuChE, respectively).**

They are two closely homologous enzymes, valuable biomarkers in diseases associated with parasympathetic malfunction.

Serum Cholinesterase dosage in COVID-19 patients

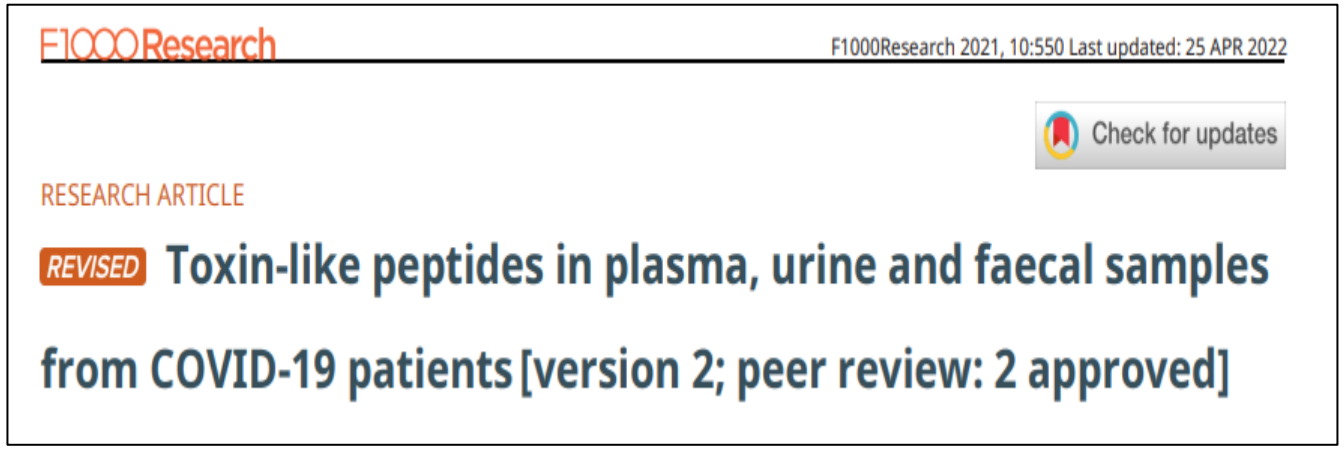
Nakajima K. et al. (*Nakajima, Kento et al. "Serum cholinesterase associated with COVID-19 pneumonia severity and mortality." The Journal of infection vol. 82,2 (2021): 282-327. doi:10.1016/j.jinf.2020.08.021*) were among the first to **emphasize the importance of assaying cholinesterases** during the admission of COVID-19 patients to the hospital to classify the degree of severity and have a **prognostic factor**.

1 .Definitions: Symptoms of the toxins on Achr (N-M)

Cardiocirculatory collapse bradycardia, hypotension, arrhythmias	M2 receptors: bradycardic effect with slowing heart activity and cardiac range reduction, followed by arrhythmic and tachycardic compensation
Heart arrhythmias	in generalized vasodilation resulting in a rapid drop in blood pressure
Ventricular tachycardia	
Body temperature alteration	
Electrolyte imbalance (particularly K+)	Eye symptoms include myosis, eye pain, conjunctive congestion, vision reduction, ciliate spasm, and eyebrow pain.
Butyrylcholinesterase low plasma levels	The effects on the Snc are confused state, ataxia, verbal confusion, reflexes loss, Cheyne-stokes breath, convulsions, coma, and respiratory paralysis.
Other nonspecific neurosensory symptoms : anosmia and dysgeusia	Blood pressure can drop to low levels due to irregularities in heart rhythm, effects caused by hypoxemia, and often antagonized by assisted ventilation lung.

Rhinorrhea and hyperemia	nausea
Chest constriction sense	vomiting
Breathlessness	abdominal cramps
Bronchus constriction	diarrhea
Bronchial secretions increase	increased gastrointestinal motility
Prolonged apnea	
Muscle fatigue and general weakness, involuntary contractions, spread fasciculations, and finally marked weakness increase and respiratory muscles paralysis	Extreme salivation, involuntary feces, and urine emission, sweating, tearing
Laryngospasm	Impaired diaphragm and intercostal muscles movements, and respiratory depression

2 . Toxin-like peptides: What they are; where they come from; why important; how to recognize them.


F1000Research F1000Research 2021, 10:550 Last updated: 25 APR 2022

Check for updates

RESEARCH ARTICLE

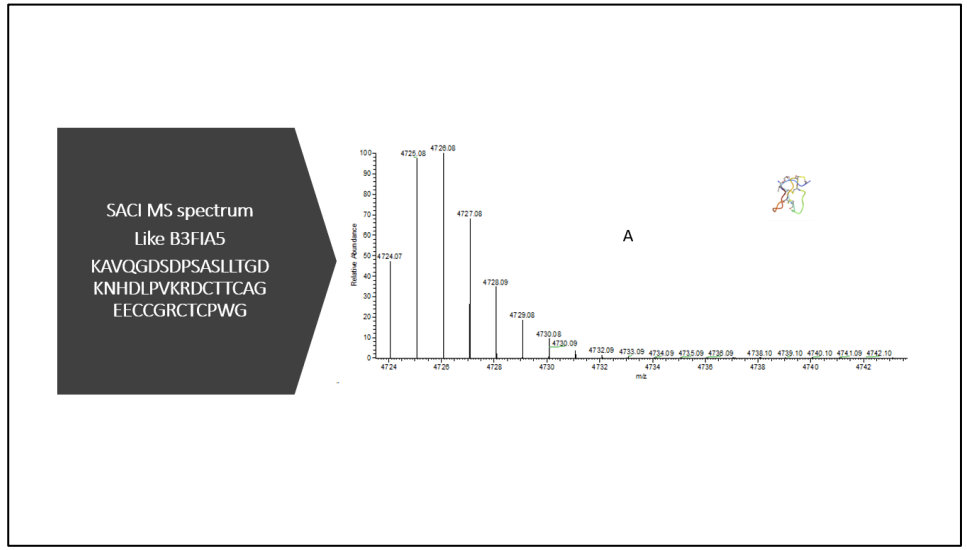
REVISED **Toxin-like peptides in plasma, urine and faecal samples from COVID-19 patients [version 2; peer review: 2 approved]**

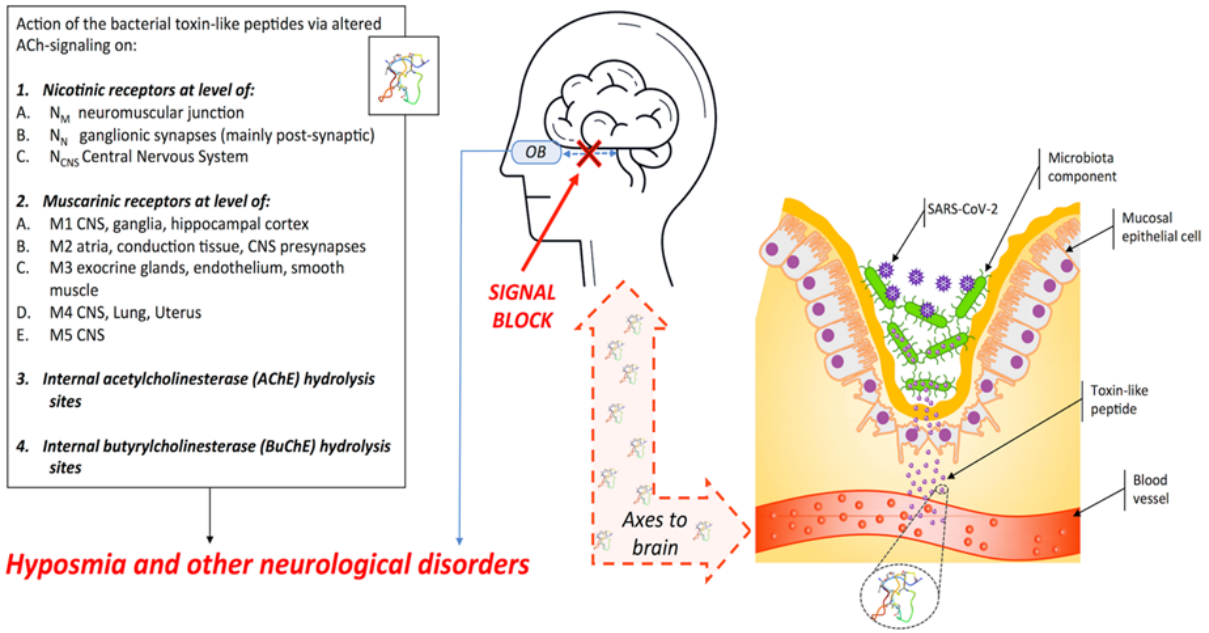
Sample Analyzed for the presence of proteins with potential toxic effects **using ion mobility mass spectrometry (LC-SACI-CIMS)** coupled with sur-face-Electrospray-NIST-activated chemical ionization (SANS), followed by a Bayesian model search (SANIST-CIMS) against the complete 'Uni-Prot KB set of manually revised venom proteins and toxins'.

Each of the samples tested showed a high number, **greater than 70,** of oligopeptides. Furthermore, analyzing the single sequences, it was evident that frequent amino acid variations within the same peptides suggested a **possible origin from a polycistronic transcription** mechanism typical of bacteria. This was confirmed by the same examination performed **in bacterial cultures** contaminated by SARS-CoV-2.

Protein	ID	Database	-log(e)	FDR p value
Conotoxin Pu6.1	D2DGD8	Uniprot	75	0.001
Conotoxin Vi15a	B3FIA5	Uniprot	89	0.005
Putative alpha-conotoxin Qc alphaL-1	A1X8B8	Uniprot	76	0.005
Conotoxin 10	Q5K0C5	Uniprot	76	0.001
Rho-conotoxin TIA	P58811	Uniprot	54	0.001
Kunitz-type serine protease inhibitor conotoxin Cal9.1a	D2Y488	Uniprot	67	0.001
Alpha-conotoxin Pu1.5	P0C8U9	Uniprot	57	0.002
Conotoxin Fla16d	V5V893	Uniprot	67	0.003
Phospholipase A2 MALT0035C	F5CPF1	Uniprot	87	0.003
Phospholipase A2 AP-PLA2-I	Q3C2C2	Uniprot	81	0.004
Acidic phospholipase A2 PePLA2	Q2PG83	Uniprot	66	0.001
Basic phospholipase A2 BFPA	A6MEY4	Uniprot	69	0.001
Basic phospholipase A2 PL-X	P06860	Uniprot	70	0.001
Complement factor B Ba fragment	Q91900	Uniprot	74	0.001
Acidic phospholipase A2 homolog textilotoxin D chain	P23028-1	Uniprot	73	0.002
Acidic phospholipase A2 homolog textilotoxin D chain	P23028-2	Uniprot	65	0.002
Venom prothrombin activator pseutarin-C non-catalytic subunit	Q7SZN0	Uniprot	60	0.002
Coagulation factor V	Q593B6	Uniprot	61	
Venom prothrombin activator oscutarin-C non-catalytic subunit	Q58L91	Uniprot	87	0.001
Short neurotoxin 4	Q9W7J9	Uniprot	69	0.001
Conotoxin Cl9.6	D6C4M3	Uniprot	58	0.002
Zinc metalloproteinase-disintegrin-like halysase	Q8AWI5	Uniprot	57	0.003
Alpha-elapitoxin-Oh2b	P82662	Uniprot	96	0.003
Sigma-conotoxin GVIIIA	P58924	Uniprot	43	0.002
Conotoxin Mr15.2	P0DM19	Uniprot	47	0.001
Conotoxin mr3g	P0C1N5	Uniprot	74	0.001
Contryphan-R	P58786	Uniprot	58	0.002
Snake venom metalloprotease inhibitor 02D01	A8YPR6	Uniprot	43	0.002
Bradykinin-potentiating and C-type natriuretic peptides	P0C7P5	Uniprot	51	0.003
Bradykinin-potentiating and C-type natriuretic peptides	Q9PW56	Uniprot	51	0.003
Zinc metalloproteinase/ disintegrin	Q698K8	Uniprot	49	0.004

2 . Toxin-like peptides: What they are; where they come from; why important; how to recognize them.





The ID-like proteins/toxins were founded, using SACI MS method, in bacteria colture and plasma of COVID-19 patients (UniProtKB/Swiss-Prot database). In yellow, the **C-CC-C- cysteine-rich motif is typical of toxin aging on Acetylcholine receptors.**
KAVQGDSDPSASLLTGDKNHDLPVKRDCTTCAGEECCGRCTCPWG

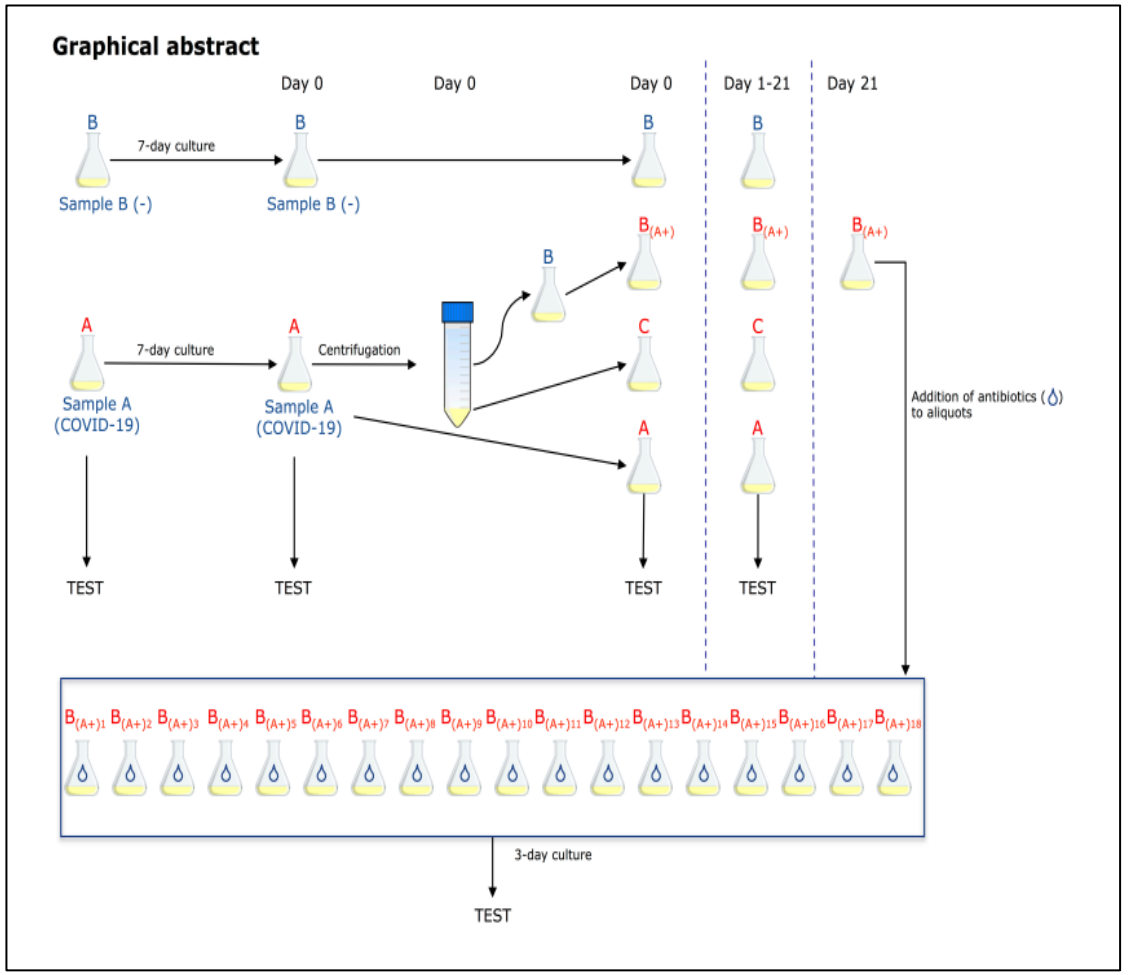
2 . Toxin-like peptides: What they are; where they come from; why important; how to recognize them.

F1000Research F1000Research 2021, 10:370 Last updated: 25 APR 2022

Check for updates

RESEARCH ARTICLE

REVISED **Increase of SARS-CoV-2 RNA load in faecal samples prompts for rethinking of SARS-CoV-2 biology and COVID-19 epidemiology [version 3; peer review: 2 approved]**

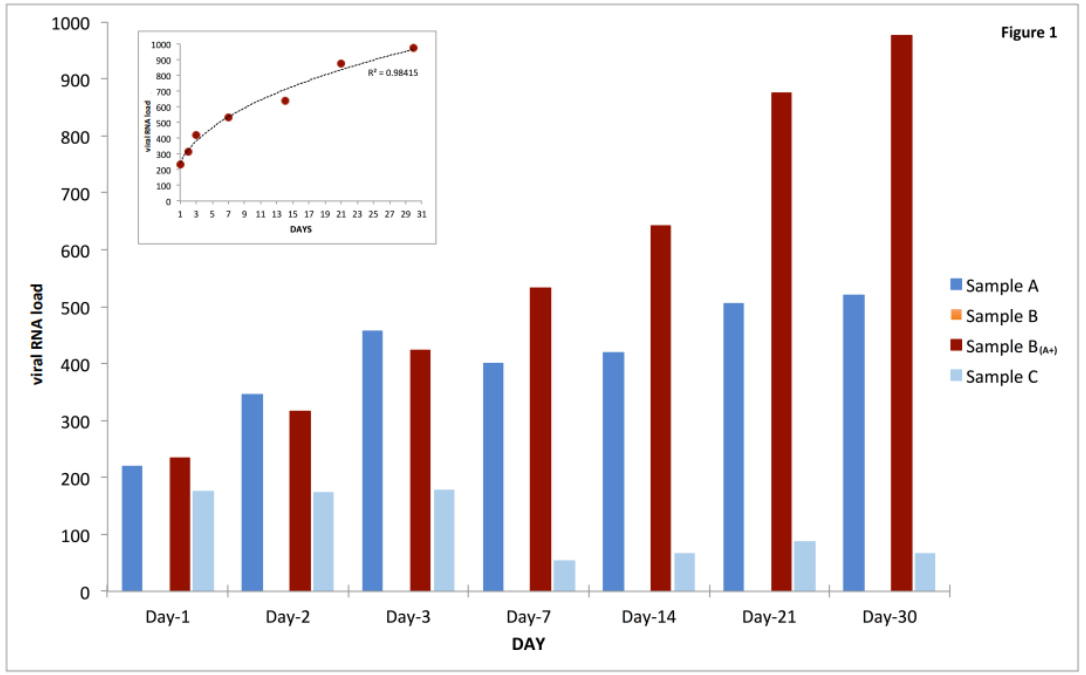


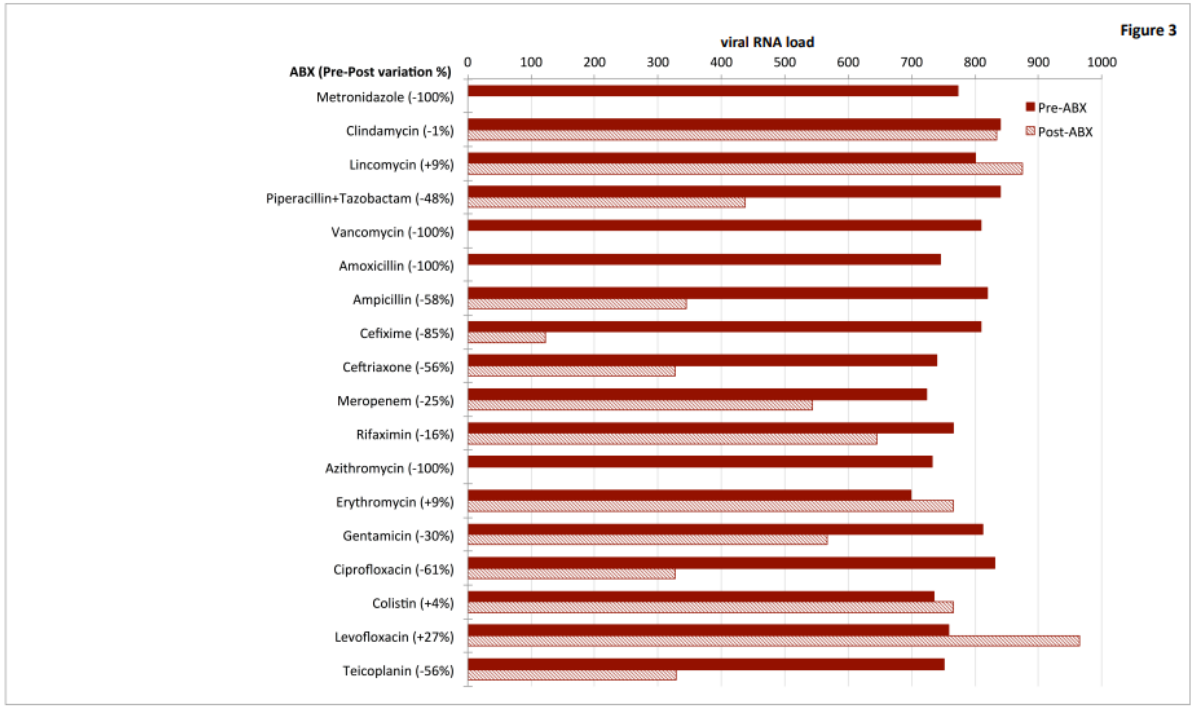
Samples A : cultures of bacteria from COVID-19 positive patients

Samples B: are cultures of bacteria from healthy persons but contaminated with supernatant from samples A

Sample C: obtained from the pellet, without supernatant from sample A, and placed in a new culture.

2 . Toxin-like peptides: What they are; where they come from; why important; how to recognize them.



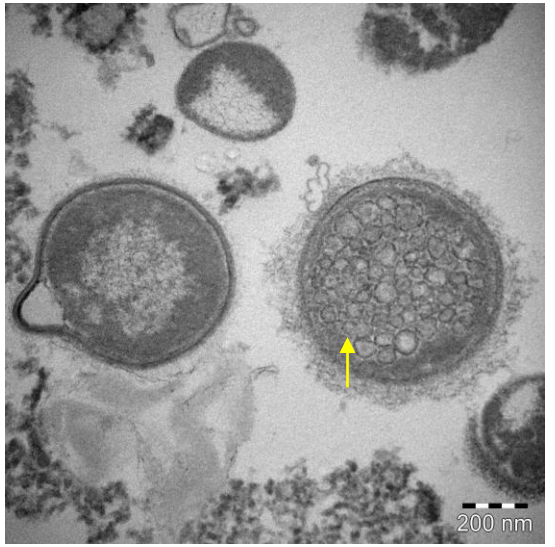


SARS-CoV-2 RNA load variation over time. SARS-CoV-2 RNA load measurements (reported as AU, see extended data)
of samples A (blue bars), B (orange bars), B(A+) (red bars), and C (azure bars) grown, all under the same conditions for thirty days from
inoculation (day 0). SARS-CoV-2 RNA load in sample B(A+) had a power increase trend over time (as shown in the small frame on top-left),
slightly increased in sample A, and decreased in sample C. As expected, sample B was found constantly negative

Amoxicillin, Azithromycin, Metronidazole, Vancomicin

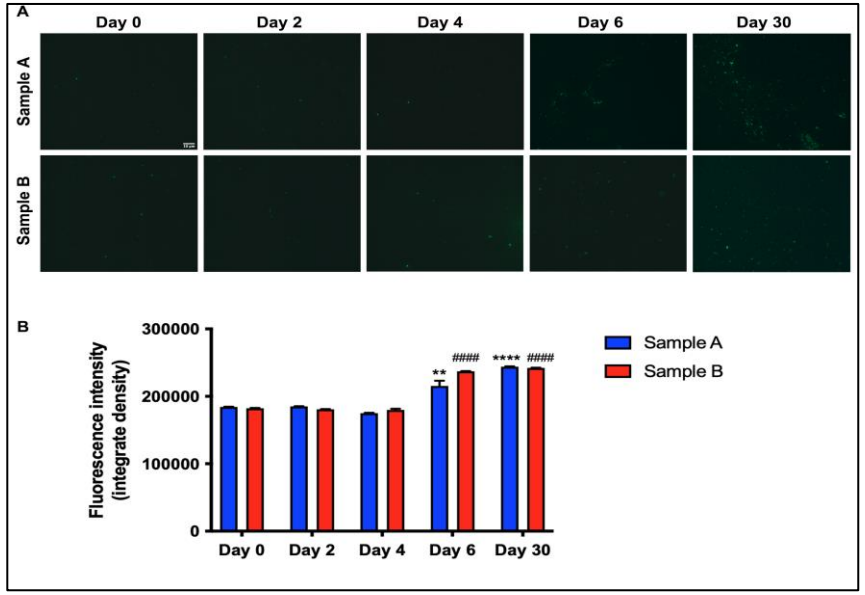
Supplementary material: Bacteriophage behavior

Brogna C, Cristoni S, Petrillo M *et al.* **The first report on detecting SARS-CoV-2 inside human fecal-oral bacteria: A case series on asymptomatic family members and a child with COVID-19** *F1000Research* 2022, **11**:135 (https://doi.org/10.12688/f1000research.77421.1)



*These papers are **still under review**. They will be a topic for the next meeting perhaps. However, it is important to point out that **our data suggest the bacteriophagic behavior of SARS-CoV-2.***

I would mention only two pieces of evidence compared to the others written in the papers



Analyze the bacteriophagic behavior of viruses. In particular, we examined **the increase of microscopic fluorescence enhancement of viral proteins of a fixed aliquot (**0.20µL) from day zero to day 30 of bacterial cultures in two samples A and B.

Supplementary material: Bacteriophage behavior

Brogna C, Cristoni S, Petrillo M *et al.* **The first report on detecting SARS-CoV-2 inside human fecal-oral bacteria: A case series on asymptomatic family members and a child with COVID-19** *F1000Research* 2022, **11**:135 (https://doi.org/10.12688/f1000research.77421.1)

*These papers are **still under review**. They will be a topic for the next meeting perhaps. However, it is important to point out that **our data suggest the bacteriophagic behavior of SARS-CoV-2.***

I would mention only two pieces of evidence compared to the others written in the papers

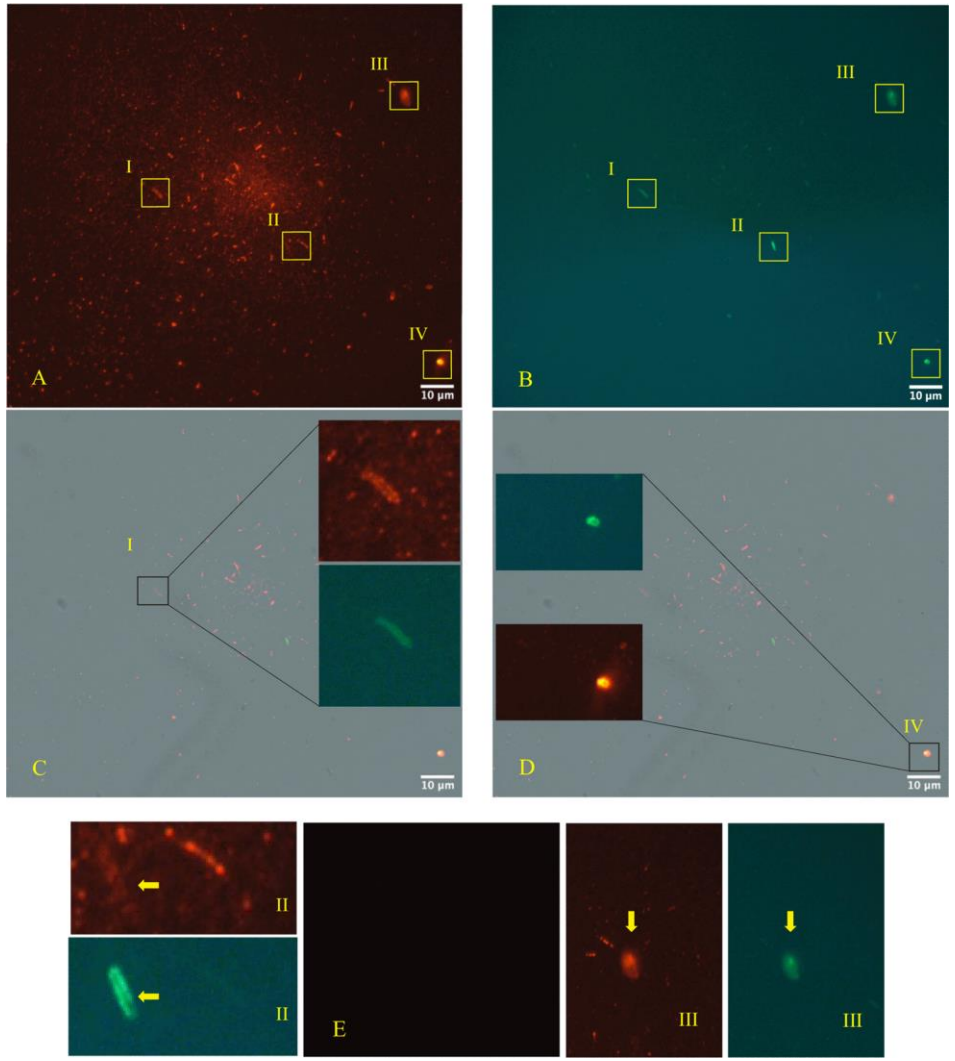


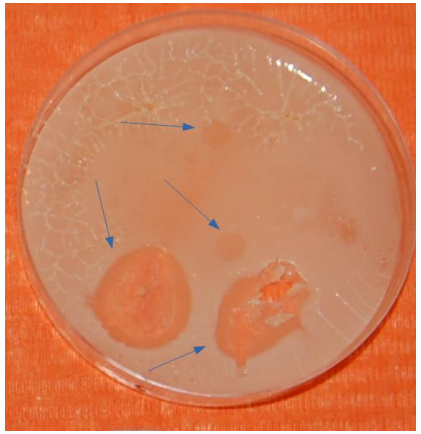
Figure 3. Fluorescence microscope images.
Panels A, B, C, D (Zeiss Axioplan 2, Axiocam 305 color, magnification 100×) show immunofluorescence staining versus SARS-CoV-2 nucleocapsid protein (red light), gram positive bacteria (green light). Panel E is the negative control. The roman numerals I,II,III,IV and yellow rectangles indicate four gram-positive bacteria (green light) infected by SARS-CoV-2 (red light)

Supplementary material: Bacteriophage behavior

DECREASE
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Bifidobacterium
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Fusicatenibacter
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;Klebsiella
Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Anaerostipes
Bacteria;Firmicutes;Bacilli;Lactobacillales;Enterococcaceae;Enterococcus
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Blautia
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Dorea
Bacteria;Firmicutes;Erysipelotrichia;Erysipelotrichales;Erysipelotrichaceae;Coprobacillus

INCREASE
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;unkn. Burkholderiales(o);unkn. Burkholderiales(o)
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;unkn. Clostridiaceae(f)
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Hungatella
Bacteria;Firmicutes;Tissierellia;Tissierellales;Peptoniphilaceae;Finegoldia
Bacteria;Firmicutes;Erysipelotrichia;Erysipelotrichales;Erysipelotrichaceae;Holdemanella
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;unkn. Ruminococcaceae(f)
Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Campylobacteraceae;Campylobacter
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;unkn. Lachnospiraceae(f)
Bacteria;Firmicutes;Clostridia;Clostridiales;Eubacteriaceae;Eubacterium
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides

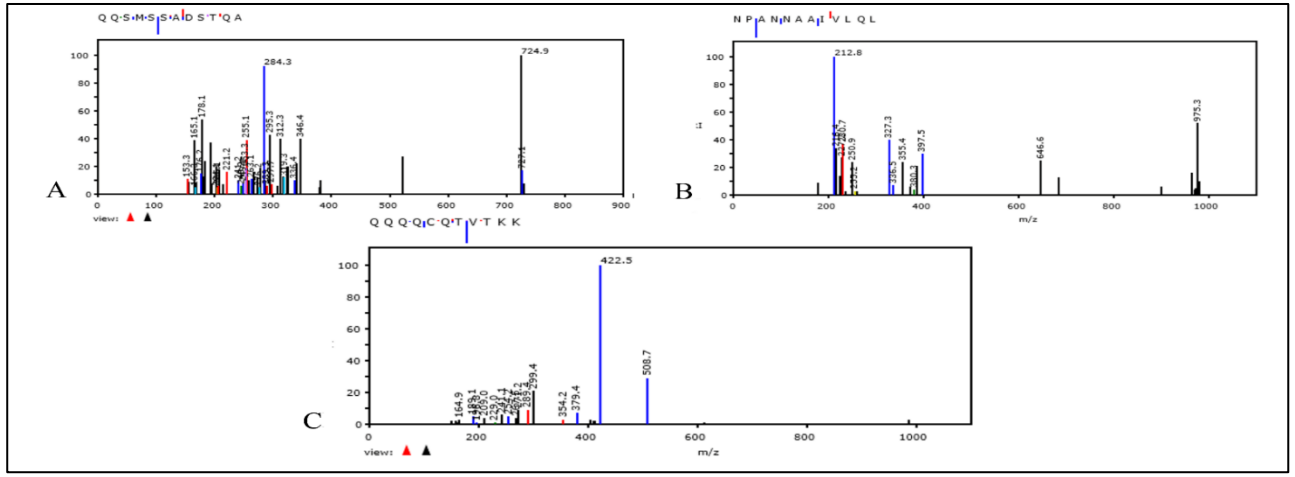
*These papers are **still under review**. They will be a topic for the next meeting perhaps. However, it is important to point out that **our data suggest the bacteriophagic behavior of SARS-CoV-2.***
I would mention only two pieces of evidence compared to the others written in the papers



A decrease in some genera of bacteria **after 30 days** of bacterial culture in the presence of virus.
This could indicate **a lytic behavior of the virus towards these bacteria.** Some Bacteria genus like *Dorea, Fusicatenibacter, Klebsiella, Streptococcusdecreased* while other bacteria genus like *Campylobacter, Prevotella, Staphylococcus, Bacteroides, and Citrobater* increased.

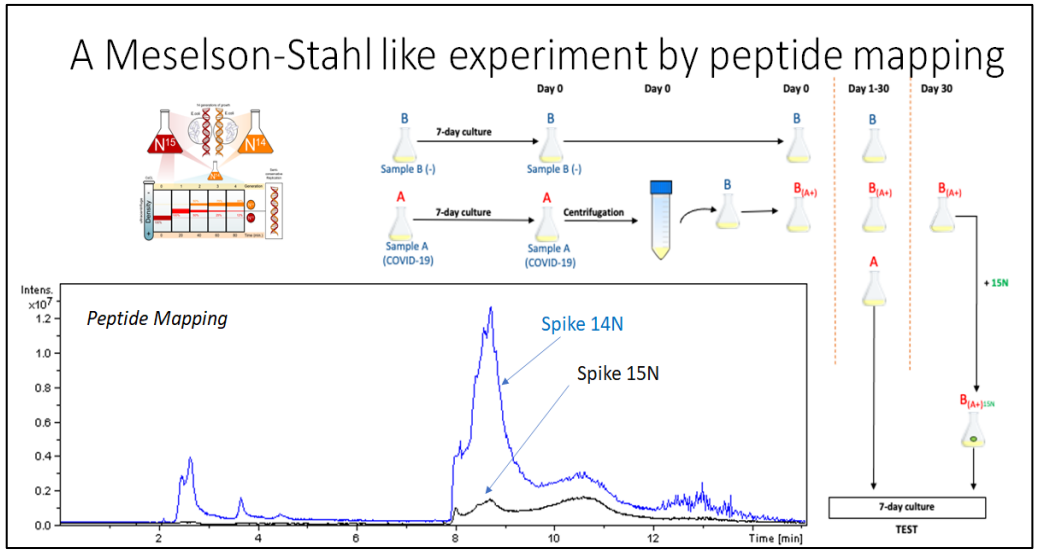
Lytic plaques on a plate where a bacterium of the genus Dorea

Supplementary material: Bacteriophage behavior



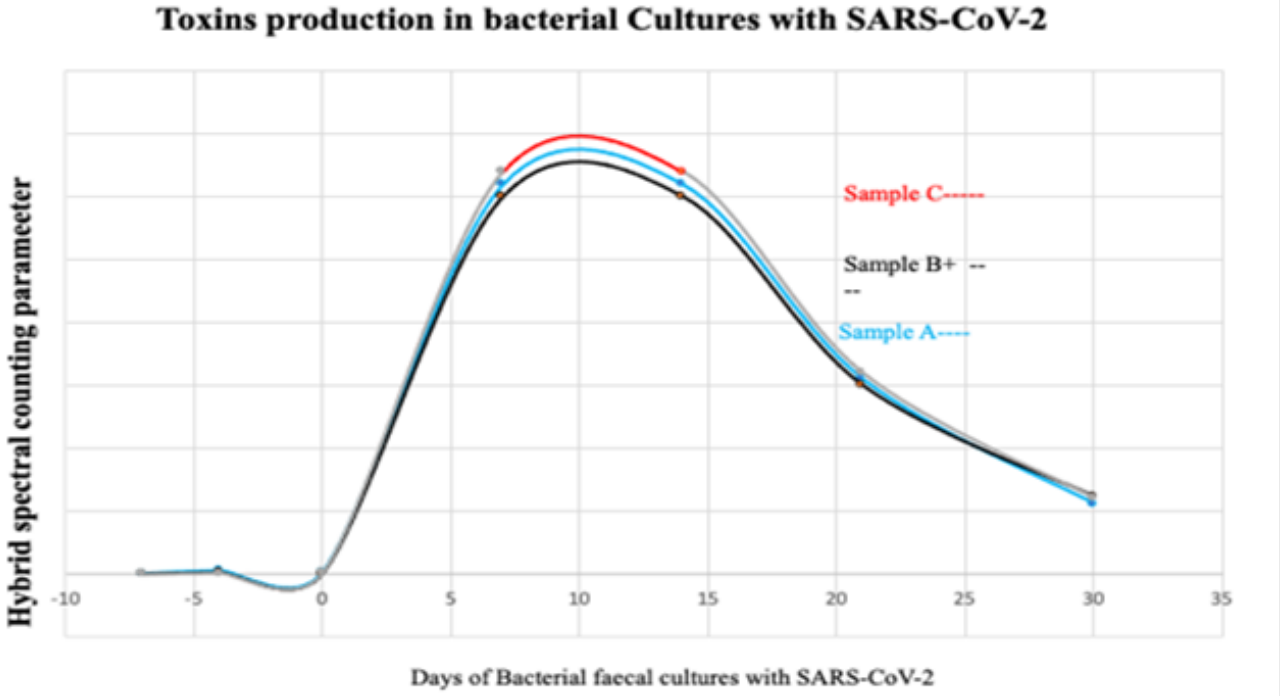
Panels A-C: shows **the MS/MS spectra of the nucleocapsid protein of SARS-CoV-2 containing the nitrogen isotope.** Panel A shows peptide seq: QQSMSSADSTQA; ID:|A0A8B1JYE4|A0A8B1JYE4_SARS2; Mods: Q408 + 1 (13C) +2 (15N), Q409 + 1 (13C) +2 (15N) . Panel B shows peptide seq: NPANNAAIVLQL ; ID: A0A7M1YDW6|A0A7M1YDW6_SARS2;; Mods: Q160+ 1 (13C) +2 (15N). Panel C shows peptide seq: QQQQCQTVTKK; ID:|A0A8B1XSI6|A0A8XSI6_SARS2; Mods: Q239+ 1 (13C) +2 (15N), Q239+Ammonia-loss, Q240+ 1 (13C) +2 (15N), Q241+ 1 (13C) +2 (15N), Q242+ 1 (13C) +2 (15N), Q244+ 1 (13C) +2 (15N).

Petrillo M, Querci M, Brogna C *et al.* **Evidence of SARS-CoV-2 bacteriophage potential in human gut microbiota** *F1000Research* 2022, **11**:292 (https://doi.org/10.12688/f1000research.109236.1)



Peptide mapping of SARS-CoV-2 spike protein were acquired by means of liquid chromatography mass spectrometry associated to 14N and 15N profiles

2 . Toxin-like peptides: What they are; where they come from; why important; how to recognize them.



We noted that by culturing bacteria from the fecal sample in which SARS-CoV-2 was removed (**sample C**), and comparing it with fecal bacterial cultures in which SARS-CoV-2 was still present, **the semiquantitative spectral concentration count** , calculated in absolute e-value , of the toxin-like peptide **produced by the bacteria remained identical up to 30 days**

We noted that in sample C the viral remained low and tended to decrease but the **toxicological concentration did not decrease**

.

Therefore, I inferred that :

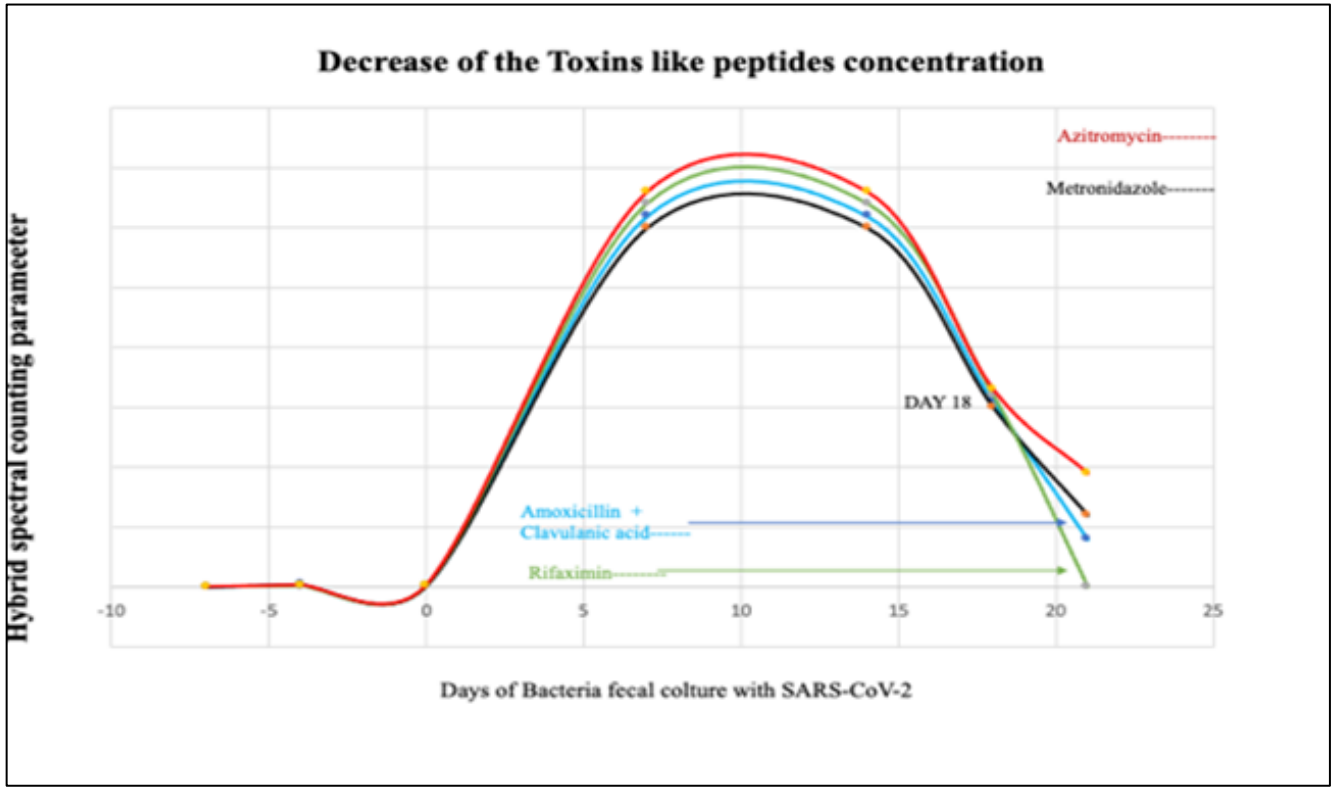
1. Maybe, the toxins produced by bacteria have their function **against viruses(?)**

2. once activated, the bacteria **stop producing toxins very slowly.**

They don’t stop.

New Data not yet published

2 . Toxin-like peptides: What they are; where they come from; why important; how to recognize them.



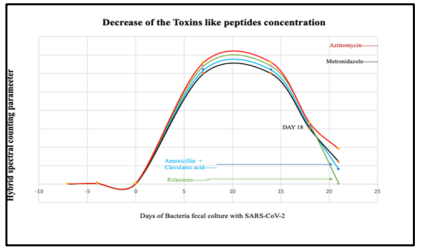
However do antibiotics that decrease viral RNA replication also work for toxicological behavior?

We have seen **Amoxicillin plus clavulanic acid and Rifaximin also turn off the toxicological production** , which azithromycin and metronidazole do not do at all.

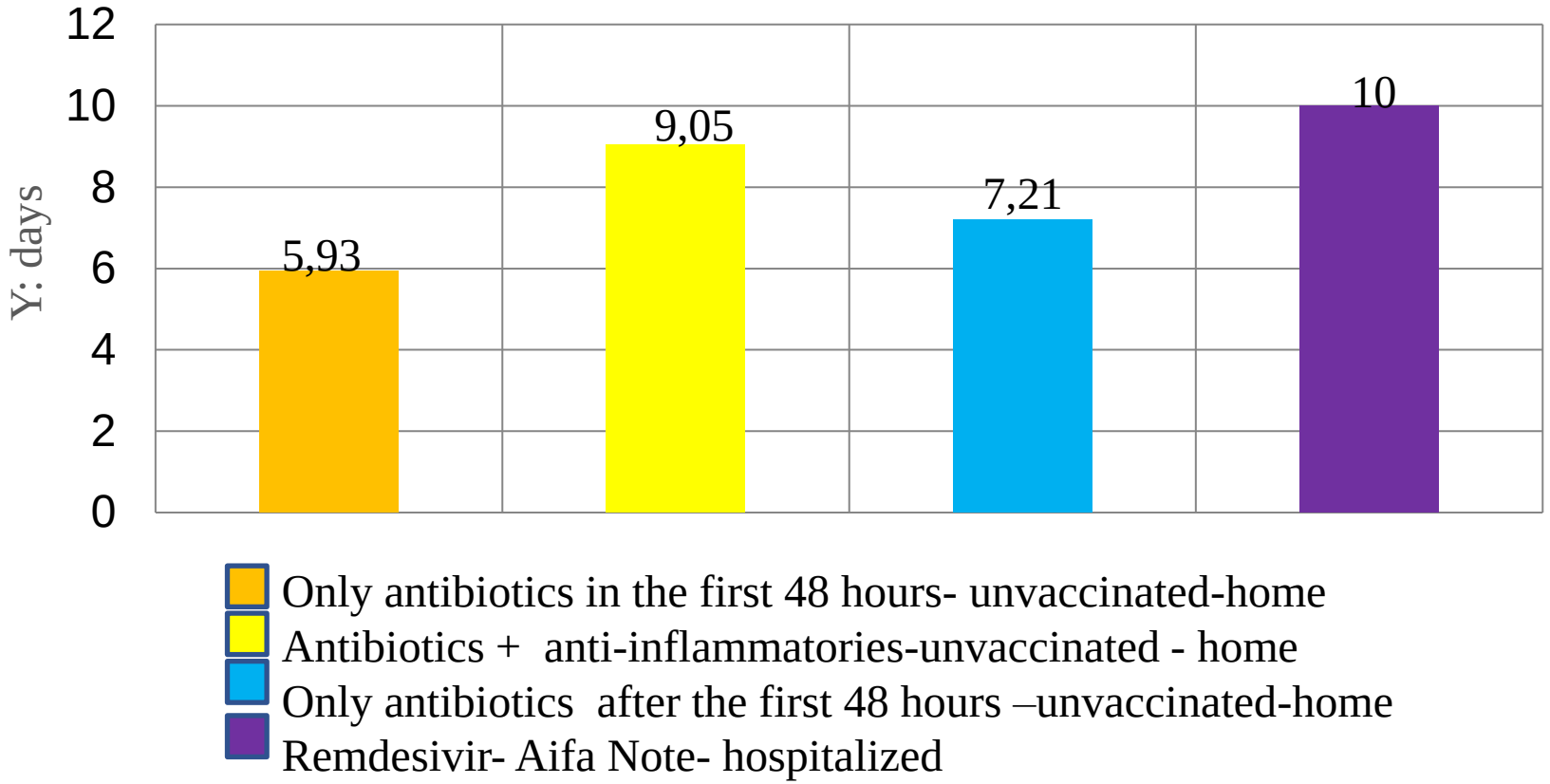
So it is likely to think that the human microbiota set acts with different tasks.

New Data not yet published

2 . Toxin-like peptides: What they are; where they come from; why important; how to recognize them.



The geometric mean value of days of antibiotics used in combination with or without anti-inflammatories in 100 COVID-19 patients- Industrial data.

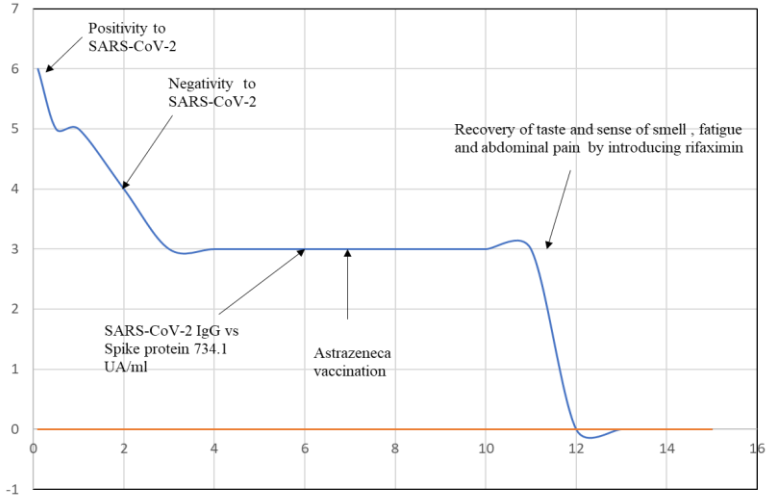


Acute SARS-CoV-2 Infection.
100 nasal swab-positive and symptomatic patients.
Assessment of time to clinical recovery from first symptoms.
Adult patients, 55% male, and 45% female.
Control data: The official cure time with Remdesivir in hospitalized

Industrial Data

2 . Toxin-like peptides: What they are; where they come from; why important; how to recognize them.

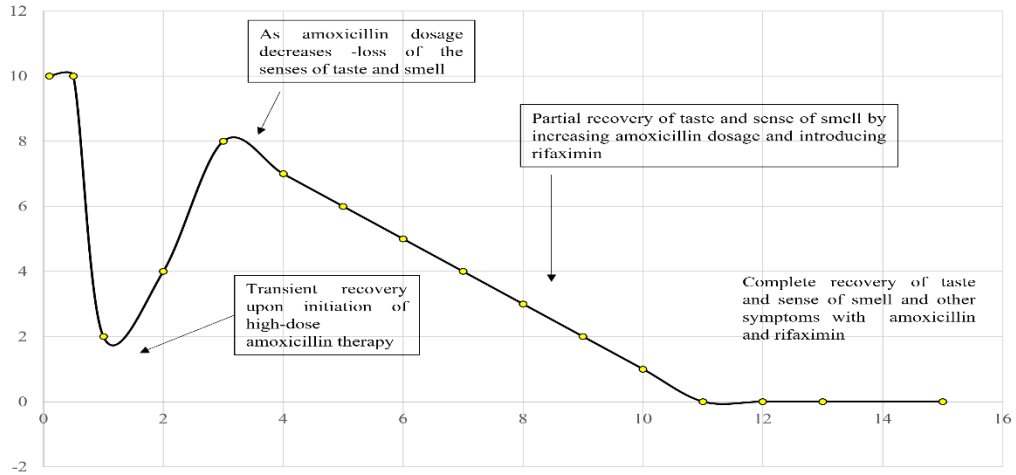
Patient F, 54 years old– Acute infection ,positive to SARS-CoV-2 rt-PCR in october 2020: treatment of Neuronal Symptoms (Ageusia-Hyposmia- abdominal pain- fatigue)



Y value
0- Nothing
1-Abdominal pain
2- Hyposmia/dysgeusia
3- Limb pain and fatigue
4- Headaches and other disorders
5- Cold
6-Fever 39°C + cough and other respiratory symptoms

X value	Period	Drugs	
0,1	10/05/20	paracetamol 2 days	
0,5	10/12/20		0
1	10/30/20		0
2	11/30/20		0
3	12/31/20		0
4	01/31/21		0
5	02/28/21		0
6	03/31/21		0
7	04/30/21		0
8	05/31/21		0
9	06/3021		0
10	07/31/21		0
11	08/10/21	Rifaximin 7 days + probiotics	
12	08/31/21		0
13	09/30/21		0
14	10/30/21		0
15	11/30/21		0

Patient F, 46 years old. – treatment of Neuronal Symptoms (Ageusia-Hyposmia- Paresthesias) After 30 days.
V.A.S (Visual Analogic scales) of the senses of taste and smell



Legend:

10---
Ageusia and anosmia

From 9 to 1 ---
Dysgeusia and hyposmia decremental

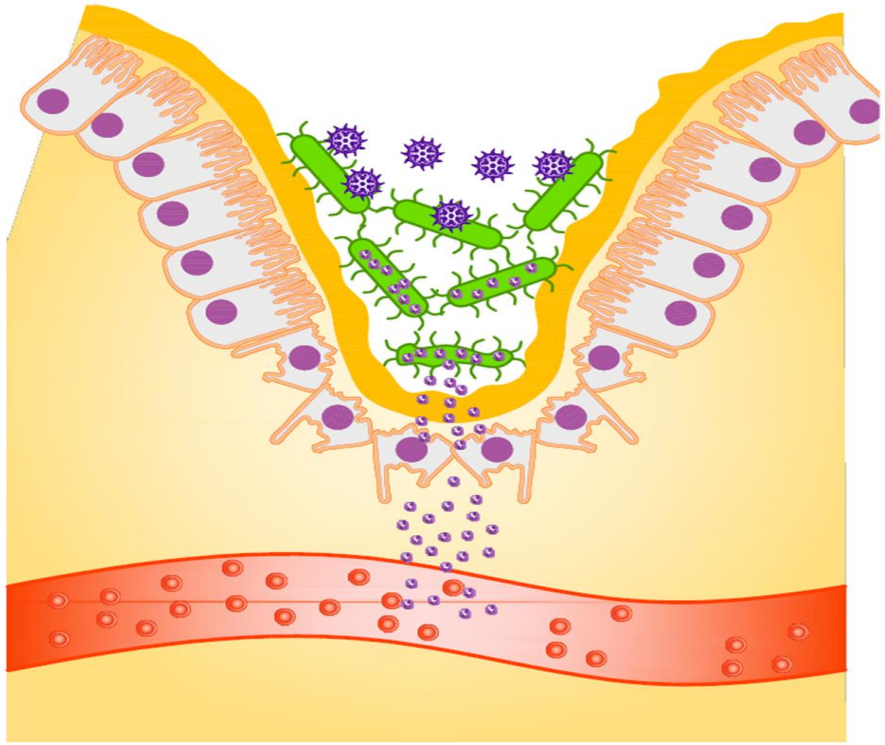
0
full recovery of taste and smell

Patient F16, 54 years old, living in XXXXXXX, healthy with negative medical history for any disorder or drugs. In October 2020 she and her family were positive to NP/OP swabs. She has a fever of 39°C for 2 days, cough, cold, diarrhea, anosmia, ageusia, abdominal pain and fatigue for 21days. Her first SO2 was 95%. She didn't assume any drugs. In April 2021 she was vaccinated with AstraZeneca vaccine despite a high antibody (IgG vs S protein SARS-CoV-2) titer observed one month before vaccination. Until 8 months later, she has abdominal pain, anosmia, and dysgeusia. In August 2021 she started to hire, rifaximin 800 mgr./die for 7 days, and the sense of smell and taste were recovered like no more abdominal pain was present.

Patient F9, 46 years old, living in XXXXXX, healthy with negative medical history for any disorder before the vaccination. After 6 hours of vaccination, she had a fever of 39°C, general malaise, asthenia, arthralgia, arm, and retrosternal pain. On the 16th of July 2021, she has also anosmia and dysgeusia and was positive to NP/OP swabs. She assumed palliative care until August 14, 2021, but retrosternal pain and other neurological symptoms were consistent. She started, on the 14 August, to hire Amoxicillin 875 mgr. x 2 /die + acid clavulanic 125 mgr. x 2/die for 7 days, rifaximin 800 mg/die for 7 days, heparin 4000U/die for 14 days, infusion of saline NaCl 0.9% and electrolytes (500 ml/die) for 10 days. At the end of August, she was healthy.

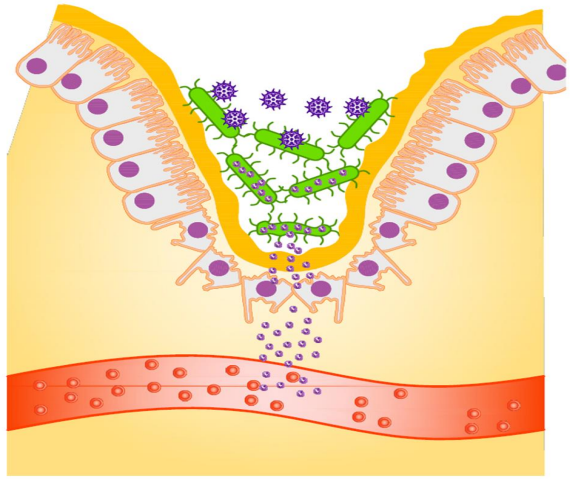
2 . Toxin-like peptides: Consideration In Acute Phase and LONG COVID

- **Accumulating toxins** saturate molecular receptors
- In the case of toxin infection, the **time factor** is crucial
- The bacteria of the microbiome represent the **first line of defense**
- They produce toxins and partly replicate the virus (**bacteriophage mechanism**)
- The bacteria **do not stop producing toxins** even when the viral load is absent
- The **immune system must work against** both viral proteins and toxins
- The gut microbiome is **the reservoir** of this phenomenon.



The toxins enter the circulation and activate the coagulation cascade and inflammation mechanisms. They also activate the autonomous nervous system by binding to nicotinic and muscarinic receptors.

2 . Toxin-like peptides: Consideration In Acute Phase and LONG COVID



If we consider the **bacteriophage mechanism** and **toxin production** by bacteria, the interaction mechanisms **of the virus and its proteins with epithelial cells become much more complex**. In addition, the activated mechanisms of the **immune system also become much more articulated.**

How is the virus really presented from bacteria to human cells?

we need to understand it!

The **clinical scenarios** that we might have are **multiple:**

From **symptomatic patients** who are **positive** on molecular testing and have antibody titers to **those** who are **negative** on molecular testing and have no antibodies.

	Asymptomatic		Symptomatic	
High Bacteria Immunity	- rtCR	+ rtPCR	- rtCR	+ rtPCR
	- IgG/IgM	+IgG/IgM	- IgG/IgM	+IgG/IgM
Low Bacteria Immunty	- rtCR	+ rtPCR	- rtCR	+ rtPCR
	- IgG/IgM	+IgG/IgM	- IgG/IgM	+IgG/IgM

2 . Toxin-like peptides: Consideration In Acute Phase and LONG COVID: Time is precious!!

So how do we act?:

1. We **regulate the toxic rate and viral replication** in the first moment: On bacteria
2. We act on **toxicological dilution** with hydration
3. Corticosteroids only if necessary and not immediately. The immunity system must work against many proteins (viral and toxigenic).
4. **Probiotics, probiotics, and antibiotics.**

What about cytokine storm?

You see, since there is not only the protein aspect of the virus at stake but also the toxins produced by the bacteria, it is **important to consider that the (mechanisms of inflammation are not only activated by the virus but also by the toxins, which certainly behave as immunogens.** Things are much more complex than we think. According to data, **stopping** the release of toxins with drugs that act on the microbiota (**Amoxicillin and Rifaximin plus probiotics**) is the first intervention to be done.

3. Future scientific and industrial implications.

Is it possible to cure the entire world with antibiotics? Certainly not.

The implications of the rise of antibiotic-resistant bacterial species would be significant..... Although so hypocritical we should not be, because many of our foods are contaminated with an excess of antibiotics.

However then what can be a different solution?

Now We have:
•Probiotics
•Antibiotics: Amoxicillin, azithromycin, rifaximin, ceftriaxone
•Nutraceuticals that act on the intestinal microbiome.
•Prevention.

The End point is:

Stop the toxins

3. Future scientific and industrial implications.

TOXINS

- Phospholipase A2 like (PLA2)
- Conotoxin like
- Mettalloproteinase like
- others

The End point is:

Stop the toxins

Quickly

1. **Stop the continuing bacteria Production:** Antibiotic plus probiotics (es: Lactobacillus Reuteri and Bacillus Clausii)

2. **Block or change their function**:

- *Saturations of Nicotinic receptor*(Es: Nicotine) ???
- *Antitoxin product against PLA 2:* Quercitin, Curcuma Longa derivates (Chethankumar M, Srinivas L. New biological activity against phospholipase A2 by Turmerin, a protein from Curcuma longa L. Biol Chem. 2008 Mar;389(3):299-303. doi: 10.1515/BC.2008.024. PMID: 18177267), others (C.A. Cotrim et al.Quercetin as an inhibitor of snake venom secretory phospholipase A2 Chem. Biol. Interact. (2011).
- *Many authors are studying a lot of natural products* (Gómez-Betancur I, Gogineni V, Salazar-Ospina A, León F. Perspective on the Therapeutics of Anti-Snake Venom. Molecules. 2019;24(18):3276. Published 2019 Sep 9. doi:10.3390/molecules24183276)

3. **Antidote** against **PLA2 and Conotoxin**?

3. Future scientific and industrial implications.

Many studies are beginning to show that **re-infections of patients may have a less severe** clinical course.

Bacteria need to meet the virus, they need to produce the right amount of toxins, but what they **don't need is to be constitutionally activated.**

It is a virus with a dual mechanism: it replicates on **both eukaryotic and prokaryotic** cells. In addition, it is a virus that induces **the expression of toxins** by bacteria.

In my opinion, the solution could be twofold. **A double vaccine**: an **antidote against toxins** (an example of the past: the antidote against diphtheria toxin) and an **attenuated virus vaccine for oral intake** (an example of the past: Sabin's polio vaccine) to **stimulate the development of immunity to both the epithelial** cell and the bacteria.

If we think back to SABIN and polio (RNA virus), what was done was accelerate the pandemic by infecting everyone with an attenuated virus.

Isn't this also the conclusion for SARS-CoV-2? So, **Is the pandemic stopped, or should it be accelerated**?

3. Future scientific and industrial implications.

How can we take action in the future by **knowing more about bacteria and the microbiome**?

In the future, by studying **the peptides of bacteria:**
we could increase our knowledge of **autoimmune diseases** (ALS, MS, PD, AD, Diabetes, and many others);
We can develop **devices or tests to** diagnose the presence of specified toxins.

In the future by **engineering bacteria**:

We will be able to **induce resistance against viruses**.
We will be able to **produce toxins and antitoxins.**
We will be able **to hasten the end of pandemics.**

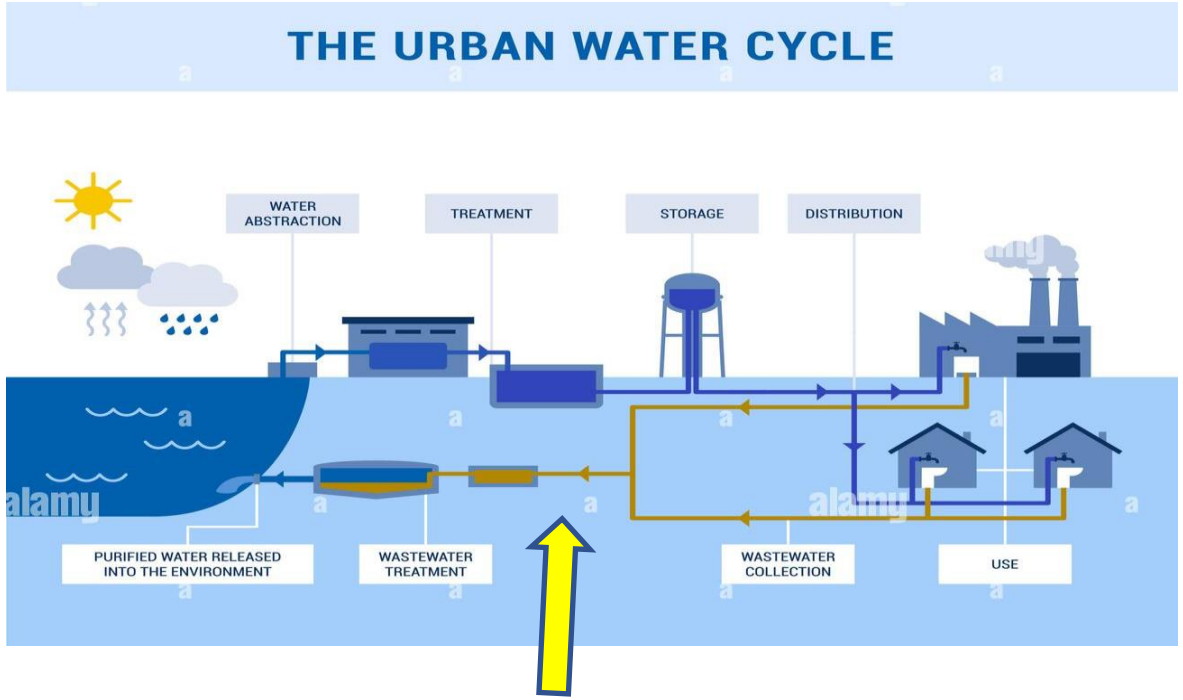
Bacteria should be seen as a huge resource and opportunity!

4. What about in terms of public health safety?

The absolute importance of purifying urban wastewater well!

1. We have seen that SARS-CoV-2 has **bacteriophagic behavior** (bacteria as an occult reservoir).
2. We observed the continuous production of the **toxins-like peptide from bacterial-virus interaction**.
3. Many studies show the presence of the virus **in the stool for many days**.
4. It is clear that there is the **cycle of urban wastewater** among the routes of spread.
5. It is also clear that after this examination, the weather determinant is not the sun or the heat but the **frequency of rainfall** and contamination of rainwater.

How to intervene?



Certainly, it is necessary to **decrease the bacterial load of the wastewater**.

Some of the material shown can be seen at the following link:
https://zenodo.org/record/6506783#.Ym0jDOhBxPY

Thank You

Dr. Carlo Brogna M.D.
Craniomed group Facility
Bresso (Mi)
Dir.brogna@craniomed.it