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## SPECIAL ISSUE
## THE COVID-19 EPIDEMIC




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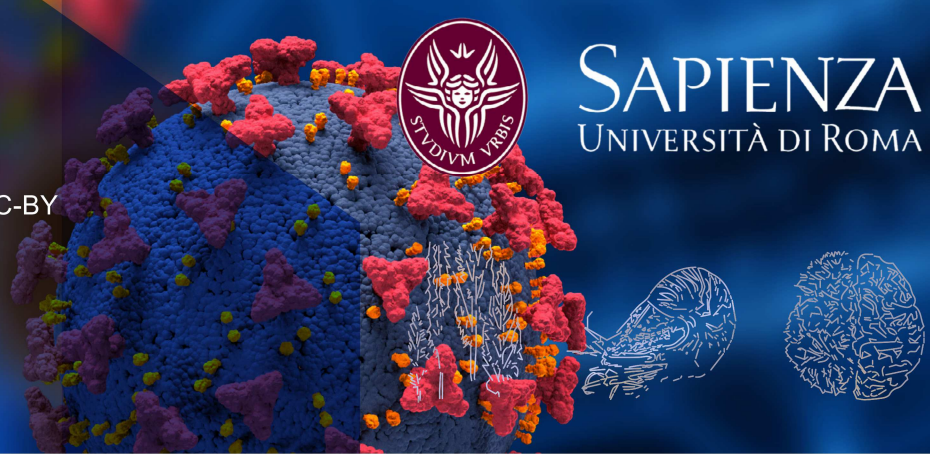


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## Editorial

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# What We Have Learned and What We Still Do Not Know about COVID-19

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On 1st January 2020, China formally notified the WHO of an outbreak of "mysterious" virus-based pneumonia in Wuhan. The outbreak of what is now called the COVID-19 epidemic was subsequently traced back to early October 2019 (Nsoesie et al., 2020). Since then, the virus has spread around the world, unleashing the worst public-health crisis in a century. Up to now more that 12 million people have been infected and 550,000 have died. The pandemic has prompted a scientific "race" (more than 30,000 scientific papers) to understand the biology of the causative agent of the COVID-19 flu-pneumonia, i.e. SARS-CoV-2, and to find reliable treatments for it.

Despite the huge effort deployed in this period, many questions and puzzles have not yet been satisfactorily answered. Here we outline some of them.

1. Although microbiologists and health officials had long warned of the pandemic potential of certain coronaviruses carried by bats (and other animals) in China, when the virus suddenly began to appear all over the world, it caught everyone unprepared. How did this happen? How (and why) was the lesson from previous pandemics forgotten (Kleinman and Watson, 2005)?

2. Computational models, cell studies and animal experiments are being used to pinpoint the viral host that kicked off the pandemic. According to Nature magazine, "There is strong evidence that the virus originated in bats. The biggest mystery remains how it got from bats to people. Researchers overwhelmingly think that it is a wild virus, which probably passed to people through an intermediate species. But no one has found the virus in the wild yet, so other explanations cannot be ruled out entirely" (Mallapaty, 2020a). This is a critical question. In the absence of evidence to explain the hypothetical spillover, other explanations, including accidental release of the virus from the Wuhan lab, must be considered. Indeed, a zoonotic spillover should not be given undue credit, because the epidemic curve is consistent with substantial human-to-human transmission (Nishiura et al., 2020). Obviously, this possibility raises a number of embarrassing concerns.

3. Furthermore, ambiguity surrounds Chinese activities in the field of transgenesis and engineering of microorganisms. We still do not know when the epidemic actually broke out, how many deaths it has caused and where the virus originated. Chinese research in transgenesis and molecular biology has long been the focus of attention for its ethical and safety implications. Over the years, Chinese researchers have shown a rather supercilious attitude to safety rules and ethical principles, sometimes incurring criticism and criminal convictions, as in the case of Dr He (Normile, 2019).

4. How deadly is COVID-19? Death rates vary for two main reasons: 1) differences in testing reliability between countries and the limited number of tests performed, which ultimately lead to underestimation of the true incidence (for instance, until recently Chinese official reports did not include numbers of asymptomatic patients); and 2) uncertainty regarding the true cause of death. Because autopsies have been limited or for-

bidden in many countries (as in Italy until May 2020), a positive COVID-19 test has been deemed "sufficient" to explain clinical outcome; other prominent comorbidities have been discarded as causes of death. It is likely that a reliable global death rate will not be established until the end of the pandemic. However, a reappraisal of true incidence rates and critical re-examination of concomitant pathologies has enabled scientists to estimate that the infection fatality rate may be significantly less than that usually reported by the media, probably averaging around 0.6 percent (de Jesus, 2020). Indeed, a recent commentary in Nature suggests that "a growing number of studies from different regions have estimated IFRs (infection fatality rates) in the range of 0.5–1%" (Mallapaty, 2020b).

5. Has management of the epidemic (political, non-pharmacological measures (i.e. quarantine), including mass-media communication) been adequate? Answers to this question will unleash a storm of controversy. Specifically, the utility of the lockdown has been questioned and is still debated (Melnick and Ioannidis, 2020), mostly because several countries (Japan, Sweden) in which such measures were not adopted (or adopted in a "mild" version, as in Denmark) did not show significant increases in incidence or fatality rates from COVID-19. Furthermore, besides generating political controversy, it is questionable whether citizens benefited from information delivered by "accredited experts", who often aired disparate and disputable opinions instead of making wise recommendations. The sad result was that people lost trust in science.

6. An intriguing feature of COVID-19 is the paucity of symptoms in the vast majority of patients (60-70%) with less than 30% requiring hospital admission. According to different reports, only 3.4 to 10% of infected patients develop severe acute respiratory distress syndrome (ARDS) and require intensive care (Grasselli et al., 2020). How can this data be explained? Attempts to correlate such findings with SARS-CoV-2 mutations or different genetic profiles of patients have failed (Parens 2020). A plausible explanation may be found by looking at COVID-19 from an "organism" viewpoint. At this level, the complex interactions taking place during the infection can be deciphered. Indeed, people dying of COVID-19 are mostly concentrated in the very elderly age group (>75 years), and already have other serious diseases (including cardiovascular diseases, hypertension, renal failure and cancer). In these patients, COVID-19 triggers an autoimmune-like response, involving a huge inflammatory reaction with venous and arterial thromboembolic complications, which ultimately unleash a cascade of events, including the so-called cytokine storm that leads to ARDS (Cecconi et al., 2020). In a nutshell, people admitted with severe COVID-19 lung complications die from a wrong/diminished/insufficient "organism" response.

These are the reasons why ORGANISMS is taking a special interest in the COVID-19 pandemic. As previously stressed by many contributors to this journal, disease cannot be "reduced" and "explained" by focusing solely on the "main causative factor". SARS-CoV-2 infection alone cannot provide a satisfactory comprehensive answer. Such an answer is provided by considering the organism as a whole with its different levels of interaction with the surrounding milieu. Here we are hosting a first special issue on COVID-19. A second one will be published in December 2020; submissions with alternative views of COVID-19 are welcomed. The Editorial Board hopes that this collective effort will contribute to a more inclusive appraisal of this challenging disease.

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## Letters

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# Safety of Hydroxychloroquine in Patients with COVID-19: The Experience in the District of Piacenza, Preliminary Data

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In December 2019, a new pathogen enveloped RNA beta-coronavirus has been identified and named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes severe pulmonary disease in about 14% of infected people (Lu 2020; Wu 2020). On March 2020, the World Health Organization (WHO) has characterized coronavirus infection disease-19 (COVID-19) as a public health emergency of international concern and defined it a pandemic (WHO, 2020). In Italy, the most involved regions by COVID-19 are Lombardy, Emilia Romagna and Veneto. The city of Piacenza (Emilia Romagna Region) is very near to the epicenter of the outbreak of COVID-19, and the catastrophic nature of Lombardy's outbreak has been widely publicized (Berrardi 2020; Horowitz 2020).

In the District of Piacenza, a week later 21 February 2020, the Emergency Department of the Piacenza Hospital was overcrowded with COVID-19 infected people, already in serious condition. Noninvasive ventilation was attempted in the majority of these patients, but the rapidity of lung deterioration in most severely affected patients was quickly worsening.

No vaccine or specific antiviral treatment for COVID-19 has yet been demonstrated to be effective in phase III randomized clinical trials, however hydroxychloroquine with or without antiviral treatment has been incorporated in national guidelines to treat COVID-19 (SIMIT 2020; Geleris 2020).

Hydroxychloroquine (HCQ) and Chloroquine (CQ) are antimalarial drugs and their role in the treatment of COVID-19 is not well defined, given that they, principally, deploy antimicrobial and anti-parasitic effects. Furthermore, these drugs have been proven beneficial in treating rheumatological and immunological diseases, because of the modulation they exert on immunity and proinflammatory cytokines (Yu 2020). However, a carefully monitoring is required when using chloroquine-based drugs, as these drugs can cause QT prolongation and might put patients at increased risk of torsade de pointes and sudden cardiac death (Geleris 2020).

We report the preliminary results on safety of the first 96 patients with COVID-19, treated at the Azienda Sanitaria of Piacenza (Northern Italy) with HCQ.

The treatment was based on a loading dose of 800 mg of hydroxychloroquine for the first day, followed by 200 mg twice daily for six more days.

In this series no arrhythmia was registered. It must be emphasized, however, that ECG was not performed as a daily monitoring program, but only when clini cally required. The median age of our patients was 56 years

| Variables | All patients N. (%) |
|---|---|
| | 96 (100%) |
| Male N. (%) | 49 (51%) |
| Female N. (%) | 47 (49%) |
| **Age medians years (range)** | 56 18-83 |
| **Survival at 30 days of treatment HCQ** | 96 (100%) |
| **Survival at 60 days of treatment HCQ** | 96 (100%) |
| **Treatment** | 800 mg of hydroxychloroquine for the first day, followed by 200 mg twice times daily for 6 more days |

**Table 1:** Clinical Demographic characteristics and outcome of the 96 COVID-19 patients treated with HCQ.

| | | |
|---|---|---|
| Patient without any adverse event | 86/96 | (89,6%) |
| Patient with adverse event possible related to the treatment (grade 1-2) | 10 /96 | (10,4%) |
| Diarrhea | 6 | (60%) |
| Abdominal pain | 3 | (30%) |
| Headache | 4 | (40%) |
| Vomiting | 2 | (20) |

**Table 2:** Adverse Events of the 96 COVID-19 patients treated with HCQ.

(range 18-83), while other clinical demographic characteristic, and outcome are reported in Table 1.

In this series of patients no deaths were registered after two months from the treatment. The majority of these patients improved after some days from the beginning of the treatment, thought several cases showed severe type of COVID-19 pneumonia.

In our experience, treatment with HCQ for patients with COVID-19 at the loading dose of 800 mg for first day followed by 200 mg twice times daily for six more days showed to be safe and beneficial. We believe that this shorten regimens used to treat COVID-19 may be suitably adopted, given the reduced rate of significant side effects (Table 2). In addition to the aforementioned cluster of Covid-19 patients, we have already reported data on 25 additional cancer patients suffering from COVID-19 infection. Treatment with HCQ of this group of high-risk patients did not entail any significant burden of side effects (Stroppa, 2020). A further observational study on additional 51 cancer patient affected by COVID-19, showed no significant side effects (Cavanna, submitted 2020).

Conclusively, Chloroquine and hydroxychloroquine are old drugs and have been widely used in the treatment of rheumatic disease and malaria. The current COVID-19 pandemic poses an urgent need to identify effective treatments. It is worth of noting that repurposing of old, off-label drugs, has gaining momentum, providing new exciting insights in pharmacology. Accordingly, HCQ has been "rediscovered" as a useful remedy in managing Covid-19 patients, thus becoming common practice among physicians (Rodriguez-Martinez 2020). Up to now, HCQ has been used in many thousands of patients with COVID-19 around the world (Geleris 2020). However, though this drug is inexpensive and readily available, a number of scientists and physicians are seriously concerned by the hypothetical burden of side effects, usually more "perceived" than actually proven. Namely, the occurrence of cardiac negative events, including the association of QT prolongation and torsade de pointes (ToP) - especially in patients with heart, hepatic or renal disease – has raised fear and suspicion. However, very recently M. Saleh (Saleh 2020) reported that effects of Chloroquine, Hydroxychloroquine and Azithromycin on the Corrected QT Interval in Patients with SARS-CoV-2 Infection are very limited. Two hundred-one patients have been treated for COVID-19 with chloroquine/hydroxychloroquine, with only seven patients (3.5%) requiring discontinuation of treatment due to QTc prolongation. Moreover, no arrhythmogenic deaths related HCQ treatment have been reported (Saleh 2020).

We are currently performing a retrospective study on additional several hundreds of COVID-19 infected patients followed and treated at home or admitted to the hospitals of the district of Piacenza (North Italy) and treated with HCQ. Preliminary data – at a glance – are unable in evidencing clinical significant side effects, as well as treatment-related deaths. We believe that HCQ can be an effective repurposed, "weapon" against COVID-19, with acceptable toxicity when administered according to our proposed model.

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Letters

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# The Relevance of Epigenetics in the SARS-CoV-2 Infection and COVID-19 Disease

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## Introduction

Almost 45% of the human genome is comprised in transposable elements, including endogenous retroviruses (ERVs). (Hurst and Magiorkinis, 2017). Although this seems to be limited to particular tissues and times, also in normal physiology, there is a real need for human endogenous retroviruses (HERV) expression (Manghera et al, 2016). HERVs ERVs belong to a number of distinct families that integrated independently during evolution (Tristem, 2000).

HERVs have the common provirus structure of coding open reading frames (ORFs) flanked by two long-terminal repeats (LTRs). LTRs are an important site for epigenetic modifications to control HERV and human gene expression. During the course of evolution and because of host defense mechanisms, most of the sequences contain INDELs or have been reduced to single LTRs by recombination (Hurst and Magiorkinis, 2017).

LTRs function as promoters both in sense and antisense orientations and can alter the expression of host genes (Dunn et al, 2006). These repeats have strong RNA Polymerase II regulatory sequences and can bind nuclear transcription factors (Thompson et al, 2016). More recently, it has been shown that LTRs are responsive to pro-inflammatory cytokines (Laurent et al, 2013). They have a pivotal role as controller of pluripotency and malignancy processes, suggesting that they regulates the expression of long-non-coding RNAs in addition to protein-coding genes, and are important sites for epigenetic modifications too. Epigenetic regulation includes the modification of both DNA and histones around which DNA is wound to create chromatin (Brookes and Shi, 2014).

These mechanisms keep often HERVs silenced, but they also could reserve unexplored functions. While histone deacetylation alone is not responsible for HERV repression, more findings underlie the importance of other factors, particularly CpG methylation, in silencing HERVs (Laska et al, 2002). The methylation of CpGs is carried out by DNMTs, with DNMT1 being the maintenance methyltransferase which is important for fidelity of methylation during DNA replication.

A microarray study analysing HERV families throughout the genome found that HERVs are heavily methylated in normal tissues. Further, the age of the HERVs correlates with their methylation status, with a loss of methylation appearing in older families (Szpakowski et al, 2009).

Krüppel associated box zinc finger proteins (KRAB-ZFP) are identified to contribute to histone methylation and heterochromatin formation early in the embryo (Thomas and Schneider, 2011).

The majority of human KRAB-ZFP binding sites were located within transposons, mainly retrotransposons including HERVs. The KRAB-ZFP bind to HERVs and silence them by burying them in heterochromatin (Imbeault at al, 2017).

Different groups of viruses will target both similar and distinct host pathways to manipulate the immune response and improve infection. Menachery et al. examined differential regulation of IFN-γ-dependent genes following infection with robust respiratory viruses, including coronaviruses. The results indicate a common mechanism utilized by H5N1-VN1203 and MERS-CoV to modulate antigen presentation and the host adaptive immune response. Particularly, epigenetic analysis suggested that DNA methylation, rather than histone modification plays a crucial role in MERS-CoV-mediated antagonism of antigen presentation gene expression; in contrast, H5N1-VN1203 likely utilizes a combination of epigenetic mechanisms to target antigen presentation (Menachery et al, 2018).

## 2. SARS-CoV-2 ad ACE2

SARS-CoV-2 belong to the Beta-coronavirus family, together with SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) (with 80% and 50% homology, respectively). Compared to other RNA virus, it is characterized by the production of transcripts encoding unknown ORFs with fusion, deletion, and/or frameshift. Therefore, in addition to the canonical genomic and nine subgenomic RNAs, SARS-CoV-2, shows a highly complex transcriptome. 41 RNA modification sites, with most frequent motif, AAGAA, were identified on viral transcripts using direct RNA sequencing techniques. Modified RNAs have shorter poly(A) tails than unmodified RNAs, suggesting a link between the modification and the 3' tail (Kim et al, 2020).

The genome sequence of SARS-CoV-2 is 82% identical to SARS-CoV. Angiotensin converting enzyme II (ACE2) was identified as the cell entry receptor for the SARS-CoV-2 spike glyco-protein, allowing the viral infection of humans, similar to SARS-CoV.

ACE2, which is located on the X chromosome, is highly expressed on the surface of lung epithelium cells. It belongs to the angiotensin-converting enzyme family and catalyzes the cleavage of angiotensin II into the vasodilator angiotensin (Shyh et al, 2020).

## 3. Epigenetics in SARS-Cov-2

SARS-CoV-2 infection is mild in the majority of individuals but progresses into severe pneumonia in a small proportion of patients. Cancer has been identified as an individual risk factor for COVID-19 severity. Likewise, ACE2 is resulted aberrantly expressed in many tumors. A bioinformatic assay showed that ACE2 overexpression and hypomethylation are present in many types of cancer, pointing out the relevance of the epigenetic factors in modulating the SARS-CoV-2 infection and outcome (Chai et al, 2020).

More generally, it has been observed that subjects with compromised immune system result more susceptible to a severe outcome of the COVID-19 disease. For example, it has been suggested that patients with systemic lupus erythematous can develop more severe COVID-19 symptoms. Also in this case, hypomethylation and overexpression of ACE2 are candidate to be responsible of this worse response (Sawalha et al, 2020). Finally, the analysis of over 700 lung transcriptome samples of patients with comorbidities associated with severe COVID-19 showed high expression of ACE2 compared to controls. The network analysis correlated ACE2 overexpression in the human lung to genes related to histone modifications, reinforcing the idea that epigenetic mechanisms can have a role in the modulation of the disease (Pinto et al, 2020).

Adults over 65 years of age represent 80% of hospitalizations due to the COVID-19 disease showing a 23-fold higher risk of death respect to younger people. As above discussed, comorbidities worsen the outcome of the disease, but are not sufficient to explain the role of age as an independent risk factor. Epigenetic hallmarks of aging are known to influence health span in older adults, possibly via mechanisms regulating the immune

system (Salimi and Hamlyn, 2020). Therefore, the well-known aging-associated changes in the epigenome may be taken into consideration in the attempt to explain the age effect in the COVID-19 course (Mueller et al, 2020). Taken together, the increased severity observed in individuals with co-morbidities and in the elderly suggests for an initial defect in the anti-viral host defense mechanisms. The long-term boosting of innate immune responses, the so-called "trained immunity," induced by live vaccines has the potential to induce protection against further infections through epigenetic, transcriptional, and functional reprogramming of innate immune cells (Netea et al, 2020).

Moreover, previous studies in our laboratories evidenced that the expression of IL-6, the main player in the so-called "cytokine storm" occurring in the most severe COVID-19 patients, is modulated by the methylation of its genes promoter (Dinicola et al., 2017). This observation suggests a further mechanism by which the epigenetics can crosstalk with the virus.

Although the studies on the SARS-CoV-2 infection and on the COVID-19 disease are still at the beginning, these observations make room for the possibility that epigenetic mechanisms can have a role both in the susceptibility to be infected and in the severity of the disease's outcome. In particular, the epigenetic control of the ACE2 gene expression seems a promising target for prevention and therapy in COVID-19.

The authors declare no conflict of interest.

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## Commentaries

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# The Claude Bernard and Conrad Waddington Legacy: Homeostasis, When Observed for a Very Long Time, is Homeoresis

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Takens theorem states a very important issue: the dynamics of any complex system, living in an *n*-dimensional phase space spanned by *n* unknown variables can be reconstructed by considering the temporal evolution of only one of these variables. This temporal evolution (in the form of series of subsequent values registered at equally spaced discrete time points) allows the reconstruction of system attractor dynamics by the method of 'embedding', i.e transforming the series into a multivariate matrix having as rows the subsequent epochs and as columns *n* time lagged copies of the original series at a fixed delay (Broomhead, D. S., & King, G. P. (1986). Extracting qualitative dynamics from experimental data. Physica D: *Nonlinear Phenomena*, 20(2-3), 217-236).

Takens theorem is strictly related to the basic notion of what an organism is: an organized system emerging from the mutual relations among n factors whose 'trajectories in time' keep track of the latent organization of the system itself. The sad news are that time, is both the most relevant and the most neglected dimension in biology. A clear symptom of this paradoxical state of affairs is the existence of a specialist journal, serving a very tiny scientific community, named *Chronobiology International* mainly focused on biological rhythms observed at population (not individual) scale. The simple fact that a *Chronophysics International* journal could not exist (time is deeply embedded in any physical approach to reality and not confined to a small specialist field) tells us of the intrinsic difficulties to face time dimension in biology. The reasons for this lack of consideration are many and go from the difficulty to make regular observations in time in a natural setting for long duration to the extreme fragmentation of biological sciences that considers time at very separated non-communicating scales going from the nano-seconds of molecular dynamics to the millions of years of evolution passing by minutes/hour of physiologic signals and the

(often sporadic) observations on development. In this paper, the authors surmount this gap and were able to build a regular embedding matrix at individual organism scale (the only physiologically relevant one) for a period going from birth to 10 years of age regularly sampled on a weekly base. The authors rely on the fact teeth grow in a way similar to the trees with a very regular pattern of deposition of dentine: laser ablation of subsequent layers of dentine and the measurement of elemental composition (zinc, copper, manganese…) on these layers allows to generate long series (around 500 equally spaced points on a weekly basis) of concentration fluctuations for each element. According to Takens theorem, the time series relative to these elements are the image in light of the 'entire metabolic dynamics' of organism (elements are tightly coupled with general metabolism intervening in many crucial reactions as such and as cofactors of essential enzymes) and their dynamics offers a unique way to study 'homeostasis in action' during development.

The authors wisely make use of non linear dynamics tools like Recurrence Quantification analysis (RQA) (Marwan, N., Romano, M. C., Thiel, M., & Kurths, J. (2007). Recurrence plots for the analysis of complex systems. *Physics reports, 438*(5-6), 237-329 and empirical phase space reconstruction (Broomhead and King, 1986) so not to impose any specific physical model to the time evolution. In this way they clearly recognize a development trajectory made of alternation of stasis (quasi-attractors) and transition to other attractors shared by all the healthy individuals analysed. This kind of dynamics was present in both development and post-adolescent (permanent teeth) phases,

When shifting to ALS (Amyotrophic Lateral Sclerosis) the authors demonstrate the patients dynamics get stuck into a single attractor and does not show the homeoresis behaviour of healthy individuals.

The predictive power of their model was very high (AUROC = 0.86) pointing to important clinical application in facing ALS before its clinical onset. Beside the specific result, the relevance of this work resides in the possibility to develop 'dynamics biomarkers' for both research and application so making temporal dimension to enter mainstream biology and filling a dramatic epistemic gap of biological sciences.

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## Commentaries

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# Yes, U.S. Farmer Suicide is Significantly Higher Than the National Average

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## Introduction

It is common knowledge that data interpretation can serve specific narratives. As seen until now, the economy of agriculture, which is at the base of human survival, has not fit into the contemporary occupational categories of a system built on financial capital. Recent U.S. farmer suicide rates, as highlighted here, demonstrate how a non-scientific research base can distort awareness about a public health crisis through subtle data misuse, and how this possibly implies a bias against rurality.

The Standard Occupational Classification (SOC) subgroup of Farmers, Ranchers, and Other Agricultural Managers in the United States, based on the Centers for Disease Control and Prevention (CDC) Morbidity and Mortality Weekly Report (MMWR) on *Suicide Rates by Major Occupational Group—17 States, 2012 and 2015* (Peterson et al. 2018), serves as the starting point for this research. Considering the CDC retraction of its earlier *Suicide Rates by Occupational Group—17 States, 2012* report (LiKamWa McIntosh et al. 2016), the following data reflect the corrected version of the CDC study published on November 16, 2018, with its corresponding erratum dated February 22, 2019 (Peterson et al. 2018; Erratum 2019).

## 1. Suicide rates by occupation

The CDC surveyed 17 out of 50 states: Alaska, Colorado, Georgia, Kentucky, Maryland, Massachusetts, New Jersey, New Mexico, North Carolina, Ohio, Oklahoma, Oregon, Rhode Island, South Carolina, Utah, Virginia, and Wisconsin (LiKamWa McIntosh et al. 2016; Peterson et al. 2018). In considering the Farmers, Ranchers, and Other Agricultural Managers occupational subgroup, the report, in both its original and corrected version, did not include several top agricultural producing states, such as California, Illinois, Indiana, Iowa, Kansas, Minnesota, Nebraska, and Texas.

Of the 17 states surveyed, the findings of the corrected version show that per 100,000 people, the major

occupational groups with the highest suicide rate were as follows: Construction and Extraction (43.6 [2012] and 53.2 [2015] among males) and Arts, Design, Entertainment, Sports, and Media (11.7 [2012] and 15.6 [2015] among females). These data are based on the 2012 and 2015 National Violent Death Reporting System (NVDRS) (Peterson et al. 2018).

In terms of federal SOC subgroups, the CDC reports the Farmers, Ranchers, and Other Agricultural Managers subgroup as follows:

> "The 2012 and 2015 male suicide rates among Farmers, Ranchers, and Other Agricultural Managers (SOC 11–9013, a subgroup of the SOC 11 Management major group) were 44.9 (CI = 34.2–57.9) and 32.2 (CI = 24.2–42.0) per 100,000, based on 59 and 54 suicides in 2012 and 2015, respectively. The 2012 and 2015 male suicide rates for Agricultural Workers (SOC 45–2000, a subgroup of the SOC 45 Farming, Fishing, and Forestry major group) were 20.4 (CI = 13.8–29.1) and 17.3 (CI = 12.1–23.9), based on 30 and 36 suicides in 2012 and 2015, respectively." (Ibid.)

## 1.1 Classifications in agriculture

Following the CDC's initial 2012 *Occupational Group* report (LiKamWa McIntosh et al. 2016) and prior to its 2012 and 2015 *Major Occupational Group* errata (Peterson et al. 2018; Erratum 2019), *The New Food Economy* (an online newsroom rebranded in 2020 as *The Counter*) published that the CDC had misclassified farmers as Triple-F (farming, fishing, and forestry) workers (Rosenberg & Wilson Stucki 2018b). In terms of classification, the authors stated that "under the federal occupational guidelines, farmers are classified as having a 'management occupation,' not a 'farming, fishing, and forestry occupation.' Yet it was the farming, fishing, and forestry, or 'Triple-F,' occupational group that had the highest suicide rate in the country: 84.5 per 100,000 people, over 4 times the overall average of 20.3 among people in the workforce. The suicide rate among managers, in contrast, was exactly average" (Rosenberg & Wilson Stucki 2018a).

From the SOC coding error, *The New Food Economy* assessed the Triple-F category as third among occupational groups (Ibid. 2018a, 2018b). The authors conjectured that if the CDC had grouped farmer suicides with Triple-F suicides, then the rate should have been "no higher than third in the study, and as low as sixth, rather than the highest" and if farmer suicides had not been grouped with Triple-F, then "the suicide rate for Triple-F workers should have been about 50 per 100,000 people—and ranked second or third highest" (Ibid. 2018a). What the authors derived from their hypothesis is that either, if correctly classified, "agricultural *workers*, not farmers, have the highest suicide rate in the country" or, if incorrectly classified, then the CDC data "provided no definitive findings" for neither farmworkers nor farmers (Ibid.).

The publication speculated that although the authors "cannot know how many of the suicides classified as Triple-F were agricultural workers, the fact that they comprise between 80 and 90 percent of the category is highly suggestive" (Ibid. 2018b). Further, the authors published an e-mail from the CDC that highlighted the misclassification of 90 farmers. Accordingly, farmers "would still fall below the rate for Triple-F workers" (Ibid.). Essentially, and reiterated here for clarity, the authors' hypothesis asserts that if the CDC's original Triple-F classification were correct, then the 2012 study "found that a group made up almost entirely of agricultural workers had the highest suicide rate in the country," and if the CDC had, in fact, misclassified farmer suicides with Triple-F suicides, then they "could not make conclusions about the respective suicide rates of Triple-F workers and farmers" (Ibid. 2018a).

## 1.2 Validity amid data misuse

Going even further, *The New Food Economy* stated that the CDC study "had nothing to do with farmers and everything to do with farm workers" (Ibid. 2018b) under the claim that the suicide crisis among farmers is "not true" and that either the CDC had made an error or the media mistook "a farmworker suicide crisis for a farmer one" (Ibid. 2018a). This message was transmitted to the media. Various publications reported the hypothetical, then CDC-confirmed, correct classification of Triple-F suicides while still projecting a lower farmer suicide rate that was still inconclusive (Clayton 2018; Norford 2018; Walrath 2018a & 2018b). The authors from the cited publications, that is, *Farm Bill Law Enterprise*, *Mother Jones*, *Progressive Farmer*, and *The New Food Economy/The Counter*, which all reported

the CDC retraction, have not revised the data following the errata nor updated their corresponding assessment of the 17-state 2012 and 2015 CDC data. Also, the National Farmers Union (Perdue 2018) and Farm Aid (Vanderpool 2018) acknowledged the CDC retraction, yet observed its inconsistency with their experience and previous studies. Farm Aid stated that "it will continue to prioritize farmer stress" based on a 30% increase in "calls to their farmer hotline and feedback from family farm partners around the country" (Ibid.).

Is *The New Food Economy*'s original hypothesis valid? Table 3 of the amended 2012 and 2015 CDC report (Peterson et al. 2018) shows that the SOC 45 Farming, Fishing, and Forestry major occupational group ranked 8th at 26.3 [2012] and 9th at 22.8 [2015]. SOC 11, the Management major occupational group, ranked 17th at 16.4 [2012] and 15th at 17.8 [2015]. According to the 2012 and 2015 report, the Farming, Fishing, and Forestry (SOC 45) major occupational group includes farm laborers and supervisors but does not include farm operators, such as self-employed farmers or farm owners. The 2012 and 2015 report placed farm operators into the Management major occupational group. Based on the report's data, a farm "manager," that is, Farmers, Ranchers, and Other Agricultural Managers subgroup (SOC 11–9013) and a farm "worker," that is, Agricultural Workers subgroup (SOC 45–2000) are, respectively, separated between the major occupational groups of SOC 11 Management and SOC 45 Farming, Fishing, and Forestry. The two groups do not overlap according to these data.

## 1.3 Significantly high farmer suicide

When looking at the CDC's national average:

> "during 2000–2016, the suicide rate among the U.S. working age population (persons aged 16–64 years) increased 34%, from 12.9 per 100,000 population to 17.3." (Ibid.)

Peterson et al. (2018) report that the Farmers, Ranchers, and Other Agricultural Managers subgroup (SOC 11–9013) male suicide rate was 44.9 [2012] and 32.2 [2015] per 100,000. These rates can be compared to the national average [(12.9 [2012] and 17.3 [2015]). Among the 17 states surveyed, suicide rates for Farmers, Ranchers, and Other Agricultural Managers were more than three times as high as the national average in 2012 and almost twice as high as the national average in 2015.

In terms of the Farmers, Ranchers, and Other Agricultural Managers subgroup (SOC 11–9013 of the Management major occupational group) in relation to the Agricultural Workers subgroup (SOC 45–2000 of the Farming, Fishing, and Forestry major occupational group), the Farmers, Ranchers, and Other Agricultural Managers subgroup (SOC 11–9013) has a suicide rate

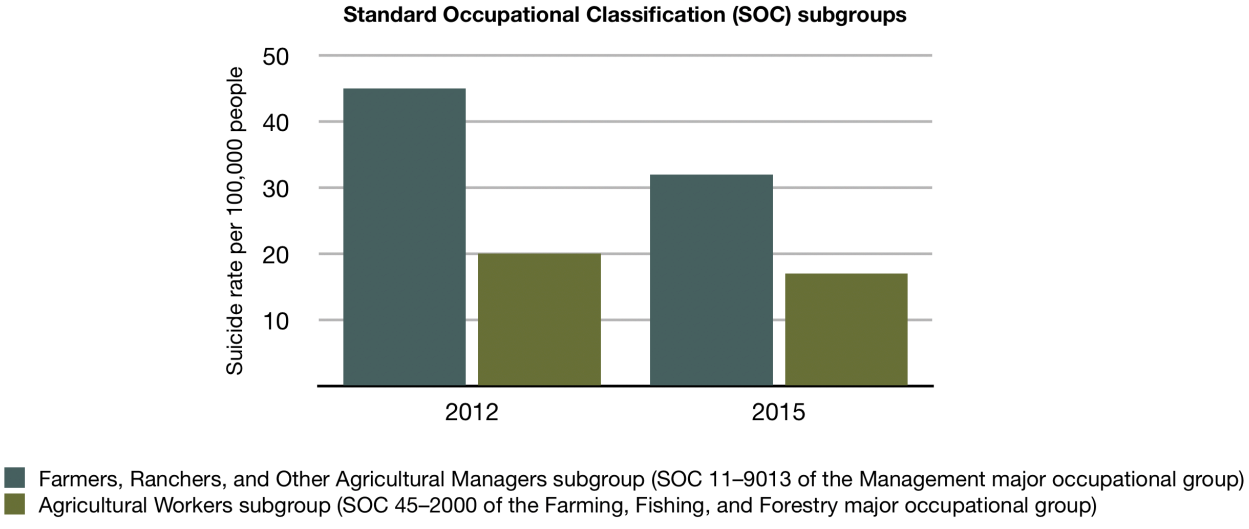


**Figure 1:** The Farmers, Ranchers, and Other Agricultural Managers subgroup (SOC 11–9013 of the Management major occupational group) compared to the Agricultural Workers subgroup (SOC 45–2000 of the Farming, Fishing, and Forestry major occupational group), based on the CDC errata, *Suicide Rates by Major Occupational Group—17 States, 2012 and 2015* (Peterson et al. 2018).

of 44.9 [2012] and 32.2 [2015] per 100,000 while the Agricultural Workers subgroup (SOC 45–2000) has a suicide rate of 20.4 [2012] and 17.3 [2015] per 100,000 (see Figure 1). Among the 17 states surveyed, Farmers, Ranchers, and Other Agricultural Managers have approximately double the rate of suicide than Agricultural Workers (Ibid.).

If comparing Farmers, Ranchers, and Other Agricultural Managers to the major occupational group with the highest suicide rate in Peterson et al. (2018), that is, Construction and Extraction (SOC 47), then in 2012, Farmers, Ranchers, and Other Agricultural Managers had a higher rate at 44.9 [2012] per 100,000 than Construction and Extraction at 43.6 [2012]. However, the proximity of these two figures lies within the statistical margin of error based on the Confidence Interval (CI). Despite Farmers, Ranchers, and Other Agricultural Managers had a rate of 44.9, this subgroup's CI was 34.2–57.9 and potentially higher or lower than that of Construction and Extraction in 2012. According to the highest estimate of 44.9, the Farmers, Ranchers, and Other Agricultural Managers subgroup (SOC 11–9013) ranks first, but it is most likely in a statistical tie with the Construction and Extraction (SOC 47) major occupational group for first.

In 2015, Farmers, Ranchers, and Other Agricultural Managers had a lower rate of 32.2 [2015] per 100,000 than Construction and Extraction at 53.2 [2015] (Ibid.). Considering the CI (24.2–42.0) was below that of Construction and Extraction, it is definite that the Farmers, Ranchers, and Other Agricultural Managers subgroup ranked lower than the 2015 Construction and Extraction major occupational group.

Comparing Farmers, Ranchers, and Other Agricultural Managers (SOC 11–9013) to Construction and Extraction (SOC 47) is problematic in that a major occupational group (SOC 47) is placed in relation to a subgroup (SOC 11–9013) of a major occupational group. However, a trend can be highlighted from this subgroup (SOC 11–9013): the farmer suicide rate was in a statistical tie with the highest suicide rate observed for any major occupational group in 2012. Farmer suicide ranked third when compared to major occupational group rates in 2015. The Peterson et al. (2018) CDC study based on 2012 and 2015 major occupational group statistics cannot provide enough detailed data to compare farmer suicide rates to every particular occupation.

## 2. Suicide rates by industry

The CDC recently published *Suicide Rates by Industry and Occupation—National Violent Death Reporting System, 32 States, 2016* (Peterson et al. 2020). This report outlines suicide among the U.S. working-age population of 16–64 years by major industry, major occupational, and detailed occupational groups. Calculated by the United States Census Bureau code for major industry groups and defined by the North American Industry Classification System (NAICS), the Agriculture, Forestry, Fishing, and Hunting group (Census code 0170–0290) had a significantly higher suicide rate among males at 36.1 (CI = 31.7–40.5). The Agriculture, Forestry, Fishing, and Hunting industry followed Mining, Quarrying, and Oil and Gas Extraction at 54.2 (CI = 44.0–64.3); Construction at 45.3 (CI = 43.4–47.2), and Other Services (e.g. automotive repair) at 39.1 (CI = 36.1–42.0) with high suicide rates among industry groups in comparison to the overall study population (see Figure 2).

In this recent, 32-state report on 2016 NVDRS data by Peterson et al. (2020), the CDC stated that "estimates for most major occupational groups are similar, although not directly comparable, to previous estimates that were based on 2015 NVDRS data from 17 states," that is, the previously outlined Peterson et al. (2018) study. According to major occupational groups, Farming, Fishing, and Forestry (Census code 6000–6130) had a male suicide rate of 31.4 (CI = 25.6–37.1) and Management (Census code 0010–0430) had a male suicide rate of 17.5 (CI = 16.4–18.6). Detailed occupational groups showed that Fishing and Hunting Workers (Census code 6100) of the Farming, Fishing, and Forestry major occupational group had an elevated suicide rate of 119.9 (CI = 60.9–215.6). Farmers, Ranchers, and Other Agricultural Managers (Census code 0205) from the Management major occupational group had a suicide rate of 43.2 (CI = 34.9–51.5). Both detailed occupational groups were "statistically higher than [the] population rate (all occupations) based on 95% CI of [the] occupational group rate not containing the total population rate point estimate" (Peterson et al. 2020).

It is also worth noting that many farmers continue working beyond 64 years of age, which is the limit of the cluster considered by the two years of 2012 and 2015,

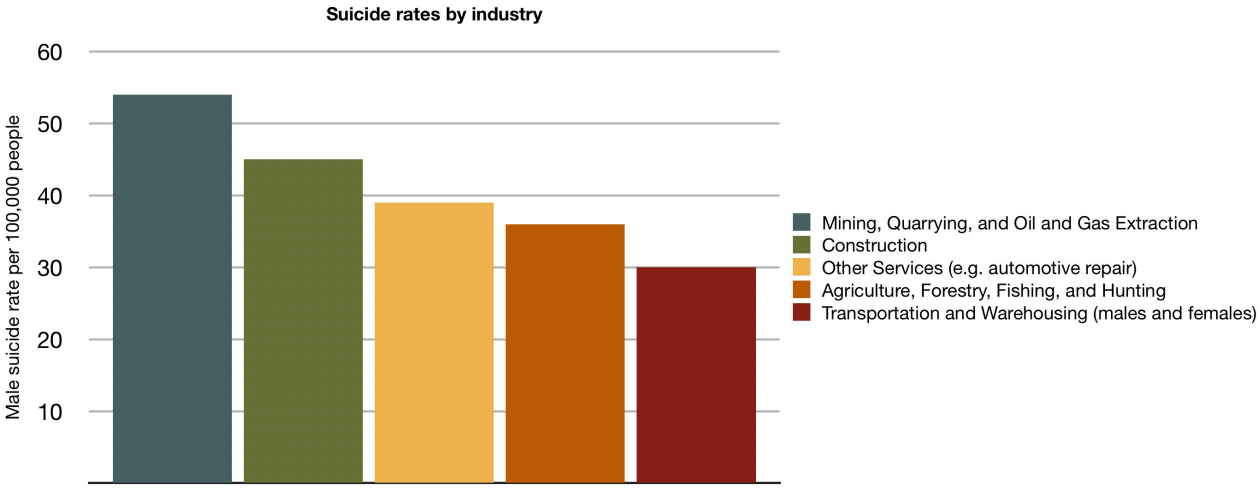


**Figure 2:** Suicide among U.S. working-age population males, 16–64 years, through major industry groups according to the CDC's *Suicide Rates by Industry and Occupation—National Violent Death Reporting System, 32 States, 2016* (Peterson et al. 2020).

as well as the 2016 CDC reports. Another study in *The Journal of Rural Health* (Ringgenberg et al. 2017, p. 6) examines the period from 1992 to 2010, indicating that 20.4% of male farmer and agriculture worker suicide occurs among those who are 65 years and older. This may signal that a relevant number of farmer suicide data have been unreported and, therefore, that actual farmer suicide figures behind the CDC study could even be underestimated. In fact, the United States Department of Agriculture's (USDA) *2017 Census of Agriculture* indicates that 1,000,534 farmers over the age of 65 are making day-to-day decisions (National Agricultural Statistics Service [NASS] 2019 p. 65). Further, 761,171 of all male producers and 392,871 of all female producers are 65 years and over (Ibid. p. 67; 69).

## 2.1 Agriculture-related work characteristics

Beyond these CDC studies, further and more comprehensive data on suicide in agriculture are outlined in *Trends and Characteristics of Occupational Suicide and Homicide in Farmers and Agriculture Workers, 1992–2010*, as published in *The Journal of Rural Health* (Ringgenberg et al. 2017). This 19-year study on all 50 states utilized data from the United States Department

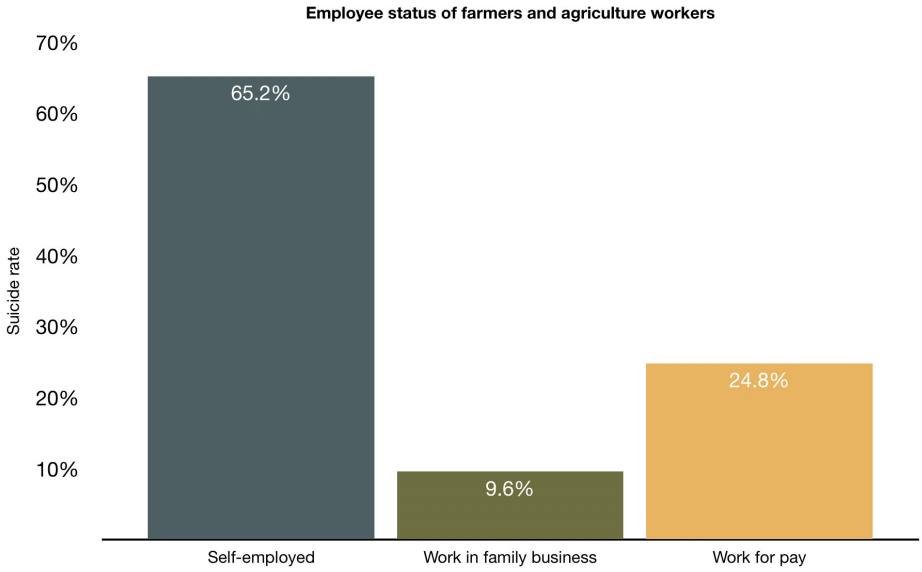


**Figure 3:** Data based on the 19-year Ringgenberg et al. (2017, p. 6) *Trends and Characteristics of Occupational Suicide and Homicide in Farmers and Agriculture Workers, 1992–2010* study.

of Labor's Bureau of Labor Statistics (BLS) Census of Fatal Occupational Injuries (CFOI). Ringgenberg et al. (2017) examine work-related suicide and homicide data on farm operators (farmers, farm owners, and farm managers) and farmworkers (agriculture workers and laborers) in comparison to the overall working population. Among these, 65.2% of suicides occur among the self-employed, 9.6% among those who work in a family business, and 24.8% among those who work for pay (Ibid. p. 6) (see Figure 3).

## 2.2 Regional relevance, national concern

Reported 2014 NASS figures (as cited in Ringgenberg et al. 2017, p. 5) show that the Midwest region (Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota, and Wisconsin based on the United States Census Bureau-designated regions and divisions) holds 50% of U.S. farmland. Ringgenberg et al. (Ibid.) observed that among agriculture-related occupational fatal injury data, the proportion of farmer suicide in this region was remarkably high at 37.4%. Such a ratio was even worse in the West (Alaska, Arizona, California, Colorado, Hawaii, Idaho, Montana, Nevada, New Mexico, Oregon, Utah, Washington, and Wyoming) which, with 16% of U.S. farms, had a proportion of 43%. While the proportion of suicide was significantly lower in the South (13.5%) and the Northeast (6.1%), there was an alarmingly high proportion of homicide in the South (44.4%) and again in the West (39.8%). These regional differences highlight a particular occupational aspect. When comparing homicide and suicide, 65% of suicide victims were self-employed, whereas 61% of homicide victims worked for pay (Ibid.).

Data from this 19-year analysis thus indicate a suicidal concern of national relevance that affects farmers in particular. Ringgenberg et al. (2017, p. 4; 6) highlight that paid farmworkers are also affected by this common tragedy yet at a suicide rate of just under three times less than farmers. Moreover, this study stresses the need for further regional data and analysis regarding the reasons for elevated suicide among farm operators and elevated homicide among farmworkers. The USDA's most recent *2017 Census of Agriculture* indicates that out of 2,042,220 total U.S. farms, 1,529,083 have no reported farm labor (NASS 2019, p. 339). Indeed, given the structure and modality of agricultural work, a division among farmers and farmworkers on such a sensitive and urgent matter can be inconsequential when considering the occupational overlap between farmer and farmworker duties. Even more, the average U.S. farm was cited at 441 acres, that is, manageable and worked by a single farmer with or without family labor and without the need for hired labor (Ibid. p. 7).

## 3. The family farm economic unit

This situation reflects a diverse social and economic status of family farms which, given the due differences, is analogous to peasantry (Bissen 2014). The peasant with no hired labor represented a theoretical issue for Karl Marx, as outlined by Alexander Chayanov. This agrarian economist and rural sociologist illuminated Marx's challenge with a specific economic figure, the peasant. Marx (as cited in Thorner 1966, p. xviii) states of the peasant: "as owner of the means of production he is capitalist, as worker he is his own wage worker," and even more, "the separation between the two is the normal relation in this [i.e., capitalist] society." According to Chayanov, the absence of one of the four elements of capitalistic entrepreneurship envisioned by classical and neoclassical economic theories, that is, "wages (of labor), interest (on capital), rent (for land), and profits (of enterprise)" makes it impossible to determine the magnitude of the remaining three, thus destroying their theoretical structure (Ibid. pp. xiii-xiv). This does not exclude farming from capitalist agriculture. However, the duality of the worker as the owner of the means of production highlights the need for another economic theory in interpreting the specific world of family farms (Chayanov 1966 [1920s], p. 42). The USDA's *2017 Census of Agriculture* (NASS 2019) mirrors this characteristic by distinguishing farmers who hire labor from farmers who do not hire labor. The latter constitute a large majority.

## 3.1 Farming's social body

Following Chayanov, the authoritative scholar of rural sociology, Teodor Shanin, detailed how family farm life has remained incongruent with narrow capitalistic

formations (Chayanov 1966 [1920s]; Shanin 1990). Shanin advanced and deepened the discourse, from the past half-century to today, on the social, economic, and political factors of peasantry and rurality. Despite the mainstream denial of peasant existence alongside myths of progress, peasantry and the family farm as a socioeconomic unit go on, even within contemporary market relations. This particular existence—and its persistence, as articulated by Shanin—stems from a pre-industrial social body that bleeds into contemporary society. Specifically, Shanin observed a resistance to industrialization during the transformation of peasants into farmers and how the farming occupation, especially when livestock husbandry is involved, differs from mechanized forms of production (Shanin 1990, pp. 25-27).

In terms of agriculture-related suicide, further examination is needed to deepen the analysis on this social and economic body. Indeed, such work is progressing through a broader and more detailed data collection process. In the meantime, however, it is clear that discrediting interpretations of actual data is a rhetorical exercise in error.

## 3.2 Survival

Yet questions remain as to why suicide is high among farmers. For over 40 years, Dr. Michael R. Rosmann has studied the purposeful drive of family farmers, developing what he refers to as the *agrarian imperative* (Rosmann 2010). Beyond personality traits, Rosmann's research highlights motivated actions and risk taking among those engaged in agriculture. The agrarian imperative is an instinct that "instills farmers to work incredibly hard, to endure unusual pain and hardship, and to take uncommon risks" (Ibid. p. 72). This lens elucidates the phenomenon of suicide among farmers not only in the U.S. but also transnationally. The survival of the human *umwelt* is at the center of this tragedy. Rosmann has found that "when the objectives of farming are not met and the loss of the farm is threatened, the same traits that motivate agricultural producers to be successful also become associated with depression and suicide" (Ibid. p. 74). Survivability requires risk: it "depends on a broad diversity of species and people" against any harm (Bissen 2017, p. 137). Rosmann emphasizes that if we lose our agrarian imperative, then we lose our survival.

Reasons as to why farmers fail to meet their basic instinct of providing food, fiber, and energy deserves further examination and may encounter a sociological and anthropological response. Isolation, compounded with a lack of alternatives, means that a sole farmer has at once neither workers to rely on for support or to exploit nor bosses to turn to or to direct responsibility. Ringgenberg et al. (2017, p. 4) highlighted owner-operator stress, particularly financial, as a trigger among self-employed farmers who may not have an off-farm income. Self-employed farm operators "take on significant responsibilities for day-to-day operations of the farm, with high work load and financial responsibilities. This increased hands-on role in both work task and management creates greater personal investment in the farm and its operations" (Ibid.). Access to lethal means, exposure to depression-linked insecticide, and poor access to mental and health care services intensify farmer vulnerability (Ibid.). Risks associated with debt demand further consideration. It is commonly held that one must borrow money to make money. If overextended, in either pursuit of more ground, that is, cash rent, or unnecessary equipment, then farmer consumption accelerates. Debt is one aspect that affects how farmers make decisions. Going beyond good measure in terms of either stress on the body or excess consumption risks losing the agrarian imperative. If farms become sheer businesses (e.g. plant, spray, and harvest without livestock and sustainable practices), then they lose their fundamental qualities to the point of working against survival.

Family farms, even in capitalistic societies, express more than pure business operations, as clearly seen when not relying on hired labor. Farming is a way of being. Acculturation and the transformation of family farms into absolute businesses and farmers into hungry consumers forge this anthropological change amid an increasingly financed urban world. This process uproots an existence, and it is at odds with human survival. Suicide may be the only way out of this indiscriminate financial market that manufactures human nature—something a modern market society has, thus far, failed to do.

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In memory of Teodor Shanin (1930–2020)

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## Editorial comment

When reading this manuscript, I was a little perplexed in the beginning: 'What does this story have to do with *Organisms*?' I was tempted to dismiss the manuscript as 'not appropriate'. Reading further, I progressively changed my mind, and I decided how this story of 'tricking by numbers while being formally correct' is very instructive for the readers of *Organisms*. Here, the trick is purely nominalist: 'How to define a given person's job?' (biologists call this problem ontology when they must assign a function to a given gene in order to interpret omics results). The author clarifies how a simple 'shift of definition' can completely change the results and the conclusions. No ontology is perfect for the simple reason (known since the Plato era) that any categorization assumes a specific viewpoint and drastically diminishes the original semantic richness of the object it describes. This problem is clearly explained by the comment that assumes a bottom-up (Aristotelian) approach to categorization. It asks, no matter the code rigor—what about the significantly higher proportion of suicides in a category that almost entirely accounts for agricultural producers?

That is the right way to do it since considering farmers as 'managers', even if legally correct, is out of touch with reality.

The thoughts on the existence of an unsurmountable fault line between post-modern society and rural life are totally correct. They warn of the dangers we are exposed to as an entire civilization if the most central human work over the past ten thousand years has no place nor social consideration in our society.

*A. Giuliani*

Reviews

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# Fibrosis: A Role for Vitamin D

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**Abstract**

Chronic inflammation leads to fibrosis and eventually organ failure. Fibrosis is defined as a wound-healing response that has gone awry. It is featured by excessive production, deposition, and accumulation of extracellular matrix components. The key mediator cells of fibrotic disorders are the myofibroblasts, derived from different precursor cells. Myofibroblasts are responsible of stiff ECM, a hallmark of fibrosis. It is mandatory understanding the molecular pathways contributing to develop the fibrotic tissue to discovery anti-fibrotic therapies. Vitamin D, the precursor of seco-steroid hormone, appears to have anti-fibrotic properties. Vitamin D deficiency may contribute to development of different fibrotic disorders in several organs. It counteracts the pro-fibrotic signals, such as TGF-β1, through several biochemical mechanisms. Counteracting TGF-β1, Vitamin D inhibits myofibroblasts activation and ECM deposition.

**Keywords:** vitamin D3; fibrosis; IL-6; TGF-β; morphogenesis; epithelial-mesenchymal-transition

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## Introduction

An injury in any organ or tissue triggers the repair process, known as wound healing. This process allows the replacement of dead or damaged cells with healthy ones of the same type. Tissue regeneration is the most common outcome of wound healing: connective tissue replacing the normal parenchyma. However, in some circumstances, these processes, can lead to an unwarranted result, as fibrosis. Several acute and chronic stimuli can trigger the repair response; however, if the injurious agents or the damages are not removed, the wound-healing mechanism may "go awry".

This can be ascribed to an increased release of inflammatory mediators and enzymes in the microenvironment. As the inflammatory response becomes chronic, thus, fibrosis occurs. Fibrosis, explained as an "out of control wound-healing response" (Wynn 2007), can eventually lead to organ failure. Fibrosis is characterized by increased accumulation of extracellular matrix (ECM) components, which disrupts the normal tissue architecture. Removing the damaging cause is paramount to avoid the development of a permanent fibrotic tissue. However, identification of fibrosis causative cues is often an uneasy task as well as their removal.

## The fibrogenic process

The fibrogenic response can be divided in the following phases: inflammation, proliferation, remodeling and maturation (Rockey et al., 2015). Following an (chemical/physical) injury, the coagulation cascade is the first process that undergoes activation.

Circulating platelets migrate into the wounded area and release a number of growth factors, such as platelet-

derived growth factor (PDGF) and transforming growth factor β1 (TGF-β1). PDGF is a potent chemoattractant for inflammatory cells, while TGF-β1 is mandatory to stimulate the formation of a provisional ECM by local fibroblasts (Barrientos et al., 2008). The provisional ECM behaves as a scaffold for migration of several inflammatory cells. Concomitantly, damaged epithelial cells also release inflammatory signals, stimulating the proliferation and recruitment of inflammatory cells in the wound. Wounded tissues further produce matrix metalloproteinase's (MMP), which disrupt the basement membrane, thus allowing and facilitating the recruitment of different kind of circulating and inflammatory cells. At this stage, chemical signals such as cytokines and chemokines, recruit endothelial cells to form new blood vessels in the wounded area. Neutrophils and macrophages are the most abundant inflammatory cells at the early stages of process. The former provides an important source of cytokines, activating additional cells to increase the immune response; the latter cleans up tissue debris and dead cells by phagocytosis. Macrophages hold both pro- and anti-fibrotic activity. On the hand, they recruit other inflammatory cells, mainly T-cells, and on the other, they prevent the development of fibrosis eliminating pro-fibrotic factors. Concomitantly, activated T-cells produce pro-fibrotic cytokines, including IL-13 and TGF-β to recruit additional fibroblasts (Li et al., 2006). Fibroblasts, the principal source of ECM components, respond to signals by proliferating and migrating toward to the site of damage. The recruited fibroblasts, in the proliferation phase, rebuild the ECM, replacing the provisional one with fibrillar collagen-rich ECM with higher mechanical strength (Van De Water et al., 2013). Besides producing ECM proteins, fibroblasts are also involved in its maintenance and reabsorption. The reciprocal crosstalk between fibroblasts and macrophages is a key element in fibrosis (Friedman, 2008). Fibroblasts produce pro-fibrotic signals to activate macrophages themselves and in turn, macrophages stimulate the fibroblast to myofibroblast activation (Pakshir and Hinz, 2018). Fibroblasts are quiescent mesenchymal cells, but upon TGF-β1 signal, they are activated into myofibroblasts. Hallmarks of myofibroblasts are high levels of a-smooth muscle actin (α-SMA) expression and a markedly enhanced contractile activity due to the incorporation of α-SMA into stress fibers (Darby et al., 1990), by generating high intracellular tension in the ECM (Hinz et al., 2001). Once activated, myofibroblasts themselves secrete TGF-β, sustaining their own activation by a positive feedback mechanism. The myofibroblasts are rarely found in healthy tissue and they can differentiate from several precursor cells (Gabbiani 2003). Among these cells, tissue-resident fibroblasts are the principal source of myofibroblasts. They can also originate from other sources, including circulating bone marrow-derived fibrocytes (Quan et al., 2006), pericytes (Kida and Duffield, 2011), epithelial and endothelial cells (Carew et al., 2012). Epithelial cells can acquire a myofibroblast phenotype undergoing to a biological process, commonly known as epithelial-mesenchymal transition (EMT). This process triggers biochemical changes in epithelial cells, which lose polarity and acquire mesenchymal features, including enhanced migratory ability, invasiveness, and mainly increased production of ECM components (Kalluri and Weinberg, 2009). It has been recently demonstrated that endothelial cells can also differentiate into myofibroblasts, through an EMT-like process, called endothelial-mesenchymal transition (EndoMT) (Piera-Velazquez et al., 2016). Both EMT and EndoMT can be induced by TGF-β (Pardali 2017). All these precursor cells amplify the pool of myofibroblasts (Hinz et al., 2007). Myofibroblasts synthesize and release elevated amount of matrix components, contributing to the excessive ECM observed in fibrotic diseases. The fibrotic matrix, in the remodeling phase, consists predominantly of fibrillar collagen types I-III (Karsdal et al., 2017), ED-A fibronectin (White et al., 2008), basement membrane collagen type IV, matricellular protein Periostin (PERST) (Kii and Hito, 2017) laminin and other less abundant elements. Myofibroblasts exert mechanical forces on ECM through binding among integrin, ECM components and cytoskeleton filaments (Zhong et al., 1998). Thus, myofibroblasts can remodel both chemical and physical properties of ECM contributing to progression of fibrosis (Hinz, 2016). The mechanical stress of ECM, due to increased tissue stiffness and decreased elasticity, enhances myofibroblasts activation and then progression of fibrosis (F. Klingberg 2013). Therefore, communication in between macrophages and myofibroblasts promotes fibrosis (Hinz, 2009). In the last phase, myofibroblasts stimulate wound contraction, process for the elimination of scar, the re-epithelialization and then the regeneration of the damaged tissue. The apoptosis of inflammatory cells and myofibroblasts is the last fundamental step completing the wound re-

pair process (Mescher 2017). However, if the controlled death fails, this leads to an unbalance between beneficial wound repair and organ fibrosis and it provokes a prolonged activity of macrophages, by activating myofibroblasts, and myofibroblasts by remodeling ECM (Sindrilaru and Scharffetter-Kochanek, 2013). The persistent accumulation and stiffening of ECM supports a positive feedback loop through biomechanical forces by sustaining activation of cells – macrophages and myofibroblasts – beyond their lifetime (Parker et al., 2014). Moreover, during fibrosis, the synthesis of new collagen by myofibroblasts exceeds the rate of its degradation, increasing the amount of matrix (Pardo and Selman, 2006). This is due to the imbalance of collagen turnover, regulated by MMP and their inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), which promote the production instead of the degradation.

## Master drivers of fibrotic response

The activated myofibroblasts are considered the main effectors of fibrosis, by producing a large amount of matrix proteins (Hinz et al., 2007). Macrophages and T-cells, instead, release biochemical signals to modulate the fibroblasts activity and the matrix metabolism. Among these signals involved in the process, TGF-β1 is considered the key mediator of the fibrotic response (Stewart et al., 2018). TGF-β belongs to a cytokines family that regulate several physiological processes. There are three different isoforms, TGF-β1, TGF-β2 and TGF-β3, but among these, TGF-β1 is the prevalent and ubiquitously one. The macrophages are responsible to regulate both the secretion and the activation of latent TGF-β1.TGF-β1 is synthesized as latent precursor complex, non-covalently bound to latency-associated protein (LAP) (Robertson et al., 2015). The latent complex is mainly stored in the matrix, covalently cross-linked to ECM proteins (Werb 1997), keeping TGF-β in an inactive form, which cannot interact with its receptors. Several proteases can catalyze the dissociation of LAP from TGF-β1; then, TGF-β1 becomes activated and it can bind to receptors (Murger et al., 1999). Upon binding to transmembrane receptors, TGF-β1 can act through two different signalling pathway: canonical or non-canonical. The canonical signalling pathway, known as Smad-dependent, involves the phosphorylation and activation of Smad2 and Smad3 that form a complex, which subsequently binds to Smad4. The activated complex can translocates to the nucleus and affect the transcription of specific target genes (Hill, 2016). In vivo studies have confirmed the TGF-β/Smad3 involvement in the fibrogenesis because Smad3-null mice became resistant to fibrotic disease (Zhao et al., 2002). However, TGF-β1 can also activate the non-canonical pathway, known as Smad-independent, which involves alternative signaling pathways, including mitogen-activated protein kinases (MAPKs) (Bhowmick et al., 2001), Wnt/β-catenin signaling, phosphatidyl-inositol 3 kinase (PI3K/AKT) (Bakin et al., 2000), p53 and Notch signaling (Zhang, 2009). TGF-β is synthesized and secreted both by macrophages and fibroblasts, thus acting through both paracrine and autocrine way. Upon TGF-β stimulation, fibroblasts are activated in acquiring myofibroblasts phenotype. As mentioned above, TGF-β increases the pool of myofibroblasts also by inducing EMT and EndoMT, respectively, in epithelial and endothelial cells (Xu et al., 2009). TGF-β signaling increases ECM synthesis, deposition, and contraction by myofibroblasts. It mainly enhances expression of collagen types I, III and VI, fibronectin and proteoglycans (Massague, 1990). TGF-β1 also inhibits ECM degradation, by inducing down-regulation of MMP expression and by increasing TIMPs expression, through the Smad3 activity (Yuan and Varga, 2001). Remodeling of ECM is paramount for the progression of fibrosis, as well as for its regression. In skin fibroblasts, TGF-β1 induces the expression of procollagen Lysyl hydroxylase 2 (PLOD2), a gene coding for an important enzyme for the hydroxylation of lysine residues in collagen (van der Slot et al., 2003). Such modification enhances the number of cross-links among pyrydoline residues that stabilize collagen fibrils, making it more difficult to degrade by enzymes (Ricard-Blum et al., 1993). Lysyl oxidase-like 2 (LOXL2) is another important enzyme for the remodeling of ECM components. It catalyzes the deamination of lysine residues of collagen monomers promoting the formation of cross-linkages (Grau-Bove et al., 2015). LOXL2 belongs to the Lysyl oxidase family, composed of five members (LOX and four LOXL variants). The cross-linkages catalyzed by LOXL2are important for collagen stabilization as well as matrix integrity and elasticity. It is noteworthy that LOXL expression and activity are enhanced in hepatic stellate cells (HSCs), involved in liver fibrosis (Liu et al., 2016). These modifications on collagen result in increased matrix stiffness, which activates the myofibroblast via mechanical forces (Ikenaga et al., 2017), thus

allowing the persistence of fibrosis (Hinz, 2016). It has been demonstrated that inhibition of LOXL2 mitigates the progression of liver fibrosis and promotes its resolution (Ikenaga et al., 2017). The expression and activity of LOXL2 are increased by Periostin (PERST) (Kumar et al., 2018), a nonstructural extracellular matrix protein. Periostin can directly interact with ECM components, such as fibronectin, collagen, elastin, and promotes the fibrillogenesis. POSTN stimulates the expression of intra- and extracellular collagen and fibronectin in HSCs, during hepatic fibrosis. Moreover, TGF-β signaling stimulates POSTN and, in turn, POSTN favors the phosphorylation of Smad2/3, even in absence of TGF-β signaling (Kumar et al., 2018).

In addition, other proteins enhance the fibrotic response downstream of TGF-β1 by increasing the contractile phenotype of fibroblasts, as well as by facilitating the release of several ECM components from activated fibroblasts. The ECM is not an inert scaffold, as it is a dynamic regulator of the cell/microenvironment cross talk. Indeed, ECM modulates traffic and activity of several signaling molecules (cytokines, growth factors, etc.) acting upon both cells and their milieu. An important ECM-related pro-fibrotic mediator is the connective tissue growth factor (CTGF), member of a small protein family, localized in the ECM (De Winter et al., 2008). CTGF is a matricellular protein (a non-structural protein found in the ECM) that modulates cell functions through cell-matrix interactions (Chen and Lau, 2009). CTGF is expressed by endothelial cells and by fibroblasts. After being secreted by cells, CTGF interacts with several molecules, mainly cytokines and growth factors. This interaction can affect - positively or negatively - the signal transduction, regulating several processes, such as cell adhesion, migration, ECM deposition and myofibroblasts activation. CTGF plays a central role in tissue remodeling and its expression seems to be correlated with observed clinical behavior. Experimental data on human fibrosis demonstrated that CTGF expression appears associated with the degree of fibrosis (Igarashi et al., 1995). CTGF induces several precursor cells to differentiate into myofibroblasts, including epithelial cells, through EMT process (Lee at al., 2010). Moreover, it increases the expression of collagen type I, fibronectin and integrin in fibroblasts and promotes their deposition and remodeling (Frazer et al., 1996). CTGF can act as an extracellular adapter by binding to TGF-β1 and helping it to bind to its receptors, potentiating its activity 2002). Thus, the overexpression of CTGF enhances the pro-fibrotic response of TGF-β1.Experimental data on transgenic mice with overexpression of CTGF in fibroblasts demonstrated a more accelerated fibrosis development without any other pro-fibrotic stimulus (Sonnylal et al., 2010). Experimental data have highlighted that TGF-β1 induces CTGF expression via Smad-dependent signaling. Smad3 binds the CTGF promoter inducing the myofibroblast differentiation and collagen synthesis (Duncan et al., 1999). Thus, CTGF is considered as a downstream mediator of the effects of TGF-β on fibroblasts (Grotendorst 1997). Moreover, CTGF can also induce the expression of TGF-β itself, triggering a positive feedback loop (Yang et al., 2010). This can contribute to the progression of fibrosis.

Beside TGF-β, other pro-fibrotic and pro-inflammatory cytokines act during fibrosis, especially by T-cells that activate macrophages and fibroblasts (Wynn, 2004) and directly stimulate collagen synthesis in fibroblasts (Oriente et al., 2000) through Smad3/TGF-β-independent mechanisms (Kaviratne et al., 2004). Several findings indicate that cytokines secreted by activated immune cells (T-cells as well as macrophages), such as IL-13, IL-4 and IL-6 can promote an inflammatory/fibrosis prone microenvironment in a TGF-β-independent way (Kaviratne et al., 2004). Studies on cytokine-deficient mice had demonstrated the important link between T-cell response and the development of fibrosis, involving IL-4 and IL-13(Chiaramonte et al., 1999), suggesting that each cytokines likely support a specific role during such process. IL-4, involved mainly in lung fibrosis (Emura et al., 1990), is almost more efficient of TGF-β as pro-fibrotic mediator (Letterio and Roberts, 1998). Fibroblasts express IL-4 Receptors (IL-4R), and upon IL-4 stimulation, they synthesize extracellular matrix components, such as collagen type I, III and fibronectin (Fertin et al. 1991), while in mice treated with IL-4 inhibitors it was demonstrated a reduced deposition of collagen and decreased development of fibrosis (Ong et al., 1998). Other studies showed that IL-13 acts as the effector cytokine of fibrosis when IL-4 is inhibited (Keane et al., 2007). Noticeably, both IL-13 and IL4 converge to the same receptor, which transduce interleukin stimulation through the STAT signaling pathway (Zurawaski et al., 1993). However, quite paradoxically, activation of the same pathway by different effectors (IL4 and IL-13 respectively) fosters the development of different outcomes and the emergence of

diverse types of pulmonary diseases (Zhu et al., 1999; Rankin et al., 1996). This conundrum can partly be explained by the fact that IL-13 may involve alternative pathways usually inaccessible to IL4 (Webb et al., 2003) (Blease et al., 2002). Moreover, IL-13 activates preferentially the TGF-β signaling – by stimulating macrophages to produce latent TGF- β – while upregulating the synthesis of those enzymes (MMP and cathepsin) that activate TGF- β through the cleavage of LAP (Lanone et al. 2002). Indeed, in transgenic mice overexpressing IL-13 and treated with an inhibitor of TGF-β development of fibrosis was markedly reduced (Lee et al., 2001). However, IL-13 can trigger fibrotic pulmonary processes even in the absence of TGF-β/SMAD signaling (Nakao et al., 2000) (Kaviratne M 2004). These controversial findings warrant a convincing explanation that probably require considering the IL-13 mediated involvement of other cytokines in fibrosis development. Noticeably, significant experimental data have been recently collected about the potential pro-fibrotic role sustained by Interleukin-6 (IL-6). IL-6 is a pro-inflammatory and pro-fibrotic cytokine, produced by several cells, including macrophages and T-cells. IL-6 binds to its receptor, IL-6R, and then this complex associates with a second receptor on the cell surface - glycoprotein 130, gp130 (Hibi et al., 1990) - to foster a number of intracellular processes (Rose-John, 2012). IL-6 has higher affinity to IL-6R than gp130, therefore only cells expressing IL-6R can respond to the signal induced by this cytokine (Jostock et al., 2001). However, IL-6R is found only in few cell types, while gp130 is ubiquitously expressed. IL-6R is mainly expressed by hepatocytes and T-cells, limiting the pool of cells able to respond to IL-6 signaling. However, IL-6 can signal through an alternative pathway, known as trans signaling, by which IL-6 recognizes and binds to a soluble form of IL-6R, i.e. sIL-6R, formed by a proteolytic cleavage (Rose-John and Heinrich, 1994). IL-6 has an affinity to sIL-6R comparable to that of IL-6R on the cell membrane. The complex IL-6/sIL-6R can bind to gp130, thus activating trans-signaling-dependent cascades (Taga et al., 1998). IL-6 trans signaling increases the pool of cells that can respond to IL-6. In both pathways, the dimerization of gp130 leads to activation of receptor-associated Janus Kinases (JAKs)(Heinrich et al., 2003), and Signal Transducers and Activators of Transcription (STATs) that, after phosphorylation, can translocate to the cell nucleus where they act as a transcription factors to regulate gene expression(Heinrich et al., 1998). IL-6 plays a role in the development of fibrosis in the lung (Le et al., 2014), by inducing the transformation of fibroblasts into myofibroblasts. Conversely, inhibition of IL-6 trans signaling attenuates pulmonary fibrosis (Le et al. 2014). Levels of IL-6 and sIL-6R are elevated in systemic sclerosis (Hasegawa et al., 1998), correlating with disease severity. In heart, liver, skin and kidney fibrosis (Tanaka et al., 2012), IL-6 induces collagen I expression, both through classical and trans-signaling manner. IL-6 trans signaling increases collagen I expression by activating STAT3 and SMAD3 and by synthesizes Gremlin-1, an antagonist of bone morphogenetic protein (BMP) (O'Reilly S 2014). Over-expression of STAT3 has been observed in tissue of patients with lung fibrosis (O'Donoghue 2012); while, deletion of IL-6 gene results in reduced lung fibrosis in animal models (Saito 2008). In scar tissue, dermal fibroblasts express high levels of gp130 on cell membrane and IL-6 signaling promotes the proliferation of fibroblasts and the increased production of ECM components, including collagen and fibronectin (Ray et al., 2013).

## Vitamin D3 biology

Vitamin D is the major regulator of calcium homeostasis and normal bone mineralization in the body (Hoenderop et al., 2005). However, in the last years it became clear that Vitamin D also plays non-calcemic effects modulating other biological functions. Vitamin D, despite its name, is not a vitamin rather it is the important precursor to the seco-steroid hormone, 1α25-dehydroxy-colecalciferol, commonly known as Calcitriol. Calcitriol mediates several biological processes in many tissues. It is obtained from dietary sources or from de novo synthesis. In the skin, the ultraviolet ray's energy converts the substrate 7-dehydrocholesterol to pre-vitamin D3 followed by thermal isomerization to itamin D3 (Dusso and Brown, 1998). The activation metabolism is characterized by two hydroxylation steps, which in turn are principally catalyzed by two P450 cytochrome enzymes (Jones and Prosser, 2014). Vitamin D3 circulates bound to the Vitamin D Binding Protein (VBP), reaching the liver where the first hydroxylation occurs, catalyzed by vitamin D-25hydroxylase (CYP2R1) to yield 25-hydroxyvitamin D3 (25(OH)D), calcidiol (Jones and Prosser, 2014). A further hydroxylation happens in the kidney, where

25(OH)D3 is hydroxylated by another member of the cytochrome P450 family - 1α-hydroxylase (CYP27B1) – to obtain calcitriol (1,25(OH)2D3), i.e. the most active form of Vitamin D3 (Jones and Prosser, 2014). Calcitriol exerts its nuclear effects by binding to the specific Vitamin D Receptor (VDR), a member of the steroid–thyroid–retinoid receptor, which is a superfamily of ligand-activated transcription factors (Christakos et al., 2016). VDR is ubiquitously present into the cytosol of a number of cells. The complex in between Vitamin D3 and its receptor regulates from 3% to 5% of the human genome, via both genomic and non-genomic mechanisms (DeLuca 2004), thus modulating several biological processes (Bouillon et al., 2008). Calcitriol binds to VDR fostering its phosphorylation. It is worth of interest that activated VDR translocates to the nucleus and then it heterodimerizes with Retinoid-X-Receptor (RXR) (Christakos et al., 2003). The RXR is a nuclear receptor activated by 9-cis retinoic acid, playing an important role in regulating retinoid signaling(Heyman et al., 1992).The Calcitriol-VDR-RXR complex recognizes and binds to VDR Element (VDRE) on promoter region of target genes and regulate their expression recruiting transcriptional co-activators or co-repressors(Pike and Meyer, 2012).

## Vitamin D and fibrosis: epidemiological and clinical data

Although fibrosis was initially thought to be an irreversible process, experimental data suggest the possibility of resolution of fibrotic diseases (Jun and Lau, 2018). The resolution occurs when the cause of injury is eliminated, but this may not be possible due to the multifactorial feature of this group of disorders. Initially, therapeutic treatments of fibrotic diseases were composed of anti-inflammatory drugs. However, these treatments lack efficacy because they block only the inflammatory cascade, but not the underlying fibrotic response. Therefore, understanding the molecular mechanisms that regulates the fibrotic cascade in every organ provides more specific target for anti-fibrotic therapy. There are three possible therapeutic targets that play a critical role in the resolution of fibrosis: 1) the myofibroblasts; 2) the fibrotic ECM; 3) the cytokines storm. As reported in literature, several compounds have been proposed as possible drugs. Among these, experimental data demonstrated that Vitamin D has anti-fibrotic properties. Epidemiological data have prompted to speculate about a direct relationship between Vitamin D3 deficiency and occurrence of fibrosis-related diseases (Holick, 2007). Beside no unanimous consensus has been reached in identifying the optimal serum levels of Vitamin D3, the cut-off has been set at 10ng/mL while the optimal range of the seco-steroid from 30 to 60 ng/mL. Namely, the role of Vitamin D3 in fibrosis has been largely demonstrated in several organs, in particularly in the liver and kidney, the two organs where vitamin D3 is metabolized. As a proof of concept, supplementation of Vitamin D assumption in patients with chronic diseases, such as kidney fibrosis, results in amelioration of medical condition (Kovesdy et al., 2008). Overall, preliminary clinical data seems to confirm an anti-fibrotic activity of vitamin D3 (Tan et al., 2007).

## Mechanisms of Vitamin D in organ fibrosis

The myofibroblast is main target of inhibitory action of 1,25(OH)2D3 during fibrosis processes (Tao et al., 2015). The Vitamin D3 interferes with the pro-fibrotic function of TGF-β1 repressing the expression of collagen in several cell types et al., 2013). Such inhibitory effect of 1,25(OH)2D3 on collagen expression has also been confirmed in experiment conducted on rats. In these experiments, rat hepatic cells treated with Vitamin D3 demonstrated down-regulation of pro-fibrotic genes induced by TGF-β1 (Abramovitch et al., 2011). Calcitriol, bound to VDR/RXR heterodimer, decreases the expression of target genes of TGF-β/Smad3 signaling. Such effect can be exerted through multiple mechanisms, supported by many different scientific evidences. Experimental analysis on hepatic fibrosis revealed the competitive binding of VDR/RXR on the Smad3 Binding Element (SBE), present on the promoter of pro-fibrotic genes. Indeed, through Chip-on-Chip analysis, it has been highlighted that Smad3 and VDR recognize the same binding sites on specific genes (Ding et al., 2013). Maybe, this competition at genomic level is due to the action of specific histone acetyltransferase (HAT) which cause a remodeling of the chromatin, making it in an open status and allowing the docking of the transcription factors (Kouzarides, 2007). For example, p300 histone acetyltransferase is a TGF-β1 target gene and is highly expressed in normal fibroblasts and myofibroblasts during fibrosis (Ghosh and Varga,

2007). Chromatin remodeling induced by p300 allows the opening of the VDR binding sites very close to those Smad3 recognizes. This is a critical step, as opening the chromatin is mandatory for any epigenetic modulation. The binding of VDR upon VDRE causes Smad3 to be dislodged from chromatin causing a blockage of pro-fibrotic genes expression, as both VDRE and SBE compete for the same binding sites. In other experimental studies on hepatic fibrosis, using co-immunoprecipitation analysis, VDR directly interacts with the phosphorylated Smad3, inhibiting the binding of Smad3 to SBE and the subsequent activation of its target genes (Ding et al., 2013). This interaction represses the TGF-β1-induced stimulatory effect on some specific features of the fibrotic process, including collagen release and myofibroblasts activation (Zerr et al., 2015). Myofibroblasts, as mentioned above, can originate from epithelial and endothelial cells, downstream of EMT activation induced by TGF-β1. This pathway is sustained by the activation of several transcription factors, including Smad and Snail (Ikushima and Miyazono, 2010). EMT is a process by which epithelial cells lose their specific markers and acquire mesenchymal traits. Epithelial cells lose cell-cell adhesions and cell-extracellular matrix junctions; in the meantime, they miss their polarity and reorganize the cytoskeleton while promoting a reprogramming of several genes. This transition generates cells with marked motility, able in degrading the extracellular matrix. Such features lead to the acquisition of a migratory and invasive phenotype. Vitamin D is a negative modulator of EMT mechanism, via the transcriptional activity of VDR (Fischer and Agrawal, 2015). Experimental data demonstrated that Vitamin D could attenuate renal fibrosis, by inhibiting the EMT process (Nieto 2011). Specifically, Vitamin D and TGF-β1 have been demonstrated to exert opposite role in respect to EMT, probably accordingly to a reciprocal feedback inhibitory loop. TGF-β, indeed, triggers the expression of VDR and VDR in turn inhibits the EMT process induced by TGF-β. Ricca and coworkers had analyzed the cross talk between the two molecules by treating normal epithelial cells with Calcitriol before, together and after TGF-β addition (Ricca et al., 2019). Calcitriol treatment represses the EMT process, through the up-regulation of epithelial marker, such as E-cadherin, and reduces the cell proliferation rate. An interesting finding is increase in VDR synthesis upon TGF-β1 stimulus. The up-regulation of VDR induced by TGF-β1 is significantly higher than that exerted by calcitriol toward VDR, i.e. its own receptor. TGF-β dependent VDR induction has been demonstrated in different cell lines, being not restricted to few cell types. Probably, the increased expression of VDR is a component of a more complex auto-inhibitory negative feedback loop, enacted by TGF-β upon its synthesis and release (Stroschein et al., 1999). Indeed, among the target genes activated by TGF-β, Smad7, in turn, negatively regulates TGF-β activity (Yan and Chen, 2011). Smad7 competes with Smad2/3 for binding to receptors, thus inhibiting the TGF-β dependent signaling (Yan and Chen, 2011). Notice that Smad7 protein levels are downregulated during kidney fibrosis, while Smad7-deficiency mice are more susceptible to fibrosis (Chung et al, 2009). This data suggests a regulatory role of Smad7 in fibrotic diseases. The auto-inhibitory regulation of TGF-β correlates with data of Ding and coworkers indicating a genomic competition for the binding sites between VDR and Smad3, which immediately increases downstream of the interaction of TGF-β with its receptor. As previously mentioned, the inhibitory action of Vitamin Don EMT occurs only if cells are treated with Vitamin D before (or together) TGF-β addition. Instead, when Vitamin D is added after TGF-β1 stimulation, no significant inhibition on TGF-β-related pathways can be observed and vitamin D result unable in antagonizing the main fibrosis-related molecular features. Vitamin D, in fact, can increase the expression of E-cadherin, and in turn may block EMT, only when is added before or simultaneously with TGF-β1. This finding suggests that, to revert the mesenchymal phenotype, Vitamin D must be already present when EMT is induced. Therefore, the supplementation of Vitamin D seems to have a protecting role against the onset of fibrotic diseases.

Liver cells usually present a low expression of VDR. Recent studies had demonstrated a high expression of the receptor in the hepatic stellate cells (HSCs). Is interesting to observe that the most relevant release of components of ECM produced during hepatic fibrosis can be ascribed to those cells. HSCs, generally, are quiescent and play the role of storage site for Vitamin A (retinoic acid) in the body (Bataller and Brenner, 2011). Following liver injury, HSCs are activated by cytokines and growth factors; then, they differentiate into myofibroblasts, beginning to proliferate, produce cytokines and release the abundant ECM components (Lee and Friedman, 2011). In hepatic fibrosis, Vitamin D represses

the production of collagen by stromal HSCs, through a VDR-mediated mechanism. In addition, Calcitriol suppresses the proliferation of hepatic stellate cells (HSCs) and influences the expression of collagen α I both at transcriptional and translational level, thus inhibiting liver fibrosis (Abramovitch et al., 2011). Indeed, VDR-null mice develop spontaneous liver fibrosis, confirming the important role of the VDR signaling to inhibit the pro-fibrotic transcriptional activity in HSCs (Ding et al., 2013). Moreover, Ding and coworkers, analyzing a mammalian model of liver fibrosis, showed that co-treatment with a Vitamin D analogue caused a reduction in the hallmarks of fibrosis. Specifically, collagen deposition and the expression of pro-fibrotic genes such as COL1A, TIMP1 and TGF-β1 (Ding et al., 2013).Vitamin D counteracts fibrosis inhibiting the expression of collagen I and III and increasing the expression of MMP8, a metalloproteinase essential in the degradation of extracellular matrix in fibrosis. The TGF-β signaling also activates HSCs, which in turn secrete the matricellular proteins, such as CTGF (Liu et al., 2013). The expression of CTGF in hepatic fibrosis increases, but upon Vitamin D treatment, its expression level decreases.

As previously mentioned, Periostin is involved in liver fibrosis, through the stimulation of LOXL2, collagen and fibronectin release by HSCs. It is worth of noting that Calcipotriol, an analog of Vitamin D, can decrease Periostin expression in HSCs (Zhang et al., 2018).

The Vitamin D is also involved in the renal fibrosis. The kidney fibrosis is a hallmark of chronic renal diseases correlating with organ failure. Paricalcitol, a Vitamin D3 analog, decreases interstitial fibrosis, the EMT process and the inflammation response (Tan et al., 2006), by inhibiting both TGF-β expression, ECM components release and the transition of tubular epithelial cells to myofibroblasts (Yang and Liu 2002).In kidney fibrosis, TGF-β and angiotensin II (Ang II) are the major pro-fibrotic drivers (Wolf, 2006). The Renin-Angiotensin System (RAS) is activated in a number of kidney diseases, thus triggering an enhanced release of AngII. The RAS is also activated in lung fibrosis and it is a pathogenic factor in this type of fibrosis (Wang et al., 2015). The RAS includes Angiotensinogen (AGT), Renin and angiotensin-converting enzyme (ACE). AngII, activated by renin and ACE, plays a crucial role in fibrosis, through its receptors, by stimulating the TGF-β pathway, ECM production and driving EMT process to myofibroblasts activation. In the kidney, myofibroblasts differentiate from tubular epithelial cells, which lose the epithelial marker, like E-cadherin, and expressα-SMA 2010). Vitamin D3 plays a protective role, by specifically antagonizing RAS, downstream of VDR activation (Li, 2010). Studies on VDR-null mice have demonstrated that the activation of RAS and the subsequent AngII overexpression play a pivotal role in renal fibrosis. Conversely, treatment with an inhibitor of the receptor of AngII blocks the fibronectin and collagen I expression. On the other hand, Vitamin D suppresses renin and AGT (Yuan et al., 2007), while VDR deletion leads to RAS activation and overproduction of AngII, which induces EMT process and enhances renal fibrosis by stimulating TGF-β synthesis (Li et al., 2002).

Moreover, VDR may regulate (directly or indirectly) other targets involved in renal fibrosis such as Wnt/β-catenin signaling (He et al., 2009). In the adult kidney, the Wnt/β-catenin signaling becomes silent after differentiation, but it can be reactivated upon injury (He et al., 2009). The Wnt signaling pathways comprises a diverse family of secreted lipid-modified signaling glycoproteins that exert their biological function via β-catenin pathway. Wnt transduces the signal through interaction with cell membrane receptor of the Frizzled family and co-receptors, LRP5/6, leading to the de-phosphorylation of β-catenin (Hwang et al., 2009). Upon de-phosphorylation, β-catenin stabilizes and translocates into the nucleus where it binds to and activates several transcription factors, thus regulating the expression of several target genes (Rao and Kühl, 2010). Besides Wnt, other molecular factors can activate β-catenin. A major regulator is the integrin-linked kinase (ILK), induced by TGF-β1, AngII, GSK3β or other pro-fibrotic factors. TGF-β1 can also fosterβ-catenin activation by up-regulating MMP-7 that trigger E-cadherin degradation, thus releasing β-catenin, which is usually linked to E-cadherin behind the cell membrane. When released from its association with E-cadherin, β-catenin can translocate to the nucleus. In addition, Wnt/β-catenin signaling regulates the expression of other critical genes, including fibronectin, MMP-7, fibroblast-specific protein 1 (Fsp1), Snail and components of RAS (Liu, 2011). The activation of these genes suggests a fundamental role of this pathway in modulating fibrosis. The induction of Fibronectin leads up to increased production and deposition of ECM components. Fsp1 is a marker of activated myofibroblasts, produced by resident fibroblasts. Upon tissue injury, β-catenin is highly up re-

gulated in renal tubular epithelial cells and sequentially it induces Snail1 expression. Snail1 is a key EMT-related transcription factor (Yoshino et al., 2007), given that it specifically represses those factors – as E-cadherin – that play a major role in assuring cell-cell adhesion and tissue architecture. Moreover, Snail1 is not only a target of β-catenin signaling but it is in turn regulated by those factors that negatively regulate β-catenin, as GSK3β. Activated Wnt inhibits GSK3βactivity, while allowing the simultaneous release of β-catenin and Snail1. This leads to synergistic effects in promoting EMT (García de Herreros and Baulida, 2012). In view of the role that Wnt/β-catenin signaling plays in kidney fibrosis, is paramount to block that pathway. Noticeably, Vitamin D can inhibit Wnt/β-catenin signaling by three different mechanisms. Firstly, Vitamin D facilitates the reciprocal VDR/β-catenin interaction, preventing the binding of β-catenin to its TCF (T-cell factors) transcriptional factors (Palmer et al., 2001). Second, Vitamin D upregulates the epithelial markers, mainly E-cadherin, thus counteracting directly EMT. Re-expression of E-cadherin allowsβ-catenin cytoplasm translocation and its subsequent interaction with E-cadherin close to the adherens junctions (Palmet et al., 2001). Third, Vitamin D triggers the expression of inhibitors of Wnt signaling, such as Dickkopf (DKK)-1. DKK-1 inhibits the Wnt signaling binding to LRP5/6, i.e. to the receptors of Wnt, thus preventing Wnt/Frizzled/LRP5/6 interaction (Semenov et al., 2001). However, inhibitory activity of Vitamin Dis in turn hampered by Snail1. Indeed, Snail1 represses VDR expression by binding to three E-boxes in the VDR gene promoter. Moreover, Snail1 reduces the half-life of VDR RNA (Palmer et al., 2004).The overexpression of Snail1 in pathological conditions, prevents the expression of E-cadherin and subsequently it blocks the action anti-fibrotic of Vitamin D. This leads to β-catenin translocation to the nucleus and the expression of related target genes. Vitamin D can act as a modulator of immune response, by attenuating inflammatory process and by downregulating the pro-inflammatory cytokine, especially IL-6 (Skrobot et al., 2018). Indeed, a few reports indicate that vitamin D exert an inhibitory role on IL6 release in fibroblasts, probably through the MAPK38 pathway (Nonn et al. 2006). Yet, studies on the relationships between vitamin D and IL6 are still on their infancy and further investigations are warranted.

However, the disparate experimental findings collected until now should be “re-interpreted” according on a very different theoretical perspective in order to gain a more comprehensive appraisal of the physiological role of vitamin D. Overall, data available claim for a relevant morphogenetic role sustained by Calcitriol in modulating the cell-microenvironment cross talk in a number of different tissues, as bone, skin, liver and immune system. To vindicate such a hypothesis, Sonnenschein and coworkers have shown that calcitriol, at physiological doses, affects the mammary gland development contributing to the proper shaping of epithelium (Hasan et al., 2019). In a model in which mechanical forces mediate cell shaping during morphogenesis, the authors observed how mammary cells embedded in type-I collagen matrix manipulate the fibers of collagen to organize the 3D structures, such as ducts and acini (Speroni et al. 2014). The organized collagen fibers constrain the cells on biological processes, including proliferation, apoptosis, and motility, whereas Vitamin D, in turn, constrains the collagen fibers organization, affecting the mechanical forces induced by the cells and ultimately cell population density in a dose-dependent manner. This study highlights that Vitamin D act as a “microenvironment organizer” of morphogenesis that affect both cells and their stroma.

## Conclusions

Vitamin D can inhibit and reduce the progression of fibrosis through various mechanisms, as reported in Fig. 1. Principally, vitamin D antagonizes TGF-β dependent pathways by interacting with Smad3, preventing its transcriptional function, or by binding to the binding sites on the target genes promoter, blocking the binding of Smad3 to the same sites. By attenuating the TGF-β pathway, Vitamin D reduces the expression of pro-fibrotic target genes, the transformation of fibroblasts in myofibroblasts and the subsequent EMT. Moreover, vitamin D also inhibits other pro-fibrotic mediators such as LOXL2, POSTN, and IL-6, blocking the excessive accumulation of several components of the extracellular matrix.

By reducing the stiffness of the matrix, Vitamin D interferes with the mechanical forces that activate fibroblasts present in tissue stroma. Overall, these evidences suggest a potential role of Vitamin D as a potential drug for the treatment of fibrotic diseases.

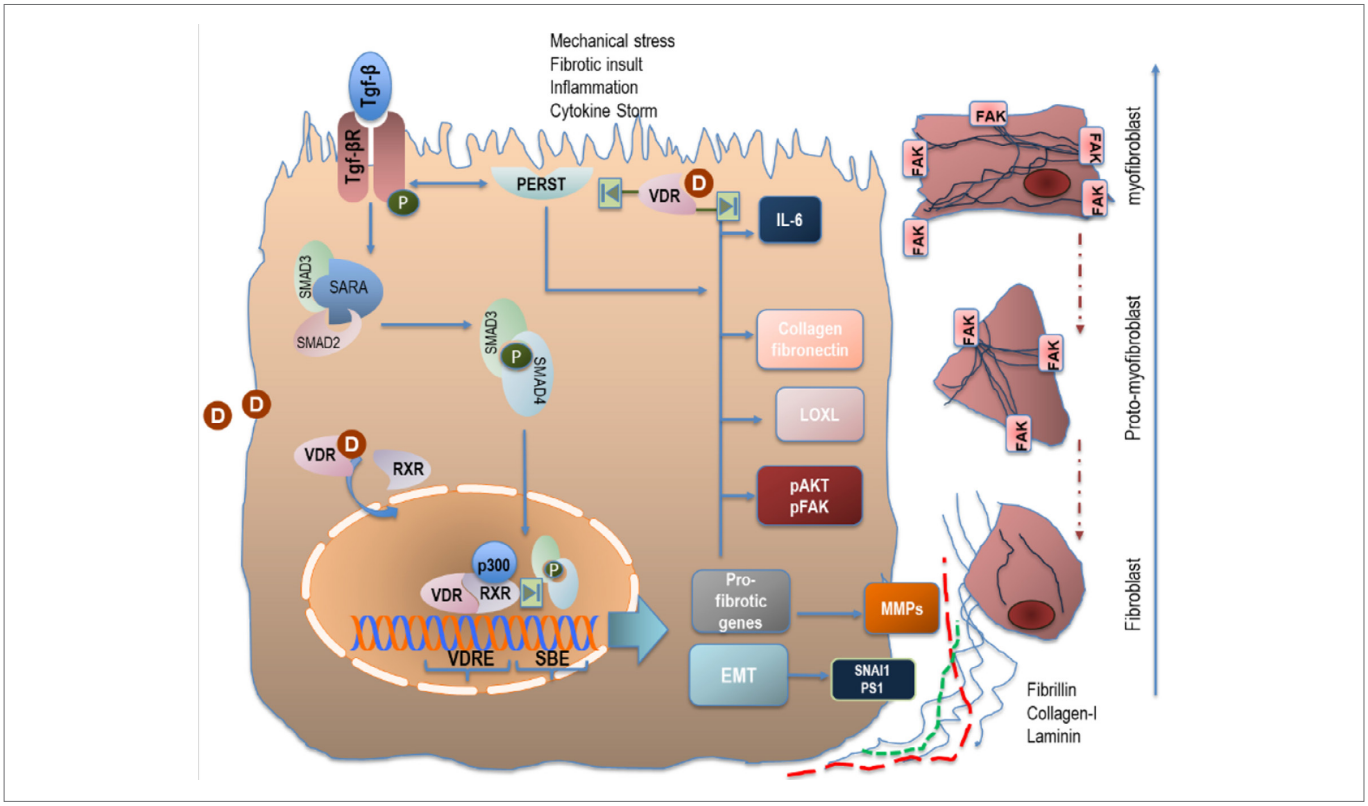


**Figure1: Interaction of vitamin D3 with fibrosis-related pathways.** Upon TGF-β interaction with its receptor, Smad3/4 complexes translocate into the nucleus and affect the transcription of specific target genes by acting upon the Smad3 Binding Element (SBE), present on the promoter of pro-fibrotic genes. Vitamin D3 compete with Smad elements for binding to the SBE, downstream of the heterodimerization of the VDR/RXR receptors, thus inhibiting the TGF-β-dependent canonical signalling pathway. Vitamin D3 can also directly inhibit Periostin (PERST) and IL-6 release, which are activated upon a variety of pro-fibrotic stimuli. Overall, these pathways promote the transformation of fibroblasts into myofibroblasts, while enacting EMT in numerous cells. Abbreviations: RXR, Retinoid-X-Receptor; Lysyl oxidase-like 2, LOXL2; Vitamin D Receptor, VDR; Epithelial Mesenchymal Transition, EMT.

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Original Articles

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# Mesoscopic Cell Mechanobiology and the Problem of Cancer

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**Abstract**

Statistically based model of the DNA ensemble evolution allowed the formulation of mechanobiological approach linking the open complex dynamics and different scenario of gene expression related to normal and cancer cell behavior. It was shown the correspondence of open complex dynamics to specific type of criticality in mesodefects (open complex) ensemble (the structural-scaling transition) in the presence of qualitative different nonlinearity of the mesoscopic potential associated with the epigenetic landscape. The role of open complexes is similar to the mesoscopic defects and provides the cell plasticity or the cell fragility depending on the structural susceptibility of the cytoskeleton structure and the types of collective modes of defects (open complexes). These modes are responsible for the DNA transformation leading to the natural cycle of gene expression and spontaneous cell division as the cancer precursor. The WTMM analysis of the phase thickness fluctuations after the Laser Interference Microscopy of living cells shows the log-normal and power law statistics for normal and cancer scenario that are linked to multi- and monofractal dynamics of open complexes.

**Keywords:** mechanobiology; DNA transformation; open complexity; Laser Interference Microscopy

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## Introduction

Cells reveal the structural organization to promptly adapt their mechanobiological environment in realization of fundamental vital cellular functions [1,2]. The mechanobiology properties of living cells are mediated by the cytoskeleton (CSK) representing a dynamic network of filamentous proteins composed of actin filaments, microtubules, and intermediate filaments [3,4]. Components of the CSK play a key role in motility, transport and cell division, providing essential scaffolding on which metabolic processes occur. Therefore, cytoskeletal morphology is thought to be a valuable indicator of cell injury and functionality [5]. Inner cytoskeleton structure also provides 'privileged' pathways along which enzymes and substrates are coherently organized and oriented, in order to optimize their interactions [6,7]. The variety of the mechanisms of structural relaxation could be associated with fundamental property of the cells qualified as the cell plasticity and the cell damage [8].

Plastic deformation as the unique mechanism of the defects induced momentum transfer and the structural memory provides specific CSK organization in mechanobiological environment. The cell plasticity can be considered as the leading mechanism providing the vital CSK properties, including the cell self-organization up to the cell division. The cell division being the vitality ground has also natural links to the defects behavior that provides the evolutionary controlled cell division (due to the preceded plasticity) or the fragility due to the pathological CSK changes leading to the spontaneous cell division. The duality of defects in the realization of the vital cellular functions (plasticity, damage, damage-

failure transitions) is stimulating for the consideration of the CSK structure as out-of-equilibrium system with defects taking into account the fundamentals of defects in the matter properties as the localization of the symmetry groups [9]. Multiscale mesoscopic approach in the simulations of biological systems with defects (biological molecules, cell and tissue) are analyzed in the mechanobiology statement to link the qualitative changes of behavior of mesoscopic systems as the specific type of criticality (structural-scaling transitions) [10]. The mesoscopic approach is considered as the "middle-out" paradigm for the description of coherent dynamic behaviors in biological systems and depends upon the choice of leading mechanisms and corresponding mesoscopic thermodynamic (and kinetic) parameters [11,12]. The mechanobiology in the combination with molecular genetic approach could provide the actual direction for objectification of pathological tendencies in the living cells in the case of cancer. The methodologies, numerical and experimental techniques coming from mechanobiological approach and combined with multi-scale signal processing are the ground of the advanced concepts of biological systems evolution related to the role of collective phenomena [13]. "In-situ" study of the cell mechanobiology by the laser interference microscopy (LIM) with the following definition of "meaningful" collective degrees of freedom allows the determination of dynamic stability of biological systems, including tissue, and they qualitative changes with "damage" accumulation with an application to cancer progression.

## Open complex dynamics: DNA and cell transformation

The open states or the bubbles appear as the areas of local denaturation due to the breaking of the H-bonds, that leads to the local opening of the base pairs, Figure 1. The open complexes develop due to the activation of specific subsets of gene expression patterns revealing the intrinsic stochasticity in gene expression, wide range of gene patterns providing different possible phenotypes [11]. The open states provides the DNA transformation as the mechanisms of the cell plasticity and the cell division.

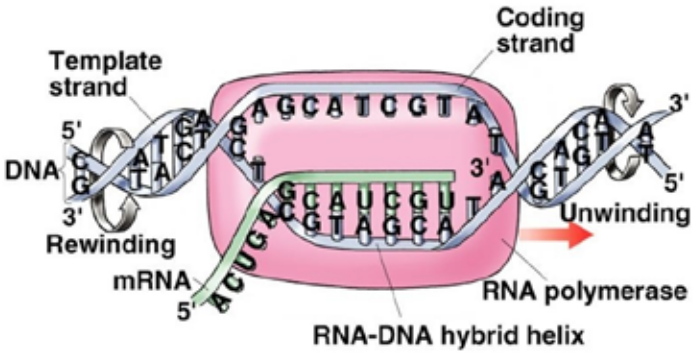



**Figure 1:** DNA structure and open complex formation.

Conformational transitions, the formation of opening states in the processes of the DNA-protein recognition are the consequence of the large-amplitude motions leading to the expression scenario [13]. Mesoscopic description of open complexes assumes the definition of internal "mesoscopic" variables reflecting the coherent behavior of the "minimal molecular set" of the breaking of the H-bonds. This set is close to 50 genes linked to the level of perturbation needed to trigger a multiscale spatial-temporal transition in the system [14]. The ensemble of "minimal set", associated with the open complexes, are responsible for the "structural memory" and realization of the cell plasticity, the cell damage, pattern formation, morphology and the shape fractality [10]. The open complexes as a typical mesoscopic defects are associated with the local unwinding, opening of the double helix. Biological processes involve ensembled molecular components (open complexes) responsible for mesoscopic properties, spatial-temporal multiscale organization according to epigenetic (thermodynamic) landscape in which system's transitions are realized [15]. Thermodynamic aspects of the open states can

be considered as the key problem for the understanding of the DNA functioning. Statistical approach to describe the collective behavior of the DNA distortion modes allowed the formulation of thermodynamics of the open complexes and the evolution equation in the generalized Ginzburg-Landau form reflecting specific type of criticality named as the structural-scaling transition [9]. Structural parameters associated with open complexes and corresponding localized untwisting modes were introduced as the localization of the symmetry group of distortion tensor. The microscopic parameter $S_{ik}$ that is associated with the open complex represents the local distortion spreading on the small segment $2d$ (about 50 base pairs) with the opening normal distortion mode $\vec{B} = B\vec{v}$ to the segment area $\vec{S}_d = S_d\vec{v}$ $(S_d \sim \pi d^2)$ with orientation $\vec{V}$. The mean value of the open complex for uni-axial case $p = \langle s_{ik} \rangle$ corresponds to the minimum of the out-of-equilibrium free energy in the Ginzburg-Landau form

$$F = \tfrac{1}{2} A(1 - \delta/\delta_*) p^2 - \tfrac{1}{4} Bp^4 + \tfrac{1}{6} C(1 - \delta/\delta_c) p^6 - D\sigma p + \chi(\nabla_l p)^2 \quad (1)$$

where $\sigma$ is the external constraint. The value of $\delta$ is the second structural parameter and represents the ratio of two characteristic scales $\delta=(d/l)^3$ the length $d$ *(d~30-50nm)* of the DNA segment of open complex nucleation and the distance l between open complexes. The bifurcation points $\delta_*=1.3$, $\delta_c=1$ separate qualitative different areas of the free energy non-linearity: transition from the uni-modal to the bimodal form at $\delta_*=1.3$ with qualitative changes of the metastability at $\delta_c=1$ from the finite to the infinite depth of the second minima). The $\delta$ - parameter and the critical values $\delta_*=1.3$, $\delta_c=1$ play the role that is similar to the characteristic temperatures in the Ginzburg-Landau theory. The gradient term in (4) describes the non-local interaction in the distortion field; *A,B,C,D* and $\chi$ are the phenomenological parameters.

## The Ginzburg-Landau free energy as the epigenetic landscape

The state space of the cells is related to the 'epigenetic landscape' due to 'causal interactions between genes and their products, which bring the phenotype into being' [11, 16, 17]. An epigenetic landscape can be interpreted as a free energy profile based on the entire ensemble of simultaneous interactions having the number for simple organism the order of $10^{7200}$, that could be reconstructed to introduce on the scale of DNA the meaningful mesoscopic variables. The idea of an 'epigenetic landscape' was proposed by Waddington [18, 19, 20] as the sequence of the valleys with the free energy minima in terms of the variables reflecting the coherent gene-by-gene behavior. These variables can significantly affect nonlinear processes, thus switching cells between distinct phenotypes, by analogy with phase transition in physical systems [21]. Collective behavior of these variables (collective modes) could provide the reprogramming of cell states, that could be not realized by interactions between genes due very high kinetic barriers of the epigenetic landscape [22,23]. The energy profiles could be considered in the framework of the Ginzburg-Landau free energy using the internal variables reflecting the coherent gene-by-gene behavior as the expression unit related to the open complex. The flatness of epigenetic landscape under transition from unimodal to bimodal energy profile is the sign of the critical point, where the sharp increase of the relaxation time corresponds to the development of a global phase transition in the whole gene expression profile (the mRNA expression [14]) with the shift in the frequency profiles to the coherent mRNA expression modes. According to these results the critical dynamics of gene expression shows the singularity induced scaling that is characteristic for out-of-equilibrium critical systems. The open complexes and the mRNA coherent expression modes appear due to the decomposition of the free energy metastability. This metastability has different nature for corresponding ranges of the "governing" structural-scaling parameter transforming the unimodal potentials into bimodal potentials with finite and infinite depth depending on the $\delta$ - range [10].

## Globally convergent dynamics of gene expression as an attractor states

Temporal development in gene-expression collective modes was studied to analyze the expression groups sorted according to normalized root-mean-square-fluctuation (*Nmsf*) and related to an early response (the first 30 min) to growth factors in a MCF-7 breast cancer cell population [22, 23 ]. The averaged values of *Nrmsf can be considered* quantitative as relationship with the mRNA expression. The hill-like distribution function marks a dynamical stable profile of expression

that in turn is defined as coherent expression state for a set of genes. As the consequence, it was assumed that *the nrmsf* can be considered as the order parameter for gene expressions stems with consolidated gene expression scales. It was noted [22] that the *nrmsf* should be related to the physical plasticity of genomic DNA: a higher nrmsf should be associated with a more pliable DNA structure. *Nrmsf* , as the spatial/temporal variance, should correspond to the degree of fluctuation/freedom in statistical thermodynamics. The log-normal law and the power law that are characteristic for the critical scenario, could link chromatin aggregation and gene expression as coordinated transitional behaviors at the chromosome level with coarse grain dynamics. Kinetics for the open complex parameter can be presented by the evolution equations for mentioned structural variables [10]:

$$\frac{dp}{dt} = -\Gamma_p \frac{\partial F}{\partial p}, \qquad \frac{d\delta}{dt} = -\Gamma_\delta \frac{\partial F}{\partial \delta}, \qquad (2)$$

where $\Gamma_p$ and $\Gamma_\delta$ are the kinetic coefficients. The path of the bifurcation point $\delta_*$ leads to penetration into the metastability area and the generation of the collective finite-amplitude distortion modes with breather ($\delta \to \delta_*$) and solitary wave ($\delta_* > \delta > \delta_c$) dynamics (Figure 2).The solitary wave solution has the form $\rho(\zeta)=\rho(x - Vt)$, where the wave amplitude, velocity and the width of the wave front are given by the self-similar solution:

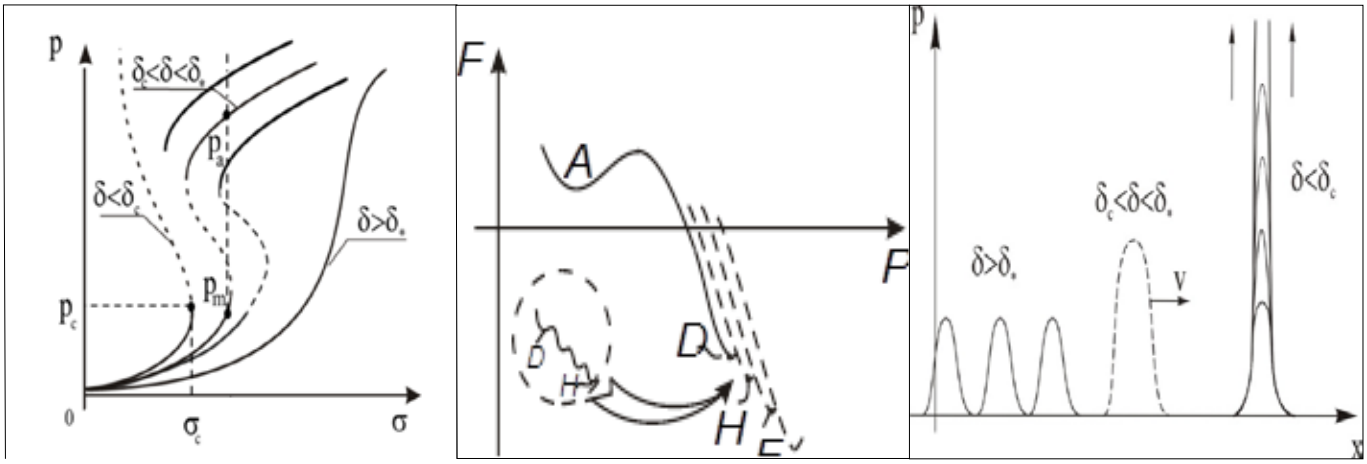
$$p = \frac{1}{2} p_a \left[1 - tahn\zeta\ L_{B^{-1}})\right], \quad L_B = \frac{4}{p_a}\left(2\frac{\chi}{A}\right)^{1/2} \qquad (3)$$

The velocity of solitary wave is $V = \chi A(p_a - p_m)/2\zeta^2$, where $(p_a - p_m)$ is the $p$-jump in the metastability area. A transition through the bifurcation point $\delta_c$ leads to the qualitative change of the double-wall potential into the form with infinite second minimum depth. These qualitative changes in the metastable potential lead to specific open complex dynamics, generation of collective distortion modes with "blow-up" kinetics [9].

Specific type of self-similar solution determines the kinetics of distortion modes for $t \to t_c$ on the set of spatial scales $L_H = kL_c, \quad k = 1,2,...K$ :

$$p(x,t) = \varphi(t)f(\zeta), \quad \zeta = \frac{x}{L_c}, \quad \varphi(t) = \Phi_0\left(1 - \frac{t}{t_c}\right)^{-m} \qquad (4)$$

where $m > o$, $\Phi_o > o$ are the parameters related to the nonlinearity of free energy release in metastability, $L_c$ and $t_c$ are spatial and temporal parameters of "blow-up" self-similar solution. The existence of the set of collective modes and consequent transformation of these modes according to the $\delta$- kinetics from the breather to solitary and blow-up dynamics illustra-




A B C

**Figure 2:** A - Phase diagram of open complex parameter $p$ versus external constraint $\sigma$ ; B – Free energy "epigenetic" landscape; C – Self-similar solutions corresponding to breathers ($S_1$), solitary wave ($S_2$), blow-up ($S_3$) attractors.

tes the globally convergent open complex dynamics in the presence of three different attractors, Figure 2. The value of structural-scaling parameter reflects the current susceptibility of the DNA ensemble to the nucleation and growth of the open complexes in the presence of the DNA constraint and the thermal bath. The kinetics of structural-scaling parameter provides the scenario of the spinodal decomposition in different areas of metastability due to the initiation of the open complex collective modes. These modes represent three types of the self-similar solutions of the evolution equations (2) for the open complex parameter $p$ in characteristic areas of structural-scaling parameter: breathers, autosolitary waves and blow-up dissipative structures. The phase spaces of these attractors are linked to the sets of mentioned collective modes. The sets of collective open complex modes (breathers, solitary and blow-up modes), that could co-exist generally in the DNA double helix structure, represent the collective variables subject the non-linear dynamics of out-of-equilibrium biological system to a few preferred global states. The solutions (3), (4) have the nature of the self-similar intermediate asymptotic that allows the consideration of mentioned collective modes as the eigenfunction spectrum of nonlinear problems [9,24,25] that explains the gene expression as the resonance pattern forming without modification of the DNA's sequences [26]. The "evolution arrow" follows from the kinetics of structural-scaling parameter $\delta$ that realizes the natural tendency for transformation of breather and solitary modes into the blow-up modes. The interpretation of biological regulation within the framework of nonlinear dynamics of open complexes can be linked to the driving factor of the $\delta$ - kinetics providing the evolution pathway though the areas of different attractors to realize the gene expression scenario and the cell evolution [27]. This analysis demonstrates temporal development of gene-expression as global phase transition [28, 29, 30]. The robust organization in cells, when the expression mechanism of thousands of genes are coordinated by a few key transcription factors can be linked to attractor states in the gene-expression landscape [31, 32, 33] providing the 'phenotypic states'. The attractor concept envisages the system as evolving toward a preferred (minimal energy) state due to appearance of 'globally convergent' solutions [34, 35, 36, 37]. These solutions attract the system dynamics in the presence of stochastic fluctuations related to a gene-by-gene interaction. Attractor states are realized in the presence of a rugged non-equilibrium free energy landscape in the terms of mentioned variables as the generalization of the Landau theory of the phase transitions [38, 39].

The generalization of the Landau theory for thermodynamics of the DNA ensemble was developed in [10] using the "effective field" approach by Leontovich [40] for the statistical thermodynamics of the out-of-equilibrium "slow driven systems". In the presence of the free energy metastability (1) the $\boldsymbol{\delta}$ kinetics plays the role of the "intelligent agents" (Maxwell's demons) that used to drive the system due to the metastability decomposition [41]. This scenario could clarify a fundamental question concerning the problem of cell dynamics controlling genome-wide expression - What is the 'driving force' that attracts the entire system toward a few preferred global states, thus making the genome act as a single integrated system? [42, 43].

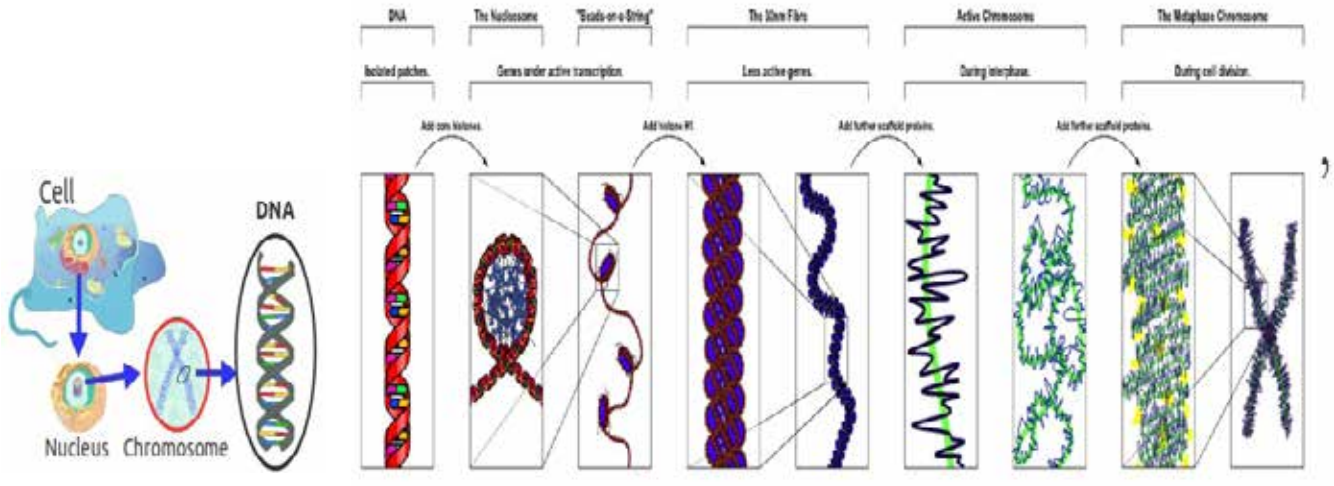



**Figure 3:** Cell structure and DNA transformation: A - cell structure, B - histone topology, C - histone package, D - cell division [https://en.wikipedia.org/wiki/Chromatin#/media/File:Chromatin_Structures.png].

The sequence of generation of these modes can be used for the explanation of the DNA transformation associated with the cell plasticity and the cell division, Figure 3 [44]. The front of autosolitary wave mode has the sharp curvature $\chi = (\partial p / \partial s)_f$ ($s$ is the longitudinal DNA axis) separating the portions of DNA strands with breather and autosolitary wave dynamics that can be associated naturally with transition to the histon topology of the DNA strand (Figure 3a) in the condition of continuously increasing curvature along the $s$-axis under $\delta \to \delta_c$.

The solitary wave dynamics of the open complexes provides the active transcription action caused by the DNA plasticity and the meeting point (centromere) of two strands with inverse curvature can be considered as analogue of "sitting dislocation" with the high energy barrier. Double helix becomes more condensed due to the histon topology dynamics. Numerous scenario of the histone topology lead to the DNA package ("histone lattice", Figure 3b, c) with less active genes and new correlation in the open complex ensemble providing the path of the $\delta_c$ critical point. It leads to the excitation of blow-up open complex dynamics inside of histone topology, rupture of the DNA strands and the formation of small $p$ chromosome arms (Figure 3d) as the precursor of the cell division [45]. The chromosome consists of the condensed structure of the DNA double-helix (10,000 times than in the normal DNA double-strand). The compact form of chromosomes has four arm structure as a pair of sister strands attached by each other at the centromere. The long $q$ - chromosome arms appear due to the tripping of some labile histon stitching (centromere) with low energy barrier after the rupture of the DNA strands in histones. Finally, due to this act of replication a chromosome consists of two sister chromatids. The DNA transformation occurs in order for the proper separation of the genetic material between daughter cells under the cell cycle (Figure 3d). Similar scenario was discussed in [23] in early response to growth factors in a MCF-7 breast cancer cell population to characterize the distinct expression domains: static, transit and dynamic domains according to the degree of temporal variation in expression.

## Laser Interference Microscopy of cell dynamics: ductile-brittle transition in CSK structure as cancer precursor

Open complexes as mesoscopic defects are responsible for two mechanisms in condensed matter associated with plasticity and damage-failure transition [44]. This is characteristic for the cells under the influence of physiological and pathological conditions through the qualitative transformation in the CSK structure. This transformation occurs as the sequence of critical events due to the path of critical points $\delta_*$ , $\delta_c$ in the attractors space. The breathers and auto-solitary modes are responsible for the cell plasticity, which indicates mechanobiological properties reflecting the natural CSK transformation and the transition to the third attractor with the phase space related to the blow-up collective modes providing the cell differentiation dynamics. This scenario of collective modes transformation reflects the duality of the open complex dynamics in the ductile-brittle CSK cycle. The structural-scaling transition events occur in the presence of the $\delta$ kinetics as the spinodal decomposition of the metastable free energy release caused by the dynamics of the open complex parameter considering the open complexes as mesodefects ensemble. The qualitative different robust statistics (the log-normal and the power statistics), is characteristic for ductile and brittle behaviors can be used for the identification of the normal and cancerous CSK states [46]. The CSK cycle of the normal cells is associated with spatial-temporal distributed collective modes of open complexes, which follow to the multifractal dynamics associated with mentioned attractor types and the log-normal statistics of finite amplitude open complex fluctuations. This stochastic multiplicativity of open complex dynamics from the breathers to auto-solitary and blow-up modes provides the evolutionary "ductile" stability of the normal cells up to the stage of the cell division. The qualitative different scenario is observed for the cancer cells with pronounced "brittle" dynamics leading to the power law statistics that is characteristic for self-organized fragmentation [47] and anomalous cell fragility for the arbitrary constraint. The cancer cell behavior can be linked to the activity of the third attractor $\delta \to \delta_c$ under the initiation of the convergent multiscale "blow-up" dissipative structures subordinated entirely the CSK dynamics. The study of the cell dynamics as the optical thickness fluctuations were conducted at

the Perm Federal Research Center of the Urals Branch of the Russian Academy of Sciences using the MIM-340 Laser Interference Microscope. Study of the nonlinear CSK dynamics using the data of the Laser Interference Microscopy (LIM) is the impact opportunity for the objectification of the cell states and cytological cancer diagnosis analyzing the time series of phase thickness fluctuations (in the "cross-sections" of the nucleus, the nucleolus, cytoplasm). The main advantages of the MIM-340 laser microscope are high resolution in the lateral plane (10-100 nm) and vertical (0.3 nm), the frequency recording of phase images (33 Hz) and the presence of an object table that allows the positioning the object (positioning accuracy is 150 nm) [48].

The results of typical measurements are presented in Figure 4 [49, 50, 51]. In these figures, the adhesion region of living cells (green color), a thin layer of cytoplasm (yellow color), nuclei (red color) and nucleolus (dark red color) are clearly visualized. High spatial and temporal resolution of the LIM pattern allowed the analysis of dynamic processes in living cells using LIM track diagrams in different cell cross-sections (Figure 5, 6). Optically dense area corresponds to the cell nuclei. These data were obtained by laser interference microscopy on 450 non-cancerous and cancer cells.

Wavelet transform maximum modulus method (WTMM) [52,53,54] was used to analyze the LIM data (Figure 5, 6) and to establish the correlation between the finite amplitude temporal fluctuation of the phase thickness (associated with the cell thickness in the nucleus cross-sections) and qualitative different CSK dynamics providing the cell plasticity and fragility. 1D realization of the WTMM method was used to process the LIM data, that allowed one to get the singularity spectrum $f(\alpha)$ corresponding to the mono- and multifractal dynamics (Figure 7) [27,50,51]. Both scenarios

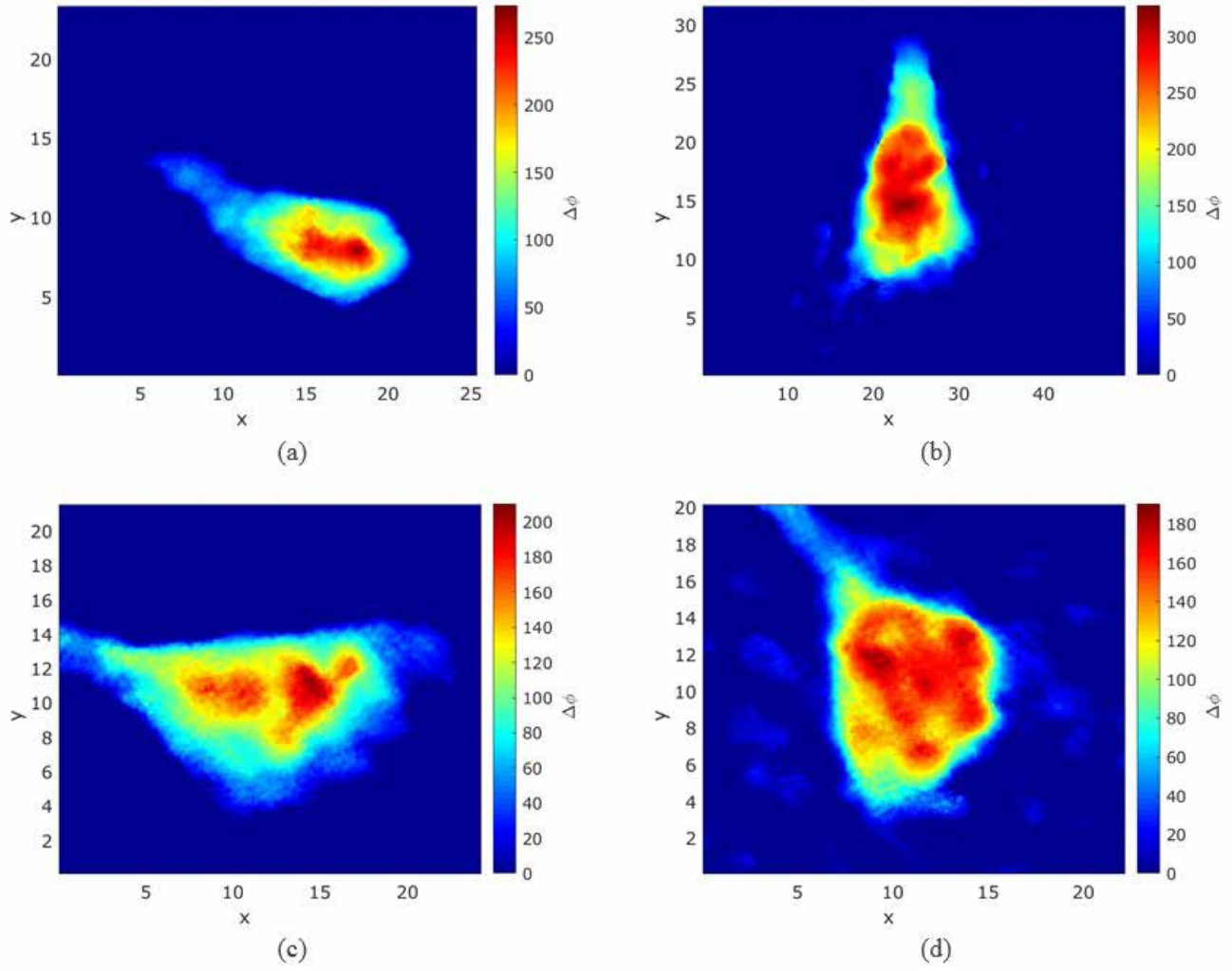



**Figure 4:** Phase images of the cells: (a) MCF-7, (b) HEK 293, (c) MCF-7, (d) HCT116. Units of measurements: x, y – mkm; ΔΦ – nm.

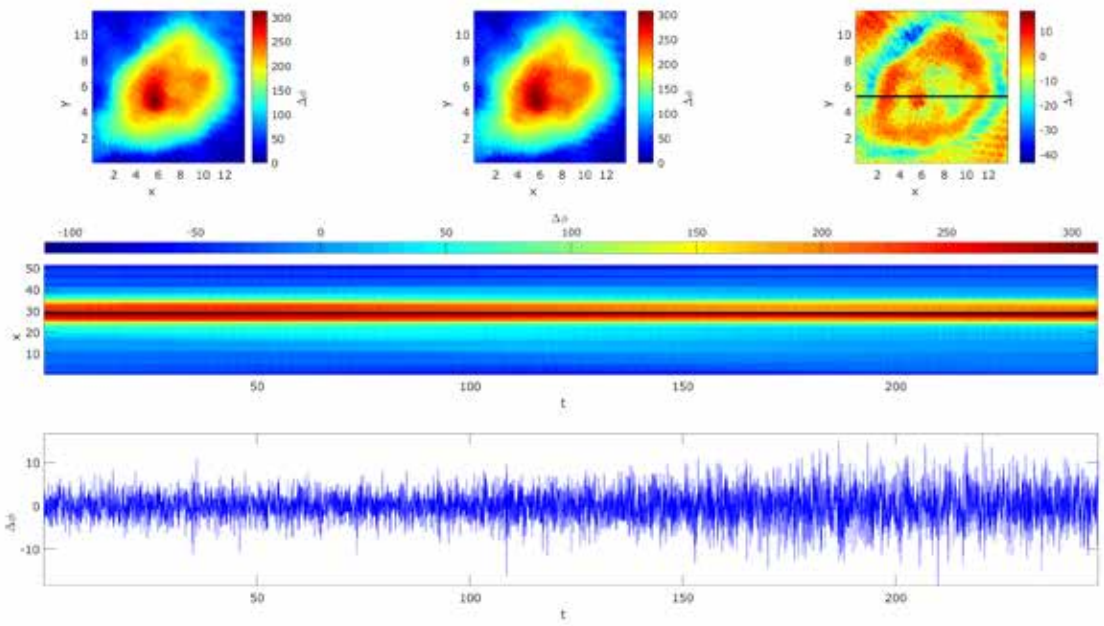


**Figure 5:** Typical phase images registered at different time, difference frame, track diagram and 1D signal of the normal human breast epithelial cell MCF-10A. Units of measurements: x, y — mkm ΔΦ — nm.

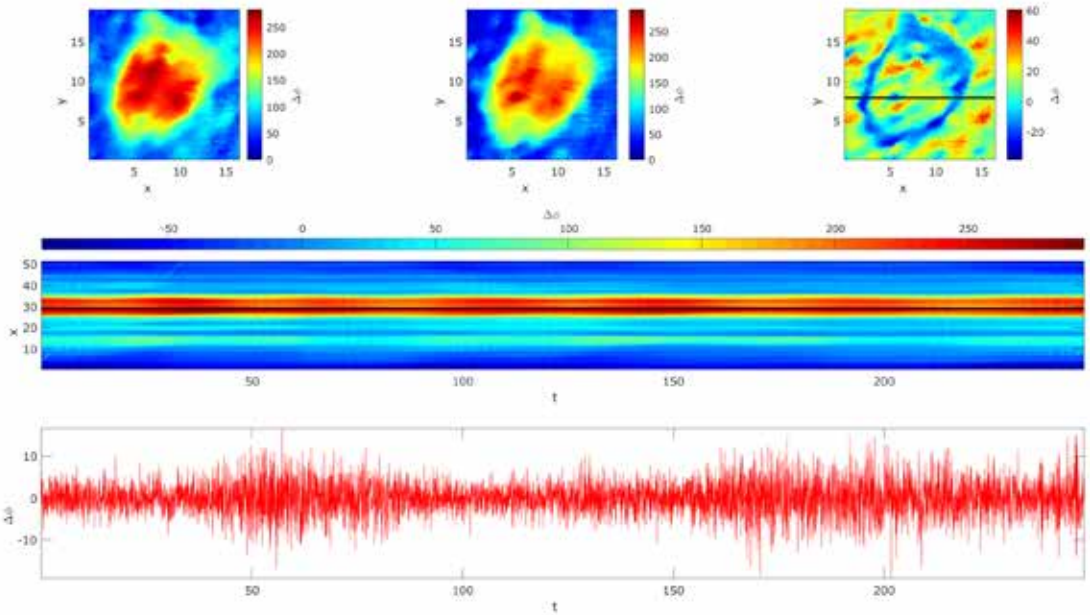


**Figure 6:** Typical phase images registered at different time, difference frame, track diagram and 1D signal of the cancer human breast epithelial cell MCF-7. Units of measurements: x, y — mkm ΔΦ — nm.

display fat-tail distributions (log-normal and power laws) that are characteristic for the critical systems dynamics subject to the collective modes providing the spinodal decomposition of free energy metastability (free energy release) [46,47, 54]. The log-normal multiplicative statistics reflects the influence on the CSK dynamics the mentioned types of collective modes in the course of the normal cell evolution as the structural-scaling transition in the open complex ensemble. The power law statistics reflects the cell fragility that is the

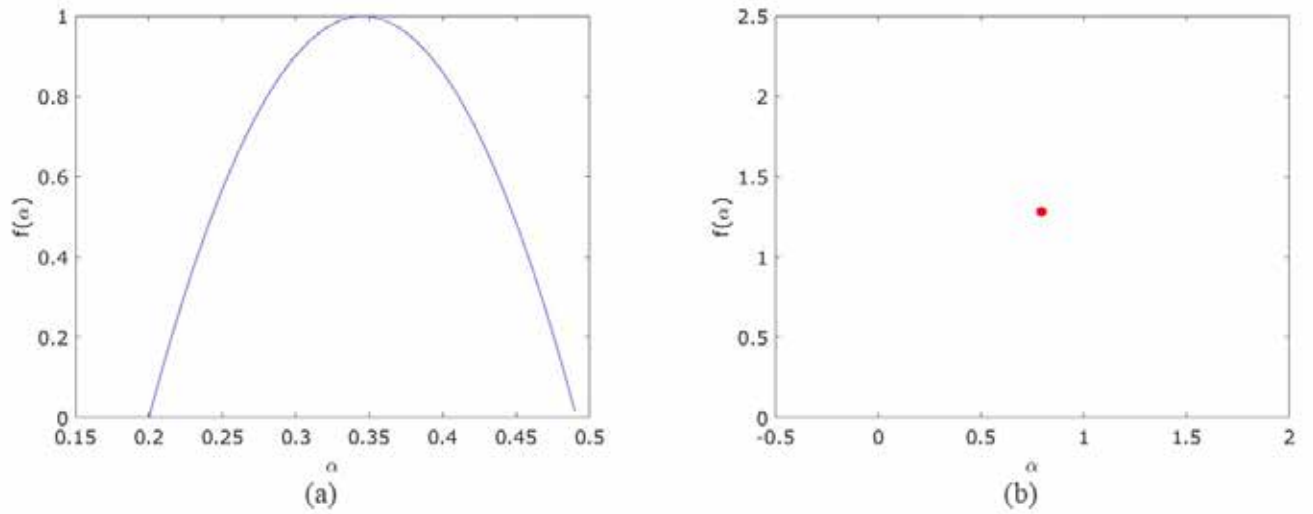



**Figure 7:** Typical multifractal spectra of LIM data: "blue" is the normal breast cell, "red" is the cancer breast cell (carcinoma).

consequence of pathological structural changes (corresponding to the range of structural-scaling parameter $\delta<\delta_c$ ) leading to the subjection of the CSK dynamics to multiscale blow-up modes. These results allow the conclusion concerning the duality of multiscale open complex dynamics responsible for the evolutionary cell transformation as the ductile structural-scaling transition in the cell division and the brittle dynamics leading to the cell fragility and the cancer development.

## Entropy: convergent and divergent cell dynamics

The structural-scaling transitions in nonlinear out-of-equilibrium systems are characterized by dynamically unstable motions in the terms of an exponential divergence on initially adjacent trajectories [55]. The measure of exponential divergence is the Kolmogorov-Sinai entropy (or the so-called K-entropy). The K-entropy is related to the average rate of divergence of initially adjacent trajectories $x_i(k)$ and hence the Lyapunov exponents $\lambda_i$

$$K = \sum_i \lambda_i , \quad \lambda_i > 0 \tag{5}$$

Instead (5) the K-entropy can be introduced as the characteristics of dynamic divergence $D(k)$ of the trajectories $x_i(k)$ [56]

$$K = \lim\left[\frac{1}{n}\sum_{i=1}^{n} \ln \frac{D(k)}{D(0)}\right], \quad D(k) = \left[x_1(k) - x_2(k)\right] \tag{6}$$

as the average time of the current trajectories divergence $k$ to the initial one. If we introduce the corresponding probability distribution of the trajectory divergence at the point in time $t$

$$f(D, t), \quad \int f(D, t)dD = 1,$$

the entropy of the system can be defined as

$$S(t) = \int 1n\, f(D, t) f(D, t)dD\,. \tag{7}$$

The distribution $f(D, t)$ can be used to introduce two characteristics of the system dynamics: an average divergence at the point in time t and the effective "volume" of the divergence

$$D(t) = \int D f(D,t)dD, \quad \Delta D(t) = \frac{1}{f(D,t)} \tag{8}$$

With small deviation of $\Delta D = D - \overline{D}$ from the average value $\overline{D}$, the entropy (7) can be determined by the analogue of the Boltzmann formula

$$S(t) = 1n\, \Delta D(t). \tag{9}$$

Using (6) and (9) we can find two equivalent relations:

$$S(t) - S(t_0) = \ln \frac{\Delta D(t)}{\Delta D(t_0)}, \quad \Delta D(t) = \Delta D(t_0)\exp\left(S(t) - S(t_0)\right), \tag{10}$$

The latter definition can be considered as the analogue of the uncertainty relation: the large trajectory divergence has the higher entropy. Using (10) the statistical analogue of the *K*-entropy can be introduced as the average rate of the entropy change in a time $(t-t_o)$

$$K_{stat} = \frac{S(t) - S(t_0)}{t - t_0} = \frac{1}{t - t_0} \ln \frac{\Delta D(t)}{\Delta D(t_0)} \qquad (11)$$

It follows from (11) that $k_{stat}$ determines the entropy production averaged over a finite time interval. Local entropy change reads as the entropy balance equation

$$\frac{dS}{dt} = \frac{d}{dt} \ln \Delta D(t) = \sigma(t) \qquad (12)$$

It is seen that the entropy production has not fixed sign and increases or decreases as the average trajectory divergence.

Development of collective open complex modes (breathers, auto-solitary, blow-up) is the consequence of the local instability due to the subordination of the cell dynamics to the set of the self-similar solutions. Singular nature of the open complex as defects allows the consideration of open complex collective modes as the string objects [25] with the interactions responsible for dramatic change in the symmetry properties and the transformation pattern in Figure 3. The first symmetry breaking occurs in the course of mutual excitation of the breathers as the expression template related to the epigenetic landscape. The scale renormalization in the breather ensemble provides the long range correlation in more coarser epigenetic landscape, the second symmetry breaking due to the metastabilty decomposition in the range $\delta_* > \delta > \delta_c$ with the generation of auto-solitary modes and realization of the transcriptional dynamics, the histone topology and the histone package. The scaling related to the auto-solitary modes leads to the second bifurcation at $\delta = \delta_c$ eading to the generation of blow-up open complex modes providing the cell division scenario. This pathway of the normal cell evolution occurs in the presence of three attractors excited consequently due to the structural-scaling transition and the convergence of trajectories related to the open complex dynamics of mentioned collective modes. As the consequence, this dynamics is characterized by the minimum of the entropy production. The multiply "resonance" excitation of the third blow-up attractor localized on the set of the fundamental lengths $L_k$ is characteristic for the cancer cells revealing the divergent dynamics and the spontaneous fragility. The resonance excitation of the multiply blow-up modes is typical for the epigenetic landscape with pronounced coarsening of the metastable potential in the presence of the infinite second minima. The mutual resonance blow-up kinetics of the open complexes leads to the spontaneous DNA fragmentation and low viscosity that is observed for the strongly coupled systems [25]. The low viscosity could provide the anomalous proliferation as the metastasis mechanism.

## Conclusion

The mechanobiology of living cells is associated with the cytoskeleton (CSK) dynamic network (filamentous proteins, actin filaments, microtubules, intermediate filaments) revealing the fundamental property of the cells qualified as the cell plasticity and the cell damage. Plasticity is the phenomenon inherently linked to the collective behavior of mesodefects in the "biological crystals" (the open complexes in the DNA double helix) and provides the unique mechanism of the defects induced momentum transfer and the structural memory as the expression scenario. The cell plasticity can be considered as the leading mechanism providing the vital CSK properties, including the DNA transformation, expression dynamics, the cell division. Open complex mechanisms of plasticity and fragility are analyzed as specific type of critical phenomena in condensed matter with mesodefects – the structural-scaling transition. The expression dynamics, the self-organization of DNA and the cells are linked to the collective modes of the open complexes (breathers, auto-solitary waves, blow-up dissipative structures) responsible for the configuration mobility of the CSK structure and the cell division. The gene expression and the cell division being the vitality ground have natural links to the defects behavior and provides the evolutionary meaningful mechanisms of self-organization for normal cell dynamics or pathological scenario of defects induced fragility as the cancer precursor. Study of the nonlinear CSK dynamics was conducted analyzing the time series of phase thickness fluctuations after the Laser Interference Microscopy in the cell "cross-sections" containing the nucleus, the

nucleolus, cytoplasm. The application of the WTMM method allowed the demonstration of the links of temporal correlations of finite-amplitude phase thickness fluctuation, dynamics of collective modes of open complexes and qualitative different CSK dynamics that are characteristic for the cell plasticity and fragility. The phase thickness fluctuations display the fat-tail distributions, the log-normal and the power laws, with multi- and monofractal singularity spectrum. The multifractal singularity spectrum in the case of the cell plasticity reflects the temporal sequences of the phase thickness fluctuation in the presence of mentioned open complex "singular" collective modes (breathers, auto-solitary, blow-up). The monofractal singularity spectrum and the power law of the phase thickness fluctuation are the consequence of the shifting of the CSK dynamics into the area of the attractor with the blow-up open complex dynamics, that leads to the spontaneous CSK fragmentation (the cell fragility). The open complex dynamics, which follows to the structural-scaling transition, allows the interpretation of the normal and cancer cell evolution scenario. The structural CSK susceptibility to both scenario is given by the values of structural-scaling parameter charactering the nonlinearity (metastability) of the epigenetic landscape and corresponding open complex kinetics. The pathological changes of the CSK structure in the presence of the "monofractal" blow-up open complex dynamics leads to the cancer progression.

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# Does Evolution Have a Target Morphology?

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**Abstract**

We suggest here that evolutionary and developmental processes differ primarily in scale. Both evolution and development are dynamical processes subject to bottom-up and top-down constraints, and both can be viewed as search processes in rugged landscapes with multiple attractors. An important aspect of regulative development and regeneration is the ability of the system to reach the same anatomical configuration from different starting points and despite perturbations – a robustness toward a specific "target morphology" as the set point of a homeostatic cycle. We propose that evolution can be viewed as a developmental process of life as a whole, and that principles of regulative development and regeneration can, therefore, be expected to be active at much larger spatio-temporal scales: the major evolutionary transitions, including endosymbiosis, multicellularity, and the emergence of social groups, can be regarded as features of a "target morphology" of organismal phylogeny that biological evolution can be expected to replicate starting from a wide range of initial states and under a wide range of environmental conditions. Each of these transitions, like anatomical homeostasis on the ontogenetic timescale, can be regarded as a solution to a single problem, the reduction of environmental uncertainty, as it is manifested at progressively larger scales.

**Keywords:** free-energy principle, major evolutionary transitions, prediction, scale-free biology

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> *When told that people say that it looked as if the sun went round the Earth, Wittgenstein asked, "what would it have looked like if it had looked as if the Earth turned on its axis?"* — Anscomb (1959, p. 151)

## Introduction

In multicellular organisms, development generally proceeds from a single-celled zygote through a succession of embryonic or immature forms until some stable, species- or variety-typical, adult morphology is achieved. In competent organisms, including starfish, planaria, salamanders, and deer, lost limbs, damaged organs, or even the entire body may be regenerated until this same stable, species- or variety-typical, adult morphology is restored (Birnbaum and Sánchez Alvarado, 2008). Thus, regeneration and regulative development are individual cases of a more general biological process: anatomical homeostasis, which is able to robustly achieve a specific large-scale geometry despite drastic perturbations such as amputation and from different starting conditions. For example, genomically-normal tadpoles altered to have their craniofacial organs in the wrong locations largely become normal frogs (Vandenberg, Adams and Levin, 2012). Similarly, some embryos can be cut in half, fused with others, or implanted with aggressive cancer cells, and still result in perfectly normal bodies (Mintz and Illmensee, 1975). These kinds of examples illustrate how anatomical homeostasis can reach the same large-scale anatomy

from diverse starting conditions and other perturbations (Pezzulo and Levin, 2016). We will refer to such a stable endpoint of development or regeneration, averaged over species- or variety-typical outcomes, as the "target morphology" of the species or variety (Levin, 2011). In regulative development and regeneration, the target morphology is operationally defined as that shape which, once achieved, causes further growth and remodeling to stop ( Figure 1). Individual organisms may alter their size once their target morphology is reached, but do not significantly alter their internal organization or external shape. Significant departures from target morphology, e.g. different numbers of digits or unusual craniofacial morphologies, are generally considered pathological, even if they may be long-term viable. Such departures must be possible, however, for morphology to evolve under natural selection; all speciation events and morphological innovations initially represented a "birth defect" relative to the parent lineage.

The concept of target morphology is clearly applicable below the scale of the whole, multicellular organism. Tissues and some organs have the capacity to restore structure at their appropriate level. Individual cells, e.g. muscle cells or neurons, have characteristic morphologies that are the typical endpoints of differentiation for cells of that type in that organism or even across a major phylogenetic lineage. Metabolic cycles and gene regulatory networks have the capacity to retain their dynamics in the face of environmental changes and some mutations. One can even view the tertiary structures of macromolecules under typical physiological conditions (and short timescales) as target morphologies. As at the whole-organism scale, significant departures from target morphology by cells or macromolecules are ge-

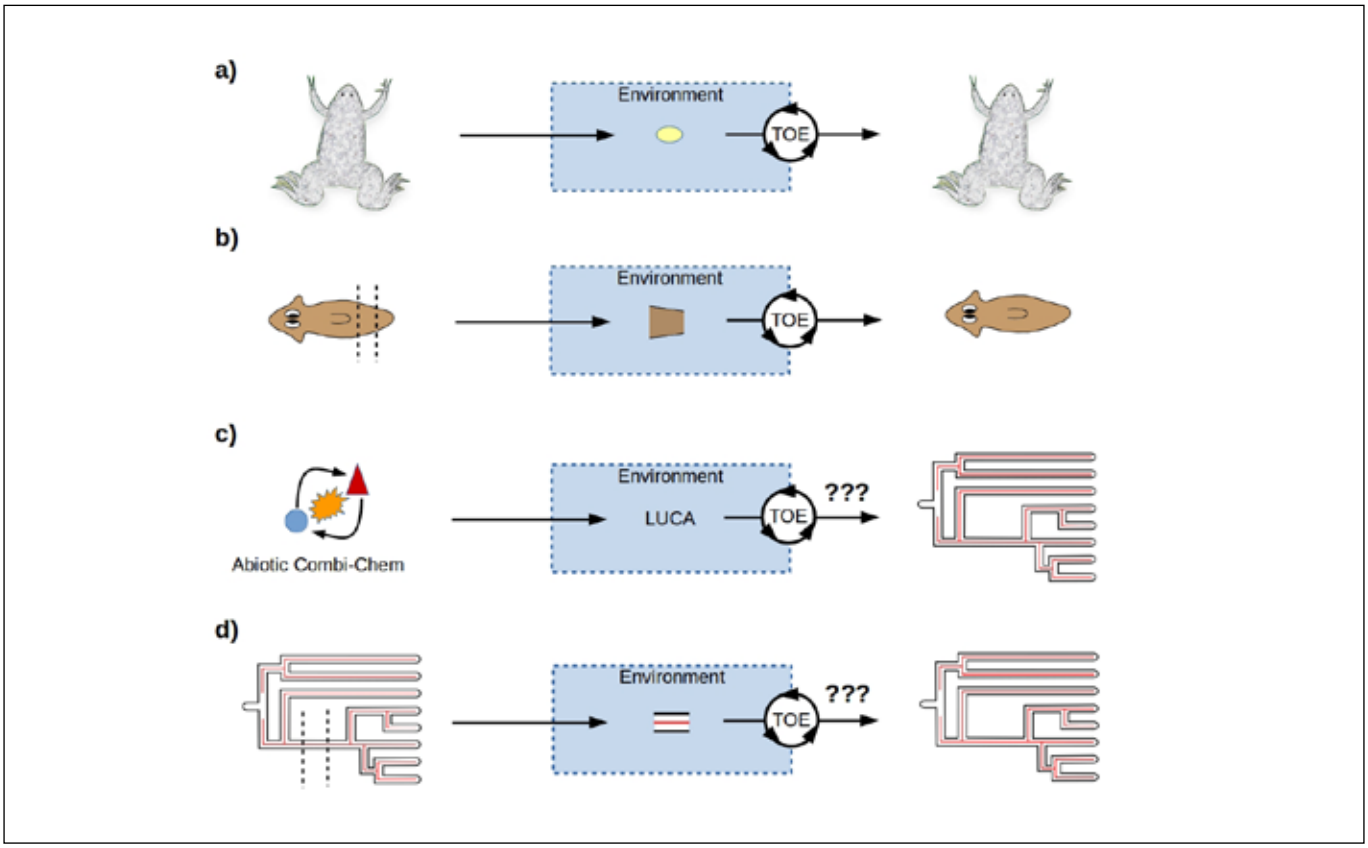


**Figure 1:** The concept of target morphology. a) In sexually-reproducing organisms such as *X. laevis*, a single zygotic cell placed in an appropriate environment develops into an adult that replicates the morphology of the parent(s). At the cellular level, development involves a test-operate-exit (TOE) cycle that finds and fixes errors, thus maintaining the integrity of the process. b) A fragment excised from a regeneration-competent animal such as the planarian *D. japonica* and placed in an appropriate environment regenerates cells and tissues to replicate the adult form. c) Can the "shape" of the phylogeny of life since the last universal common ancestor (LUCA), including its major features such as endosymbiosis, multicellularity, and social organization be regarded as a "target morphology" of evolution? d) If some sample of the phylogeny of life since LUCA were to be placed in an appropriate planetary environment, would it "regenerate" a phylogenetic outcome with the same general characteristics?

nerally considered pathological, and indeed generally disrupt "normal" function. But what about higher levels of organization and temporal scales *larger* than the lifetime of the individual organism?

Here, we ask whether the concept of target morphology could be usefully applied above the spatio-temporal scale characteristic of an individual organism. Can the typical sizes and organizations of ant hills, wolf packs, or human social groups, for example, be considered "targets" of inter-organism social interactions? Can climax communities be considered targets of inter-population interactions that constitute ecosystem-scale succession? Can biosphere-scale evolution, in particular, be considered to have a target morphology? What constraints did the structures of the last universal common ancestor (LUCA) and the environment in which it lived place on the evolutionary process that produced life as we know it? What constraints do molecular and cell biology place on evolution, and conversely, what aspects of information-processing at different scales facilitate robustness toward specific outcomes despite noise and uncertainty at lower scales? If the "tape" of Terrestrial evolution could, in Gould's (1989) metaphor, be run again, could we expect to see major transitions such as the rise of eukaryotes or multicellularity (Maynard Smith and Szathmáry, 1995; Szathmáry, 2015) replicated? Could we expect to see a "cognitive niche" (Pinker, 2010) occupied by an omnivorous, social generalist?

The idea that evolution might, like organismal development, be directed toward some target is obviously controversial and often rejected out of hand. Evolution must, after all, cope with unpredictable events such as bolide impacts causing mass extinctions (e.g. Schulte et al., 2010). It is important, however, to examine limitations on our observations which shape our intuitions on such questions. As Wittgenstein put it on being told that people say that it looked as if the sun went round the Earth, "what would it have looked like if it had looked as if the Earth turned on its axis?" (Anscomb, 1959, p. 151).

Consider an observer gathering data at the individual cell level during embryogenesis. Imagine this observer had never heard of development and did not already know the fact, so obvious to us that we rarely question it, that it always ends up making the same large-scale anatomy. Seeing the amount of stochastic behavior, frequent failures, diversity of even genetically identical cells' behaviors, and variability in biochemical and biomechanical properties, would they be able to infer that despite all the noise, there is a single morphological attractor at which all of this messy activity will inevitably arrive, even if significantly perturbed? Would the #1 fact of embryogenesis – its invariant outcome – be apparent at a small scale of spatio-temporal observations? No such small-scale observations, we suggest, would reveal this emergent property, just as small-scale observations of gas molecules in the interior of a large but unobserved container would not reveal the gas pressure measurable outside the container or the inevitable large-scale outcomes of thermodynamic manipulations. Could it be that our observations of individual biological species and small – even with paleontology – numbers of generations are likewise too constrained by their small scale to make plausible the idea that evolutionary processes may also operate over a space with very strong attractors, to which populations converge despite the stochastic events at the mutation and selection levels? Are major transitions such as multicellularity, in particular, attractors in the landscape of evolution?

This controversy goes to the heart of evolutionary theory: it concerns whether the core evolutionary processes of variation and selection are mechanistically coupled. In organismal development, these processes clearly are coupled: only specific cell types are produced at any given stage, and these cells assemble into specific micro-environments into which later-developing cells are born or migrate and must afterwards function. Variation and selection are, in contrast, uncoupled in Darwinian evolution; while Darwin (1859) rejected "chance" variation in favor of unknown causes (e.g. p. 131), these causes are presented as prior to and hence independent of selection. The Neo-Darwinian movement of the mid-20th century largely discredited "orthogenetic" conceptions of evolutionary variation as somehow directed or constrained (see Ulett, 2014 for a historical review), replacing them with a conception of variation as strictly random. Selection, in this case, "does all the work" in evolution; as Monod (1972) puts it, "from a source of noise natural selection alone and unaided [draws] all the music of the biosphere" (p. 118). While the past five decades have yielded an increasingly mechanistic and non-random understanding of variation at the level of the genome (e.g. Kitts et al., 1982; Lichten and Golsman, 1995; Hall et al., 1999; Foster, 2000; Callinan et al., 2005; Lemons and McGinnis, 2006; Mitchell et al., 2009; Stern and Orgogozo, 2009; Uller et al., 2018), if these mechanisms are both mutually uncorrelated

and uncorrelated with later-acting selection, variation remains effectively random and evolutionary history remains purely contingent, producing diversity at all scales with selection as the sole constraint (McShea, 1994; McShea and Brandon, 2010). This Neo-Darwinian conclusion has its prominent critics, e.g. Conway-Morris (2003; 2010) and Orgogozo (2015) who point out that it cannot adequately explain observed cases of convergent evolution, but it nonetheless remains the dominant view within evolutionary biology. Astro/exobiologists and artificial life researchers, in contrast, make it their business to attempt predictions of how evolution might proceed from various initial conditions (e.g. DesMarais and Walter, 1999; Chyba and Hand, 2005; Kaltenegger, 2017; Lenton et al., 2018). The operational assumption in these fields is that evolution is not *purely* contingent, but can rather be characterized as a dynamical system governed by both bottom-up (e.g. variation) and top-down (e.g. selection) constraints. Given reasonable assumptions about the dynamics and the constraints, the possibility of some level of predictability is simply taken for granted.

Here we introduce two lines of argument for a deep mechanistic coupling between variation and selection, and hence for a view of evolutionary processes as search processes in a space with invariant attractors. The first is that the processes we characterize as "evolution" and "development" differ primarily in scale. Indeed as pointed out previously (Hermida, 2016; Fields and Levin, 2018; Mariscal and Doolittle, 2018), all of life since LUCA can be viewed as a single, continuous cell lineage; hence evolution can be viewed as a developmental process with LUCA as the "zygotic" founder cell. Viewing evolution as a developmental process naturally raises the questions of what variants are possible at each stage, and of how such variants might be expected to survive under selective constraints largely imposed by other organisms (Fields and Levin, 2020a). The second line of argument builds on Friston's (2013) observation that all living systems face a thermodynamic requirement to minimize the variational free energy (VFE) – effectively, the unpredictability – of their environments. We have argued previously that the transition to multicellularity can be viewed as a VFE minimization strategy (Fields and Levin, 2019). Briefly, reproductive (i.e. stem) cells can be expected to produce "bodies" comprising non-reproductive (i.e. somatic) progeny as protection against sufficiently-challenging environments. If the transition to multicellularity can be understood in effectively thermodynamic terms, as a general response to selective pressures that works independently of minor details or contingencies, might not the other major transitions be similarly understandable? Could we not expect any evolutionary "tape" run long enough to generate social multicellulars and a cognitive niche? Could we not expect that morphogenetic mechanisms developed at the cellular scale, e.g. developmental bioelectricity (Levin and Martyniuk, 2018), would be co-opted into whole-organism scale mechanisms, e.g. bioelectric mechanisms for controlling organism-scale behavior such as nervous systems (Fields, Bischof and Levin, 2020)?

In what follows, we first review target morphologies at the molecular (§2.1), organismal (§2.2) and ecosystem (§2.3) scales. In each case, we consider where and how the information specifying the target morphology is stored and how this information is accessed and expressed as the target morphology is being generated. We discuss, in each case, how top-down constraints arising at larger scales regulate processes at the scale of interest. We then address the question posed in our title, focusing specifically on the major transitions to cellularity (§3.1), endosymbiosis (§3.2), multicellularity (§3.3), social groups (§3.4), and finally the emergence of a cognitive niche (§3.5). In contrast to the standard, multilevel-evolution view that these transitions represent selection for increased cooperation (Buss, 1987; Maynard Smith and Szathmáry, 1995; Michod, 1999; Szathmáry, 2015), we suggest that in each transition cooperative structures develop once a toolkit of pre-adapted communication and regulation capabilities has been assembled. In line with our previous model of multicellularity (Fields and Levin, 2019), we suggest that the driver of this process is in every case VFE minimization, i.e. that the major transitions are thermodynamic attractors. We outline, in §4, a new "picture" of evolution as a sequence of phase transitions that each incorporate smaller-scale systems into larger-scale organizations. As the larger-scale organizations are, in every case, micro-environments that both provide new resources and impose new selective constraints, the products of this evolutionary process are multi-scale, heterogeneous communities, i.e. holobionts (Bordenstein and Theis,

2015). We conclude in §5 by suggesting experimental approaches that could test these ideas.

## 1. Target morphologies: from macromolecules to ecosystems

Toward the end of *Wonderful Life* (1989), Gould speculates that had the early chordate *Pikaia* not survived the Middle Cambrian, "we are wiped out of future history – all of us, from shark to robin to orangutan" (p. 323). A tiny change in evolutionary history, in other words, could produce an entirely different later outcome. With no ability to rewind the "tape" of evolution of life on Earth and run it again, we are left with "just history" to explain the landscape of organisms and their relations that we see around us.

Contrast this with organismal development, where tiny changes occasionally produce informative monstrosities, but typically result in either no difference at all, minor variants, or lethality. We know this because we have, in fact, observed the tape run many times. Given only a genome, a zygote, or even an early embryo and required to employ first principles, not comparative methods, we might be no better off in predicting the adult form than we would be trying to predict humans from *Pikaia*. Yet developmental biology has the goal of achieving an understanding that supports such predictability. Why, as Conway-Morris (2010) asks, has evolutionary biology seemingly abandoned that goal?

It is perhaps useful to compare the situation in evolutionary biology to that in physical cosmology, another setting in which "rerunning the tape" is not possible. Like the evolution of life, the evolution of the physical universe is characterized by a sequence of major transitions (e.g. from pure radiation to elementary particles to atoms) that progressively generate a hierarchy of scale-specific structures (Hawking, 1988; Smolin, 1997). Theoretical models of this process postulate, at each scale, local physical interactions constrained by global boundary conditions. Central to these models are formal notions of complexity that capture organizational structure instead of, or in addition to, diversity of form (Lineweaver, Davies and Ruse, 2013). Such models have generated significant empirical predictions, many of which have been extensively tested (e.g. Cyburt et al., 2016). The success of such models suggests that a similar, multiscale approach may be useful for studying evolution. They demonstrate, for example, that "noise" on one scale may resolve into predictability at a smaller scale, or self-organize into predictability, in response to overlying constraints, at a larger scale.

In the sections that follow, we consider three scales at which biological processes uncontroversially generate target morphologies. All of these processes are obviously evolved processes, and the details of the morphologies that they produce have obviously been shaped by selection. The claim that they are evolved processes, however, tells us nothing about how they work. Understanding how they work, and indeed, understanding how selection might have shaped them, requires understanding them as combinations of underlying dynamics and overlying constraints, as emphasized by Polanyi (1968), Rosen (1986), Kaufmann (1993), McShea (2016), and many others. In such systems, the dynamics may be governed by large-scale attractors – i.e. target morphologies – that are undetectable by small-scale, local observations but are obvious when the system is observed at the scale of its overlying constraints. In none of the cases considered here has such an understanding of the coupling between dynamics and constraints been fully achieved, and it remains unknown whether it can be fully achieved. It has, however, in every case been partially achieved, and even this partial achievement yields substantial predictive power.

### 1.1 Macromolecular tertiary structure

Both RNAs and proteins fold into complex, sequence-specific secondary and tertiary structures as they are being synthesized. These structures are essential to function, and their integrity is maintained through the course of often-complex conformational changes involved in reversible binding and catalysis. It is reasonable to regard these tertiary structures as "morphologies" and to regard the "correct" functional structure into which an RNA or protein polymer folds as the "target morphology" of that polymer. One can then ask what information specifies this target morphology and how that information is deployed to correctly construct the target morphology. Predictive answers to these questions not only have explanatory power for natural systems, but also clear medical and technological relevance.

Theoretical studies of RNA folding began with the assumption that the final structure was specified, under physiological conditions, by the RNA sequence (e.g. Ti-

noco, Uhlenbeck and Levine, 1971; DeLisi and Crothers, 1971). In the ensuing decades it has become clear that the sequence information is "read" in stages during folding, and that parts of the sequence can encode different "instructions" in the context of different partially-folded structures (Chen, 2008). It is also now clear that "bottom-up" sequence information is insufficient to specify the mature structure of most functional RNAs; a myriad of proteins, some specific to particular RNAs or classes of RNAs, are also required for correct folding (Pan and Sosnick, 2006). These ancillary proteins are, therefore, also contributors of instructive information to the folding process. From the RNA's perspective, they are parts of the environment that provide top-down information or, in the more usual language, constraints.

Early research on protein folding similarly assumed that the information specifying the final structure resided in the sequence (e.g. Kuntz, 1972; Nagano, 1973). The most straightforward interpretation of this assumption, that protein folding minimizes the free energy of interaction of its amino acids, is computationally intractable (e.g. Fraenkel, 1993), raising the question of how Nature could efficiently solve this problem. There is now considerable evidence that proteins fold incrementally, with already-folded domains providing higher-level constraints on the folding of later domains as well as on domain assembly (Dill and MacCallum, 2012). Additional high-level constraints may be provided by chaperone proteins (Saibil, 2013) or other cofactors present in the "typical" environment. Hence as in the case of RNA, information at multiple scales is required to achieve the molecular-scale target morphology.

## 1.2 Organismal morphology

By considering the genome to be the sole carrier of inherited information, Modern Synthesis evolutionary theory committed itself to the genome as the sole driver of development. Hence we have, for example, "[d]evelopmental biology can be seen as the study of how information in the genome is translated into adult structure, and evolutionary biology of how the information came to be there in the first place" (Szathmáry and Maynard Smith, 1995, p. 231). This extreme view has been criticized from multiple perspectives (e.g. Pigliucci and Müller, 2010; Danchin et al., 2011; Laland et al., 2015; Booth, Mariscal and Doolittle, 2016; Gawne, McKenna and Nijhout, 2018) but still remains prominent.

That supra-genomic information can be inherited has been known at least since the work of Beisson and Sonneborn (1965) demonstrating inheritance of experimentally-induced alterations of cortical pattern in *Paramecium* (see Harold, 2005; Fields and Levin, 2018 for reviews of multiple studies along these lines that illustrate the stable and yet re-writable nature of target morphology on a single cell level). Indeed it is clear that any daughter cell, even if produced by asymmetric cleavage, inherits not only multiple active cytosolic molecules, including mRNAs, but also intact cytoskeletal components, an organized cell membrane, and organelles from its parent. We have previously termed this spatially-organized, functionally-intact information the "architectome" and shown that it is inherited in addition to the genome, transcriptome, and proteome (Fields and Levin, 2018). Evolution is, therefore, not just the evolution of the genome and its products, but also the evolution of the architectome. The genome and architectome scales are coupled bottom-up over evolutionary time by the incorporation of evolved gene products into the evolving architectome; they can also be expected to be coupled top-down through constraints on gene expression imposed by the architectome (Pezzulo and Levin, 2016).

Bioelectricity provides one mechanism for top-down control of gene expression. Bioelectric fields have long been known to influence morphological changes in single cells and developmental processes in multicellular organisms (Matthews, 1903; Burr and Northrop, 1935; Lund, 1947; Waris, 1950). More recently, electric circuits in non-neural cell groups have been revealed as containing instructive information for organ morphogenesis and axial patterning in a wide range of animal models and human channelopathies (Bates, 2015; see Levin, Pezzulo and Finkelstein, 2017; McLaughlin and Levin, 2018 for more recent reviews). It has now been shown, using regenerating planaria (*Dugesia*) as a model system, that stable tissue-wide bioelectric prepatterns can drive a global change of the bodyplan to a two-headed symmetrical form, and are heritable without genetic change (Durant et al, 2017; Durant et al, 2019). Additional examples of the target morphology being specifically re-written include trophic memory in deer antlers and crab claws (reviewed in Lobo et al., 2014), as well as the results of repeated amputations in salamanders (Bryant et al., 2017).

Single genomes can support multiple target morphologies, in unicells (e.g. amoeboid and flagellate forms in *Naegleria gruberi*, Brunet and King, 2017) and facultative multicellulars (e.g. choanoflagellates) as well as in obligate multicellulars including metazoa. Target morphology can be preserved despite significant genetic change, e.g. in planaria (*Dugesia*) which maintained a fixed morphology and behavioral repertoire over 20 years of asexual reproduction despite the accumulation of non-synonymous codon substitutions in 74% of predicted genes (Nishimura et al., 2015). On the other hand, small changes in the genome, or in environmental conditions including bioelectric signaling, diet, toxins, parasites, and commensal bacteria etc. can produce large changes in final morphology as well as function. Homeotic transformations can move substantial components of intact, functional morphology from one part of the body to another through the localized co-regulation of large numbers of genes (e.g. Gehring and Hiromi, 1986). In planaria, homeotic replacements of posterior structures with anterior structures can be effected by either genetic or bioelectric manipulations (Lobo and Levin, 2015), with bioelectrically-induced replacements typically more accurately-scaled that genetically-induced replacements (Durant et al., 2019).

The development of mechanistic models, typically incorporating assumptions about physical forces as well as biochemical and/or cellular communication processes, has been a mainstay of developmental biology for decades (e.g. Thompson, 1942; Turing, 1952; Wolpert, 1969; Gierer and Meinhardt, 1972) and has flourished more recently as sophisticated gene-regulatory network (GRN, e.g. Engler et al., 2009; Hecker et al., 2009; Briscoe and Kicheva, 2017; Herrera-Delgado et al., 2018) and organism-scale cell-cell communication (e.g. Pietak and Levin, 2016) models have become feasible. Such models all incorporate, either explicitly or implicitly, both bottom-up and top-down constraints on the developmental dynamics at the scale of interest. While the assumptions and mechanisms implemented by such models are inevitably simplified compared to the actual biology, they are nonetheless capable of correctly predicting the outcomes of not-yet performed experiments, and hence of motivating and directing experimental work (e.g. Raspopovic et al., 2014; Chernet, Fields and Levin, 2015; Lobo and Levin, 2015; Pai et al., 2018; Streichan et al., 2018; Lee, Richtsmeier and Kraft, 2019; Pietak et al., 2019).

## 1.3 Ecosystem-level structure

Recognizable large-scale ecosystems such as grasslands or forests have been known to develop by long-term successional processes for over a century (for a historical review, see Connell and Slayter, 1977). The stable, "climax" endpoints of such processes have well-defined structures and internal self-stabilizing dynamics, and can be considered "target morphologies" in a natural sense. Smaller-scale multi-organism communities that incorporate abiotic materials in specific, reproducible configurations, such as termite mounds, have well-defined target morphologies in an even more obvious sense, and have been proposed to be individual "extended organisms" with heterogeneous genomes (Turner, 2004). With the discovery of ubiquitous, obligate microbiomes and the rise of the holobiont concept (Guerrero, Margulis and Berlanga, 2013; Gilbert, 2014; Bordenstein and Theis, 2015), it is now clear that all multicellular organisms are "ecosystems" in some sense, making the analogy between organismal morphology and ecosystem morphology even more direct. Stereotypical changes in microbiome structure as the "host" body ages (e.g. Miller, 2016) suggest that the concepts of "succession" and "development" are strongly coupled within holobionts. For example, the activity of commensal microbiota can strongly influence the morphogenesis of its host organism, such as the induction of second heads and alteration of visual system structure by bacteria in regenerating planaria (Williams et al., 2020).

As in the case of organismal development, studies of ecosystem succession have relied on mathematical modeling almost since their inception (Connell and Slayter, 1977). Such models typically employ abstract "spaces" with dimensions, e.g. principal components, representing populations or subpopulations or their properties (Lockwood and Lockwood, 1993; Levin et al., 1997; Logofet and Lesnaya, 2000; Fukami et al., 2005). "Morphology" in this case corresponds to a probability distribution, or a stable dynamics over such distributions in a system such as Lotka-Volterra, in this abstract space. In considering the possibility of a target morphology for an evolutionary process, we can expect this more abstract, organizational sense of morphology to be more relevant than the idea of a three-dimensional shape.

## 2. Are major evolutionary transitions predictable?

The central idea of cladistics is that any correct phylogeny depicts an organismal lineage. As noted earlier, if we think of a phylogeny of life on Earth as depicting a cell lineage, it becomes clear that all of life can be considered a single, spatio-temporally extended living entity. This entity has, in particular, a spatio-temporally continuous cytoplasm enclosed by a spatio-temporally continuous membrane (Fields and Levin, 2018). This is illustrated in Figure 2. The analogy with a cell lineage describing a developmental process is obvious.

When we think of life as a single entity in this way, evolution becomes an interaction between processes internal to this entity, including variation, competition, and cooperation, and processes external to this entity. In this it is fully analogous to development, where variation is differentiation, cooperation is well recognized – to the extent of *defining* biological individuals as units of maximum cooperation (Queller and Strassmann, 2009; Strassmann and Queller, 2010) – and competition is increasingly being demonstrated (Gogna, Shee and Moreno, 2015; Madan, Gogna and Moreno, 2018; Gawne, McKenna and Levin, 2020). While the developmental processes of individual organisms are "evolved" while evolution itself clearly is not, this distinction as noted above has no explanatory power. Evolution is, moreover, increasingly recognized to be a learning process, one that results not only in adaptation, but also in increased evolvability (Watson and Szathmáry, 2016; Kouvaris et al., 2017).

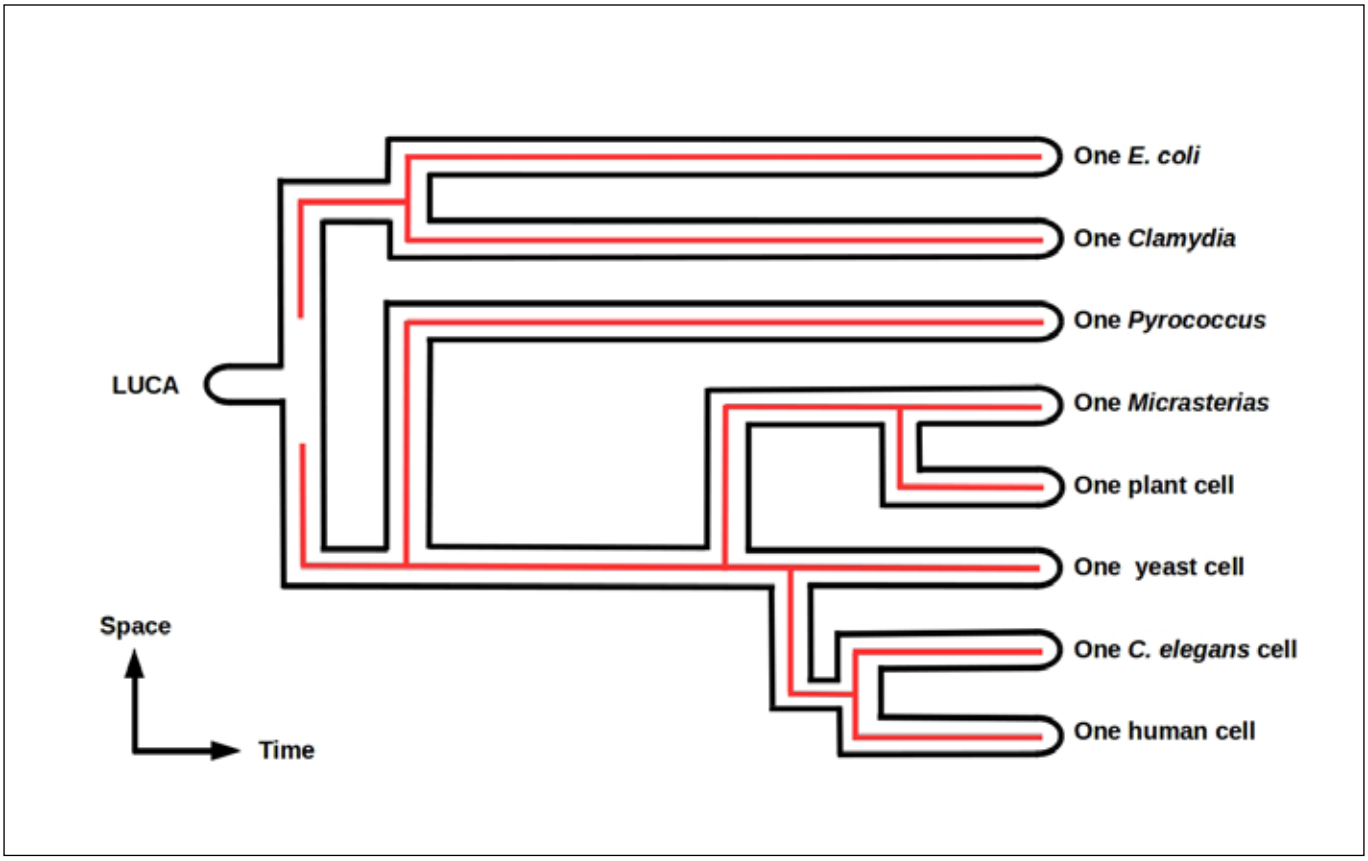


**Figure 2:** Representation of a sample of phylogeny as a cell lineage starting from LUCA. Whether LUCA had a DNA genome (red lines) is left open. Endosymbiotic events are not shown; see Fields and Levin (2018) for lineage diagrams that include them.

Viewing evolution as a space- and time-dependent interaction between a living entity and its environment allows powerful and general information-theoretic tools to be brought to bear on the question of its predictability. Friston (2013) argued that all living systems face a thermodynamic requirement to minimize the variational free energy (VFE) – effectively, the unpredictability – of their environments. Environmental VFE is, moreover, defined at a specific locus: the boundary through which the system interacts with its environment, characterized in mathematical terms as a Markov blanket (MB, Pearl, 1988; see Friston, 2013; Friston et al., 2015; Fields and Levin, 2018 for further discussion). Kuchling et al. (2019) showed that MBs can be defined, and VFE minimization within the MB characterized in terms of approximate Bayesian inference, in systems satisfying very general physical assumptions; these assumptions can be generalized still further when the system-environment interaction is describing using quantum instead of classical physics (Fields and Marcianò, 2019; Fields and Glazebrook, 2020). We have shown previously that under appropriate environmental conditions, MBs can support phase transitions to more complex internal organizations (Fields and Levin, 2019). We argue in what follows that such conditions occur ubiquitously and at multiple scales over evolutionary history, and drive a sequence of phase transitions to larger and more complex organizational structures.

## 2.1 Cellularity

Origin-of-life proposals are notoriously diverse and controversial, and the structure of the biosphere prior to LUCA remains primarily a topic of speculation (Cornish-Bowden and Cárdenas, 2017; Bartlett and Wong, 2020). Whether life had one origin or many, and whether LUCA was the product of a single lineage or many both remain unclear. The structure of LUCA itself is largely unknown, though it seems reasonable to assume that LUCA was a membrane-bound cell (or protocell, Szathmáry, 2015) with both nucleic acids and proteins.

Selection clearly favored cellular life. Are there, however, principles on the basis of which we could expect cellular life to develop an any suitable environment? Friston (2013) offers a heuristic "proof" that any system with an MB will approach a stable, self-sustaining dynamics within the MB, concluding that as living systems at least approximately satisfy the conditions required to maintain an MB, life is "(almost) inevitable" (p. 1). An MB, however, is a set of *states*, not a physical structure such as a membrane. Hence given Friston's result, the key question becomes that of principles on the basis of which we could expect the emergence of physical boundaries, the states of which constitute MBs for whatever systems the boundaries enclose. All current cells are bounded by membranes, but it cannot be ruled out that earlier "cells" – ancestors of LUCA – were bounded by protein capsids, other non-lipid biotic structures, or even abiotic structures.

It is useful to think of this question of boundaries in more abstract, cybernetic terms. Homeostasis can be considered a form of memory, a record of what has worked in the past (Ashby, 1956). The processes that maintain homeostasis can, as Friston (2013) emphasizes, be considered inferential: they are processes that compare external conditions to the memory and adjust one or the other, what Friston calls "active inference." The most fundamental requirement of any such process is that "external conditions" and "memory" be separately accessible. Maintaining homeostasis, therefore, requires a boundary. Some approaches to the origin of life postulate abiotic boundaries, e.g. mineral surfaces (Szathmáry, 2015), but any free-living life form requires a boundary that it can regenerate, particularly following replication. Hence the origin of the chemistry required to regenerate boundaries may be the principle problem to be solved by emergent protocellular life forms. As Cornish-Bowden and Cárdenas (2017) put it, "Understanding how the transition to an organism with a large coding capacity can have happened is a more challenging problem than understanding how LUCA could have evolved to *Homo sapiens*" (p. 72).

Importantly, we are just starting to understand how a sufficiently protected not-yet-cellular system could have been both stable and sufficiently robust to explore the space of possibilities leading to cellularity. Recent work has highlighted the capabilities of subcellular components, such as molecular networks that show learning and adaptation (Watson et al., 2010; Herrera-Delgado et al., 2018), cell-free systems that show complex self-assembly of cytoskeletal structures (Cheng and Ferrell, 2018), and dynamic, responsive motile behavior of cell fragments (Albrecht-Buehler, 1980; Euteneuer and Schliwa, 1984; Sun et al., 2013). Moreover, syncytial systems like Physarum (Vallverdú et al., 2018), giant cells such as algae (Coneva and Chitwood, 2015), Acetabula-

ria (Mandoli, 1998), and glass sponges (Leys, 2015) demonstrate how flexible the idea of a "cell" is. Evolution clearly pushes the limits of cellularity to make it look and behave like multicellularity.

## 2.2 Endosymbiosis

Once regenerable boundaries become available, the logic of VFE minimization is sufficient to drive increases in complexity. One need only postulate a sufficiently variable environment and an ability of cells to exchange information.

Within a VFE minimization or active inference framework, the primary driver of evolution is predictability (Friston, 2013; Friston et al., 2015; Kuchling et al., 2019). For a cell equipped with a memory, the most predictable state is the state of its own memory: homeostasis is precisely the process of keeping this state fixed. If cells are capable of both communicating the states of their memories to other cells and receiving such communications, then the states of other cells also become predictable. Cell-surface markers and diffusible signals are such means of communication. As a means of communicating not just the state of the memory, but a functional component of the memory, lateral gene transfer (LGT) is an even more efficient means of increasing mutual predictability, one that microbes make particular use of in challenging environments (Robbins, Krishtalka and Wooley, 2016). Indeed LGT can be viewed as "endosymbiosis" at the scale of the genome.

If the states of other cells are more predictable than the state of the open environment, any cell that associates closely with other cells achieves an increase in predictive success, i.e. a decrease in VFE. Hence facultative multicellularity is a direct prediction of the VFE minimization framework. Any evolutionary process capable of producing cellular life can be expected to generate facultatively multicellular life. The appearance of microbial stromatolites 3,500 million years ago (MYA), i.e. shortly after LUCA (Stal, 2012), is therefore not surprising.

Facultatively-multicellular microbial systems exhibit division of labor, even in single-species systems such as Myxobacteria (Muñoz-Dorado et al., 2016). In many systems, division of labor includes both division of metabolic labor and differential exposure to the open environment (Stal, 2012; Ereshefsky and Pedroso, 2015). If such systems are considered "individuals" as Ereshefsky and Pedroso (2015) suggest, their "internal" protected components have many of the features of endosymbionts: internal location, specific metabolic functions, and only partial reproductive independence (Booth and Doolittle, 2015). Such facultative endosymbiosis appears both quite common and very old.

The transition from facultative endosymbiosis to the obligate, cellular endosymbiosis found in eukaryotes represents, from a VFE perspective, an increase in predictive power. Cellular symbiosis renders the presence and contribution of the metabolic partner secure from the "host" perspective, and the availability of protection from the open environment secure from the endosymbiont's perspective. Hence this transition can be expected in any evolutionary process driven by VFE minimization. As Booth and Doolittle (2015) point out, the idea that eukaryogenesis was unique and highly improbable may largely be the result of ascertainment bias.

By coupling reproductive cycles, obligate endosymbiosis assures that components that work well together stay together. From an information-processing perspective, this represents an increase in computational power, one that enables more efficient search of fitness landscapes that are rugged on multiple scales (Watson and Pollack, 2003). A capability for more efficient search is, effectively, evolvability. Hence one can expect evolutionary processes to generate, via endosymbiotic or other reproductive-coupling processes, systems that are progressively more evolvable. Multicellular organisms possessing obligate, endosymbiotic microbiomes, and hence living and reproducing as holobionts, are not surprising from this perspective.

## 2.3 Multicellularity

We extend the above considerations of the advantages for predictability of facultative multicellularity to the case of obligate multicellularity in Fields and Levin (2019). In obligate multicellulars, there is an asymmetry in the benefits conferred by communication, one also observed in facultative multicellulars such as *Myxobacteria* and *Dictyostelium*: only a fraction of the cells involved get to reproduce. This fraction ranges from roughly 30% in asexual planaria (Elliott and Sánchez Alvarado, 2012) to roughly 5% in C. elegans hermaphrodites (Sulston and Horvitz, 1977) to much less than 1% in insects or vertebrates.

Why would evolution generate large, complex, multicellular systems in which most of the component cells have zero individual reproductive fitness? We suggested

in Fields and Levin (2019) that reproductive (i.e. stem) cells faced with suitably-challenging environments assemble somatic bodies out of expendable, reproductively-suppressed progeny to keep the environment at bay while avoiding the risk of competition for their protected status and reproductive fitness. This "imperial" model of multicellularity requires a means of enforcing reproductive suppression over long distances, a problem for which specialized signaling systems including neurons provide a solution (Fields, Bischof and Levin, 2020; Fields and Levin, 2020b). Here again, VFE minimization and hence the preservation of memory correlates with signaling capability. As in the case of facultative multicellulars that limit reproduction to only some cells, the division of labor between stem and somatic cells is extreme from a fitness point of view, and the signaling can be regarded as coercive instead of cooperative.

## 2.4 Social groups

Microbial stromatolites are arguably the first social groups; indeed any facultative multicellular can be regarded as a "social group" at the cellular level. Such groups are held together by specific forms of communication – in this case, intercellular signaling with emergent "conventions" such as quorum sensing – and typically exhibit division of labor.

Beyond the cellular level, a VFE minimization framework favors social group formation whenever it increases net predictability, i.e. whenever the states or behavior of other in-group members are more predictable, by the average in-group member, than the states or behaviors of out-group members, including the open environment. While increased predictability is expected to be the case in general within a species, predictability is also high in "extended organism" cooperatives (e.g. Turner, 2004) and in the vast array of symbiotic, mutualist, and facilitated arrangements between disparate species (e.g. Bronstein, 2009). From this perspective, non-social organisms are the exception requiring explanation, e.g. in terms of required range size or hunting style for solitary carnivores.

## 2.5 The cognitive niche

When interactions between cells and multicellular organisms are conceptualized in terms of memory, information processing, and communication, it is natural to regard them as "cognitive" (Pattee, 1982; Stewart, 1996; Baluška and Levin, 2016; Levin, 2019). Indeed, the idea that VFE minimization implements approximate Bayesian inference originated in cognitive neuroscience (Friston, 2010). If evolution is viewed as driven at multiple scales by VFE minimation as suggested in the previous sections, all of life can be regarded as occupying a cognitive niche, an idea reminiscent of both the Gaia hypothesis (Lovelock and Margulis, 1974; Lenton et al., 2018) and biosemiotic thinking (Maturana and Varela, 1980; Kull et al., 2011).

The term "cognitive niche" is nonetheless applied primarily to the niche we humans occupy, one that demands abstraction, analogical reasoning, and planning as well as memory, perceptual processing, and situation-appropriate action. It is often identified specifically with human-like generative language capabilities (Pinker, 2010). Can we expect such a niche to be occupied, eventually, in a generic evolutionary scenario allowed to run long enough?

As higher cognitive capabilities are clearly useful for reducing environmental uncertainty, including uncertainty about what other organisms and particularly conspecifics (Adolphs, 2009) are likely to do next, one might expect an "advanced" cognitive niche to arise and be filled purely on VFE minimization grounds. However, one would also expect to see substantial pre-adaptation in organisms occupying niches that required lesser, but still significant, cognitive capabilities. Studies in both cognitive ethology, e.g. of analogical reasoning in tool use (Fields, 2011), and comparative genetics, e.g. of the role of *FOXP2* in communication ability (Fisher and Scharff, 2009) provide compelling evidence for such pre-adaptation. Both molecular and bioelectric signaling, for example, enormously pre-date their employment by neurons. The earliest function of neurons, moreover, may have been the control of cell proliferation and differentiation, functions that neurons still provide today (Fields, Bischof and Levin, 2020). Hence nervous systems themselves may be a pre-adaptation for complex behaviors and hence general intelligence.

# 3. Reassessing evolutionary "direction"

As Orgogozo (2015) emphasizes, the question of the predictability of evolution can be posed at different scales and levels of abstraction. Here we have posed the

question both abstractly and at large scale: are the major transitions of Terrestrial evolution predictable? Would we expect a generic evolutionary process running anywhere to produce cells, facultative multicellulars, endosymbionts, obligate multicellulars, and social groups? If we regard the predicted outcome as a target morphology, the "morphology" being targeted in this case is a multi-scale organizational structure. We are asking, effectively, if we can expect a generic evolutionary process to produce smart, social holobionts.

As discussed above, the basic ingredients needed to get such a process off the ground are boundaries, memory, information processing, and communication. The boundary must be impermeable to whatever implements the memory but permeable to whatever implements communication: these are the conditions that define an MB. Within the MB, it is sufficient that the information processing system implement VFE minimization, i.e. that its fundamental goal is to increase predictability.

Given such a starting point – a bounded "cell" that can talk to other cells – an evolutionary process will display major transitions if it is able to replicate this basic organizational structure on larger and larger scales. The key to achieving larger scales is, however, built into the system. Aggregating small entities will produce a large entity, and small entities can be expected to aggregate for protection from their environment. The pre-adaptation needed by the small entities to act as a larger unit is communication. This communication can be cooperative, but can also be coercive. Both communication styles were discovered, on Earth, by bacteria. We would expect them to be discovered at an early stage in any evolutionary process.

These considerations suggest that the "direction" of evolution is not toward higher complexity per se as often believed, but rather toward larger scales. Dynamics at larger scales is not more complex than dynamics at smaller scales; large-scale dynamics rather replicates smaller-scale dynamics using larger components. Complexity at the whole-system level increases due to the hierarchization resulting from this embedding (McShea, 2016).

The basic algorithm driving both evolution and development, VFE minimization, remains at least approximately fixed across scales. Evidence that phenomena as diverse as GRNs and metabolic networks (Agrawal, 2002; Barabási and Oltvai, 2004), functional networks in the mammalian brain (Bassett and Bullmore, 2006), and human social networks (Newman, 2001) all share the same small-world architecture suggests that the architecture of memory may also be fixed across scales. Whether communication capabilities are similarly fixed, e.g. whether cell-cell communication systems have a "grammar" with structural properties resembling those of human languages, remains to be determined.

## 4. Future work and predictions

These ideas suggest a number of experimental approaches. First, it will be important to develop multiscale computer models that include both developmental and evolutionary scales. Some of this has been done in the field of artificial life (via "artificial embryogeny" e.g. Stanley and Miikkulainen, 2003; Andersen, Newman and Otter, 2006; 2009; Stanley, 2007; Cussat-Blanc et al., 2010; Pollack and Lowell, 2018) but further advances will require richer, more biorealistic virtual environments specifically including cells as allostatic agents pursuing infotaxis and surprise minimization, and the ability to form multicellular collectives whose large-scale shape and behavior are subject to selection. In such simulations, we predict the scaling of simple, adaptive homeostatic loops at the cellular level to multicellular anatomical homeostasis, and the discovery of similar plasticity toward system-level targets on multiple scales including the physiological, anatomical, and even evolutionary.

Second, wetlab experiments in synthetic morphology, especially those incorporating evolutionary dynamics (Kriegman et al., 2020), and model systems used for the study of origins of multicellularity (Ratcliff et al., 2012; Libby et al., 2016) should enable specific tests of our hypothesis regarding the stability of specific evolutionary transitions to the vagaries and noise of events at the lower levels. An especially interesting context is the use of bioelectric dynamics in bacterial biofilms (Prindle et al., 2015; Ratcliff et al., 2015; Humphries et al., 2017; Yang et al, 2020), suggesting experiments in repeated evolution of bacterial and yeast populations to determine how frequently discoveries, such as using bioelectrics to organize structure and physiology in such "proto-bodies", occur despite variable genetic and environmental conditions.

## 5. Conclusions

We have suggested here that reconceptualizing evolutionary biology to look more like developmental biology leads to novel insights and predictions (see also Fields and Levin, 2020b) and that such a reconceptualization is indeed underway already. Target morphologies in the form of large-scale attractors are to be expected in this setting; we suggest that the major evolutionary transitions are such attractors, and that their replication in multiple "rounds" of evolution could be expected. New theoretical and experimental technologies offer the possibility of testing evolutionary processes in controlled settings with known initial states and adjustable constraints. Both physics and computer science, in particular, have well-developed theoretical vocabularies and toolkits that have yet to be applied extensively to biological problems.

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# Stochastic or Deterministic? That is the Question

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**Commentary on:** Kupiec, JJ 2020, A probabilist theory for cell differentiation, embryonic mortality and DNA C-value paradox, *Organisms: Journal of Biological Sciences*, vol. 4, no. 1 pp. 80-85. DOI: 10.13133/2532-5876/16955

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Scientific progress depends essentially on new ideas. Nevertheless, in biology, it is usually difficult to trace back with precision their origin. There are multiple reasons for this. Perhaps the most important of them is the simple fact, that truly new ideas are usually met with doubt and receive little attention at the moment of their publication, usually in a specialized journal. The paper entitled "A probabilist theory for cell differentiation, embryonic mortality and DNA C-value paradox" by J.J Kupiec reproduced in this issue of "Organisms" is a good example. It was published in 1983 and represents the first step toward a new theory of cell differentiation, the theory called ontophylogenesis. According to the main propositions of the paper differential gene expression during cell differentiation and embryonal development is provoked by random interactions between molecules. The apparently predetermined gene expression patterns that characterise the defined cell phenotypes are the results of a selective stabilization of some patterns through interactions between the cells. This way of framing one of the modern biology's central questions calls for the same reasoning Charles Darwin proposed to explain the evolution of biological species. The idea that spontaneous variation followed by selective stabilization of some of these variants can account for the emergence of new cell types during ontogenesis places the evolution of the species and cell types on the same theoretical ground.

The theory outlined in the 1983 paper and developed further in his subsequent publications by J.J. Kupiec found a favorable echo in the community of theoretical biologists and philosophers and stimulated further thinking and discussions. This was not the case in the community of experimental biologists. The paper remained virtually undetected for many years. With hindsight, this is not surprising. Such a theory could not gain high popularity during the heyday of the molecular genetics. The latter considers that embryonal development is a sequence of molecular and cellular events programmed by the genome and there is no place for random changes in development. Such a deterministic framing of the issue is closely related to the pre-Darwinian view of biological diversity and has been criticized many times. Yet, a softer than the original version of the genetic program narrative is still dominating the scientific literature. This version acknowledges the existence of some variations during ontogenesis, but considers them as environmentally- induced that are counteracted by the robustness of the DNA-encoded program. The molecular genetic vision makes predictions on individual cells, genes and molecules on the basis of the averages measured experimentally on populations. The variation between cells or molecules is deliberately ignored because considered irrelevant. While variation is in the *blind spot* of molecular genetics, it is the most important element of the probabilistic model proposed

by Kupiec. Until recently, it was technically challenging to measure variation among the cells. This is now changing; the resolution of the analytical techniques is increasing. It is becoming easy to detect single molecular events in individual cells, analyze the mRNA or protein composition of single cells or measure the dynamic changes of individual cells in vivo. Now, almost 40 years after its publication we realize that the basic assumptions and predictions formulated in Kupiec's 1983 and later papers (Kupiec 1996, 1997, 2000, 2009) are confirmed without exception.

The first such assumption is stochastic gene expression. Transcription of the genes, as any other biochemical reaction in the cell, depends on the interaction between molecular species, each represented by a small number of copies in the cell. For example, there are usually only two copies of a given gene in eukaryotes. The number of transcription factor molecules of a given type is much lower than the number of binding sites of that factor. Therefore, the rate of gene transcription is limited by their diffusion and diffusion is a random process. Although sporadic data had been published earlier (Hume, 2000), the scientific community became aware of the inherent randomness of gene expression after the publication of a key paper (Elowitz et al 2002). This report, using fluorescent microscopy, provided visual demonstration of the stochasticity in living cells. Since then, the phenomenon gained substantial interest and the ubiquity of the stochastic nature of gene expression is not a surprise anymore. A direct consequence of the randomness of gene expression is the spontaneous generation of phenotypic variability. Indeed, substantial differences between cells within the same tissue or clonal populations have been detected. The variation was much higher than expected. In the classical view, such variability is a nuisance that serves as an impediment to reliable behavior (Raj & Oudernardeen 2008 Cell). In Kupiec's framing it is the opposite; spontaneous variation is essential to maintain the capacity of the cell to respond to environmental changes and, eventually, to differentiate. The requirement of continuous phenotypic fluctuation is the second important assumption of the model.

However, if variation is an obstacle that the living cell must overcome to achieve normal function, as considered by the deterministic vision, then there should be evolved mechanisms by which the effects of noise are minimized. Many studies were conducted to investigate the effect of regulatory feed-back or forward loops, cascades and networks on "noise" propagation and a number of interesting individual examples were described (Eldar & Elowitz 2010). Nevertheless, one of the most important discoveries was the demonstration that the capacity of the living cell to reduce molecular fluctuations is fundamentally limited (Lestas 2010). This study demonstrated that the noise (variation in abundancies of the molecular components) decreases with the quadratic root of the number of signaling events. In other terms, it requires sixteen times more investment to simply double the accuracy of a regulatory process. The cell simply can't afford such a high energetic cost required to reduce the fluctuations to a level where regulatory systems can work in a deterministic way. Stability is not an intrinsic property of an individual cell, constant variation is. Therefore, stochastic variation represents a constraint that can't be dismissed, they have to be part of the explanatory scheme of the stability of biological systems.

The third assumption of the Kupiec model states precisely, that stability of cell states in multicellular organisms is the result of cell-to-cell or cell-environment interactions. In this way, multicellular organisms are analogous to an ecosystem where cells are individuals and cell types are species. In fact, the role of cell-to-cell interactions in embryonal development is already well known. The only difference between the deterministic and probabilistic explanation is that the former considers cell-to-cell interactions as an inducer of changes, contrary to the latter, that sees them as stabilizing force. Some recent observations however, are not only compatible with the probabilistic model, but directly refute the deterministic interpretation. For example, in the Drosophila embryo early-expressed genes exhibit the same degree of transcriptional variability. Precise expression profile in the embryo is generated by spatiotemporal averaging (Little et al. 2013).

An explicit prediction of the probabilistic model formulated by Kupiec is the transitory increase of cell-to-cell variation. This prediction is now firmly confirmed. The first observations on hematopoietic stem cells were published as early as 1997 (Hu et al. 1997). They showed that before committing to a cell fate, these cells go through a period of disordered gene expression, when many different genes typical for mutually exclusive cell fates are co-expressed. The authors called this state as "multilineage-primed". This observation has been

confirmed several times (Pina et al. 2012; Moussy et al 2017). Similar observations were reported on other experimental cell systems also (Richard et al. 2016, Mojtahedi et al 2016). Recently, the rise-then-fall profile of the transcriptional variation has been reported to be a universal feature of cellular differentiation (Gao et al 2020).

The 1983 paper was the first step on a long road. Now, 40 years later, far beyond the initial theoretical speculations and conjectures, with a substantial body of evidence as support, the probabilist theory of cell differentiation is on the way to become an alternative to the deterministic view of ontogenesis and to help definitively getting rid of the cryptic finalism hidden in it.

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# A Probabilistic Theory for Cell Differentiation, Embryonic Mortality and DNA C-value Paradox

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**Abstract**
A probabilistic theory for cell differentiation is proposed in which it is postulated that differential gene expression is provoked by random events. An analysis of determinist theories is made, and two predictions based on the probabilistic theory are compared to experimental fact. A probabilistic model of gene regulation is also given. This theory can account for several phenomena: differential gene expression, embryonic mortality, DNA C-value paradox; and it does not need t refer to a wide diversity of specific regulators.

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## Introduction

In this article a theory for cell differentiation is presented in which differential gene expression in the different cell lines constituting an organism is the result of random events which occur within the cells at the level of interactions between regulatory molecules and genes. In the frame of this theory, interactions between the different cell types do not play any inductive role in differential gene expression, but intervene secondarily to control and co-ordinate the development of the different tissues.

Three elements have led me to elaborate this theory: (i) the idea that cybernetic concepts cannot describe biological reality because it is radically different from the machine world. (ii) The analysis of determinist models of cell differentiation. (iii) The work of Geissler et al. (Geissler et al., 1977), which shows that the survival probability of an embryo is independent of that of the other embryos carried by the same female. This will be analyzed in the third part of this article.

Since the experiments of Spemann and Mangold in the early twenties, which gave rise to the concept of induction, most of the models that have been proposed to explain cell differentiation have a common basis: it is considered that a cell is determined to differentiate because it has received specific information. This information is generally thought to come from another cell in the form of a regulatory molecule ( Figure1).

Differences between models depend on: (i) the chemical nature of the regulatory molecules; (ii) the type of control of the regulatory molecules (activation or repression) (iii ) the direct action of these regulatory molecules at the chromatin level, or by the intermediary of membrane signals.

These models contain a contradiction since the cell, which transmits the information (Cell 1 in Figure 1), is already different from the one that receives the infor-

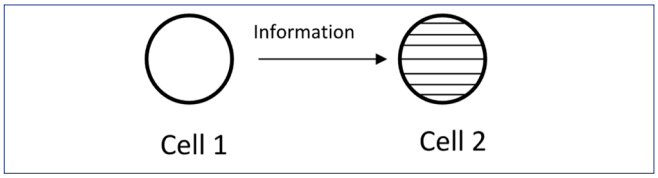


**Figure 1**

(cell 2 of Figure 1) at the beginning or the process because it synthesizes one or several informative molecules not synthesized by the other cell. In order to resolve this contradiction, the morphogenetic gradient theory is usually used (Davidson and Britten, 1971).

The implicit negative hypothesis of these models is to consider that embryonic cells, when left to themselves, are in a stable state, that they replicate identically and that they cannot express other genes without the action of an inductor.

This hypothesis has never been (and maybe cannot be) experimentally demonstrated. The fact that in certain cases cell differentiation or a specific gene expression can be induced chemically (by hormones, for example) proves that cells are inducible for certain genes, but it does not prove that this is true for all genes and that, in general, cells cannot express different genes without an induction.

On the contrary, the probabilistic theory's initial hypothesis assumes that, because of internal and random events which cause certain genes to be activated or repressed, eukaryotic cells can differentiate without the intervention of external signals.

The theoretical framework, which is thus defined, allows us to conceive that two cells become different from each other even though they are identical at the beginning of the process: in Figure 2 below, cells 1 and 2 are identical. According to whether the random event a or b occurs in either of them, they are transformed into a type A or B cell respectively.

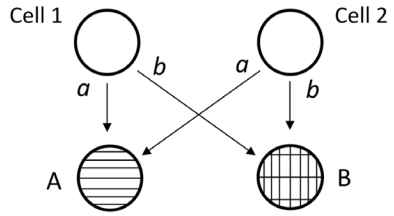


**Figure 2**

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Before raising the question or the nature of the random events who provoke cell differentiation, two predictions based on this hypothesis can be made and compared to experimental facts.

First Prediction: If the appearance of the different cell types that constitute an organism depends on the occurrence of random events, this phenomenon must only have a certain probability to succeed each time that it occurs. Therefore, it must also have a certain failure probability.

Second Prediction: In order to succeed, each of the random events leading to a cell type must occur at least once within one of the cells that constitute the embryo. If this does not happen, one or several cell types will be missing and it will be a failure.

The failure probability should be a function of, on the one hand, the probabilities of the random events in each cell leading to the different cell ty pes; and, on the other hand, the number of cells, which constitute the embryo the greater this number, the smaller the failure probability, will be.

These predictions are compatible with some experimental facts. There is always a certain failure rate in embryogenesis. This phenomenon has mainly been studied by agro-biologists with the purpose of increasing the profitability of stock. According to Vandeplassche (Vandeplassche,1968) "All species have +/- 25 % embryonic mortality which occurs before, during and shortly after implantation, so that one is inclined to believe that embryonic mortality is, at least to a certain extent, a normal phenomenon."

Several hypotheses (either genetic or physiological) have been made to explain the cause or embryonic mortality (Bishop,1964). Various factors may influence it: breed, age, genotype, temperature (for reviews on this subject (Edey, 1969; Ayalon, 1978; Fechheimer, 1979; Gustavsson, 1979). But no definitive explanation bas been given for this phenomenon. The probabilist theory provides a simple and coherent explanation since it predicts a failure probability for cell differentiation. Moreover it can give an explanation for certain experimental results that classical theories can hardly account for. Allison (Allison, 1975) has shown that in sheep there is a relationship between the survival probability of an embryo and the number of embryos carried simultaneously by the gestating female. When the number of embryos carried simultaneously by the female increases, the survival probability of each embryo decreases. Geissler et al. (Geissler et al., 1976) have done a mathematical analysis of these results. They have shown that the survival of a fertilised ovum depends only on the number of ova carried with it and is independent of the survival or death of those carried with it.

Now, if the survival probability of one embryo is independent of those of the other embryos carried by the same female, this indicates that the determining cause of this probability is internal and not external to the embryo.

The hypotheses that can be made within the frame of classical theories to explain embryonic mortality are hardly able to account for this result. In the case of hypotheses which postulate that in given conditions, there is a limit to the number of embryos that can develop inside the same female (Vandeplassche, 1968) - this limit being determined by factors such as the physical space of the uterus, nutritive conditions, hormonal imbalance, etc. -the survival probabilities of the embryos should be dependent. (For example, from the moment that the maximum number of embryos having a normal development is reached the survival probability of the remaining embryos should become zero.)

In the case of genetical hypotheses: at least an important part of mutations, chromosomal abnormalities and aberrations result from errors that occur before fecundation and there is no reason for the number of these errors to increase when the number of released ova increases. However one might postulate that the number of remaining errors occurring during or shortly after fecundation increases if the number of released ova increases. But such a hypothesis is incompatible with the results of Gates (Gates, 1956) who, by the transplantation of blastocysts from females which had been induced to super- ovulate in to non-treated females, found that these blastocysts were genetically and physiologically normal.

The probabilistic theory explains this result in the following way: when the number of embryos carried by the same female increases, the number of cells constituting each embryo in each phase of its development decreases (This hypothesis is plausible and can be tested). Now, the survival probability is as we have previously seen (second prediction) dependent on the number of cells which constitute the embryo. If this number decreases, the survival probability will also decrease. But in this case, the survival probability of one embryo remains independent of the survival probability of the other embryos since it is only dependent on the number of cells and not on the failure or survival of the other embryos. The fact that this probability decreases has for its ultimate effect that, statistically, the number of surviving embryos decreases if the number of embryos carried by the same gestating female increases.

A relation between the number of cells and the viability of embryos has already been described (Wu, 1976).

## Probabilistic theory of gene regulation in eucaryotic cells

Determinist models of gene regulation in eukaryotic cells presuppose the existence of specific regulators which specifically activate or repress different genes during cell differentiation. These models are usually elaborated by a re-thinking of the Monod-Jacob model of the lactose operon of E. Coli. Here again, this is an undemonstrated hypothesis because these models must presuppose a wide diversity of regulators, not yet disco-

vered experimentally in order to explain the diversity of tissues which constitute an organism.

Probabilistic model of gene regulation in eukaryotic cells. This model is based on two basic principles: 1) the molecules which interact with DNA and activate or repress genes are non-specific regulators. Each regulator in the cell's nucleus is present in a quantity smaller than the N number or DNA sequences with which it can interact.

The choice of the q DNA sequences among the possible N sequences, with which the q regulatory molecules interact in a cell, occurs in a stochastic way.

There is therefore a combination of distributional possibilities of the q molecules, taken the N DNA sequences. Each of these distributions corresponds to the activation or repression of q different genes (or set of genes).

At the time of DNA replication, in each cell the q regulatory molecules are redistributed over the N sequences and a different distribution or the q molecules over the N sequences may result. However, the transition of one distribution to another one is not equiprobable for all possible distributions.

The probability of transition f rom one distribution to another is a function of the relative positions of the different regulatory sequences of DNA (Different parameters might intervene to determine this probability so that it is not necessarily directly proportional to the distance separating the DNA sequences. With a view to simplicity, I have only considered distance along the DNA chain as determining the probability of transition in the following examples. This does not change the logic of the theory).

Let us consider, for example, a very simple case in which q= 1 and N = 4 ( Figure 3). When the regulatory molecule is in N1, it has an equal probability of moving into N2 or N5.

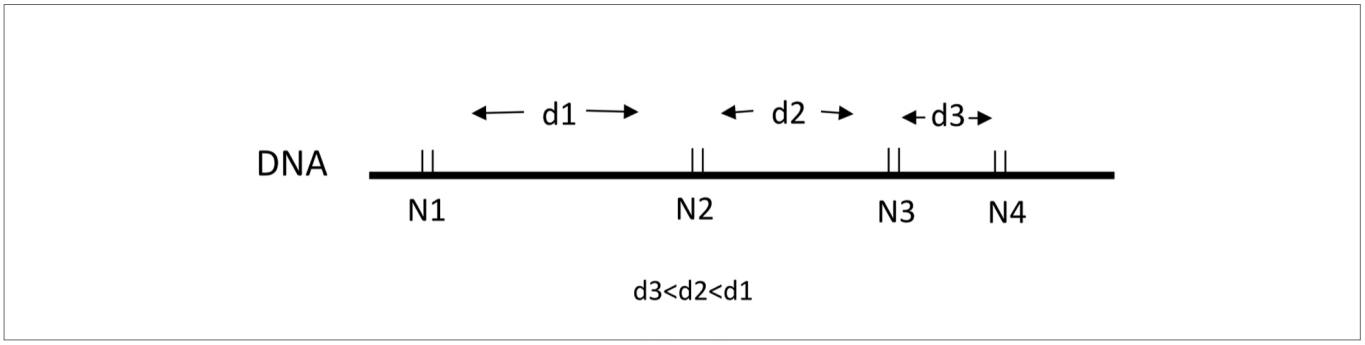


**Figure 3**

The four DNA sequences Nl, N2, N3 and N4 with which the regulatory molecule can interact have a distance of dl, d2, d3, between them. However these three distances are not equal so that: d3 < d2 < dl. When the regulatory molecule is in Nl, the probability for it to move into N2 at the time of DNA replication is higher than the probability for it to move into N3 which is further away, and even higher than the probability for it to move into N4 which is still further away.

When the regulator is in N2, the nearest sequence is N3. Therefore, the highest probability is that it will move into N3.

Similarly, when the regulator is in N3, the highest probability is that it will move into N4.

In an embryonic cell which starts dividing and in which the regulatory molecule is in N1, the successive transition into N2, N3 and N4 is most probable and each of these situations will cause different genes to be activated or repressed. So that the relative position of the four sequences Nl, N2, N3 and N4 in relation to one another determines the differentiation program which has the highest probability of being achieved.

With more genes or more regulators one can obtain several cell lines.

For example, let us now consider the case in which one regulatory molecule can interact with seven regulatory sequences ( Figure 4). These different sequences are separated from each other by the distances d1, d 2, d3, d4, d5 and d6. N1 is equidistant from N2 and N5: d l = d4. d3 < d 2 < dl, d6 < d5 < d4.

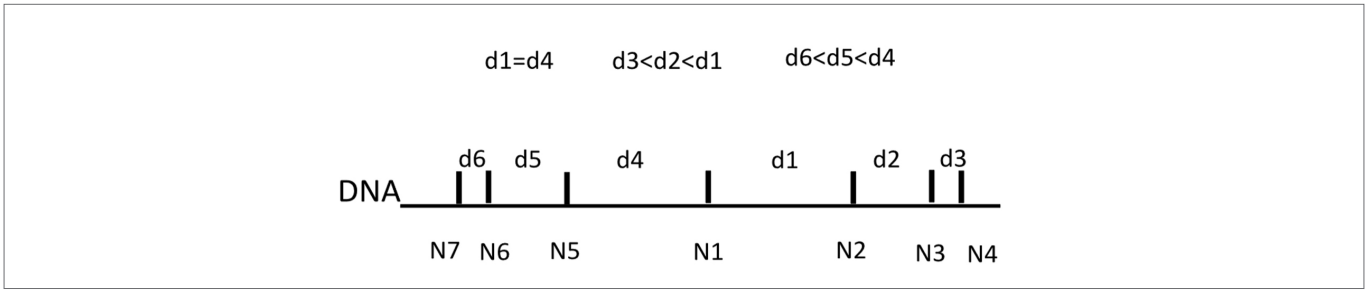


**Figure 4**

Among the embryonic cells which are in the process of dividing, in certain cases the regulator will move into N2 and in other cases into N5. In the cells where the regulator has moved into N2, the probability to move on will be highest in N3 and N4 successively; in the cells where it has moved into N5, the probability to move on will be highest in N6and N7 successively. In this case, the initial cell where the regulator was in N1 will have given rise to two different cell lines.

The relative position of the different sequences along the DNA chain defines a kind of "supercode" which determines the cell differentiation program. This differentiation program has a certain probability to be achieved, that is, it also has a certain reverse probability not to be achieved.

As we have seen previously, this proposal, which may seem paradoxical, is not incompatible with experimental facts.

Numerous models of varying complexity can be constructed with the same operating mode. These models would differ from each other by the value of q and N, the number of non-specific regulators involved, the type of control and the chemical nature of the regulators.

In reality. it is unlikely that there is a single model, valid for all phases of cell differentiation and for all species.

To my knowledge, there is as yet no direct experimental proof in favor of this type of gene regulation. However certain interesting consequences of these models can be considered.

If the relative position of the different regulatory sequences determines the differentiation program, the portions of DNA between these sequences, including the non-coding portion s, play an essential role because they determine the probability of transition from one sequence to another of the regulatory molecules.

This could partly explain why the eucaryotes have an excess of DNA which has been called the "C value paradox", to which no clearly defined function has been assigned, and which has given rise to the concept of "selfish DNA" (Orgel and Crick, 1980).

The role or certain portions of non-coding DNA might be to keep the genes at a certain distance from each other in order to maintain the relative position of these genes, that is the differentiation program.

From an evolutionist point of view, it may also be advantageous for organisms to have bits of non-coding DNA in reserve which, by changing their position in the DNA molecule, would modify the relative position of the genes, causing the differentiation program to vary.

Moreover, if certain of the N genes, which can be regulated by regulatory molecules are repeated several times, they have a higher probability to be activated or repressed. The repetition of a gene that plays an important role during cell life or at a certain stage of development, gives this gene a higher probability to be expressed or repressed than if it only occurs once in the genome.

## Role of cell interactions

During development, cells not only differentiate but also give rise to the organized structure, which constitutes an adult organism. This implies a co-ordination in the development of the different tissues.

In the frame of the probabilistic theory this is achieved by means of cell interactions which intervene secondarily to coordinate the development of the different tissues which first emerged in a random manner (It is not a new idea that cell interactions play a role in development, however, in the frame of the probabilistic theory these interactions have no primary inductive role in cell differentiation).

This means that differentiation is achieved in two steps (at each phase). Gene regulation occurs in a stochastic manner (cf. section 4). Different cell types emerge but they might change their determination at each replication. They remain totipotent. No organization

can emerge. Different cell types interact and the result of this interaction is that they become monopotent. They cannot change their determination any longer. An organization can appear. In this way, the multiplication of one cell type cannot occur independently of the multiplication of the other cell types: there is a coordination in the development or the different tissues.

## Conclusion

It may seem paradoxical to explain a phenomenon such as cell differentiation, which seems to be repeated in exactly the same way each time that it occurs, by a theory based on the occurrence of random events. The probabilistic theory explains this paradox in the following way: when we think of, or look at, cell differentiation, we usually only consider cases that succeed, so that it seems always to be an identical process. But if we consider the cases when embryonic mortality occurs, we must consider that this process is not always the same. Only one (or a very few) out of the different manners in which the differentiation program may occur leads to a viable embryo.

In the future, the probabilistic theory of cell differentiation presented here should be complemented by a theory or cell interactions, in order to account for the whole process of embryonic development.

However, the probabilistic theory already presents different characteristics and advantages: (a) it explains cell differentiation without referring to a preformationist concept such as the morphogenetic gradient; (b) it does not need a wide diversity of specific regulators; (c) it can explain the DNA C-value paradox ; and (d) it can explain embryonic mortality.

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Methods and Techniques

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# A Challenging Task—How to Successfully Separate Theca and Granulosa cells: A Mandatory Step for Investigating Ovary Steroidogenesis

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## The relevance of Theca/Granulosa cell studies

The reciprocal interaction in between theca (TCs) and granulosa (GCs) cells plays a pivotal role in allowing ovary to display a wide range of physiological functions, including steroidogenesis and reproduction capabilities. For instance, changes in the respective proportion among GCs and TCS have been correlated to progression or recovery from Polycystic Ovary Syndrome (Bevilacqua, et al. 2019). To investigate how these cells cooperate, it is important to understand how they work in isolation. Therefore, segregation of the two cell clusters constitutes a pre-requisite for planning functional studies on the ovary.

GCs are a homogeneous population of cells, a few differences between cumulus and mural cells notwithstanding. On the contrary, theca cells surrounding the antral follicle are a mixed population, composed of an inner (theca interna) and an outer layer of cells (theca externa) harbouring some appreciable morphological and functional features. The theca interna contains cells committed to endocrine function. The theca externa is a fibrous, connective tissue layer derived from fibroblast-like cells. In many mammalian species, theca cells associated with follicles undergoing atresia, still survive and remain in the ovarian stroma as nests of endocrine cells, known as secondary interstitial glands (Erickson, et al. 1985). Therefore, hereafter we shall refer to these isolated populations as theca/interstitial cells (TICs), given that both cell populations have been demonstrated to express high levels of enzymes involved in androstenedione synthesis, as 3β-Hydroxysteroid dehydrogenase (3β-HSDH) (Magoffin and Erickson 1988). Complete separation of these clusters is deprived of physiological utility, while a number of methodological hurdles make almost unpractical to actually separate them.

Indeed, to address such difficulties, several different approaches have been so far published, as briefly sketched in the followings:

1. Magoffin et al. isolate GCs and Theca/interstitial cells (TICs) from hypophysectomized immature rat ovaries. Four days after hypophysectomy ovaries are

collected, cut into 4-6 pieces, and digested in a collagenase-DNase solution (4 mg/ml of collagenase, 10 pg/d of DNase, 10 mg/ml of BSA in Medium 199 (Magoffin and Erickson 1988).

The cell suspension thus obtained is carefully layered on top of a linear (20-70%) or a discontinuous Percoll gradient. and centrifuged (4°C) at 400 x g for 20 min. The different cell populations are separated in different bands depending on their specific density. After measuring the positions of the cell bands, the bands are individually collected by aspiration.

After separation with a linear Percoll gradient, the purity of the cells is low (65%). For better cell purity cells were separated by a discontinuous gradient. This is important because the discontinuous gradient generates density steps which act as a thermodynamic barrier for the single cells. For this, the cells are blocked in the specific band and it is possible to isolate them with a higher level of purity (93%).

Each cell fraction is then washed with medium and resuspended in a known volume of medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin sulfate and 2 mM L-glutamine.

However, even though the cell purity is above 90%, the use of hypophysectomized rats is a "complicated" method for studying the endocrine regulation of ovarian TIC differentiation and not differentiated preovulatory TI cells.

The successful purity rates achieved by the Magoffin's method notwithstanding, this technique of cell segregation has been left aside by the next generation of studies. Likely, this method was discarded as the investigation was focused on human ovary cells, usually showing larger sizes than those provided by rodents, for which a direct mechanical approach was found equally reliable.

2. In the paper by Liu (Liu et al. 2017), cells are isolated from 6-week-old mice. In this study, GCs are obtained by puncturing the antral follicles with hypodermic needles, while the remaining tissue is digested with collagenase. With this method, GCs result in an almost pure population. However, as already highlighted (Ma and Hao 2018), this approach does not allow to obtain TICs with enough purity, i.e. TICs resulted usually contaminated in high proportion by GCs from secondary and preantral follicles. Likely, this unwarranted contamination arises as large follicles are mixed together with small follicles, in which GCs can hardly be segregated from TICs.

Such shortcomings hinder the reliability of the technique and the possibility to recruit a number of TICs with sufficient purity for planning successive function-related studies.

## Isolation of Theca/interstitial and Granulosa cells from antral follicles

To address these issues, herein we describe an improved method for the isolation of TICs and GCs from the murine ovary.

The method is in fact an in-depth revised version of one already described technique (Innocenti, et al. 2017), with some modifications.

We isolated ovaries from adult (6/8-week-old) female CD1 mice 48 h after subcutaneous injection with PMSG (Pregnant Mare Serum Gonadotropin) to stimulate folliculogenesis.

After 48 hours of PMSG treatment, the animals were killed using CO2. All animal procedures have been approved by the local ethics committee for animal research.

Ovaries were removed, placed in CMF (Calcium-Magnesium free PBS) and freed from the surrounding tissues under a stereomicroscope. To avoid contamination by small follicles, we first dissected large follicles away from the ovarian stroma, and then GCs were collected by puncturing and gently pressing these follicles with 25 gauge-needles in M2 medium supplemented with 0.3% BSA. GC suspension was then centrifuged at 1000 rpm for 8 min and resuspended in DMEM with 5% FBS, 1% Glutamine, 1% P/S and Gentamicin 0.4 mg/mL. After three washes with the same medium, GCs were cultured at a density of 1 x 106 cells in 60 mm dish.

The residual tissue (TICs) was smoothly scraped to eliminate adherent GCs and digested by incubation with a collagenase-DNase solution containing 4mg/mL collagenase IV, 10mg/mL DNase and 10mg/mL BSA in DMEM for 1 hour under agitation. At the end of this period, we blocked collagenase with medium supplemented with 5% FBS, filtered the cell suspension, washed the dispersed cells three times with DMEM with 5% FBS, 1% Glutamine, 1% P/S and Gentamicin 0.4 mg/mL. The cells were then cultured in 60 mm dishes.

## Identification of theca/interstitial and Granulosa cells

### Morphology

Isolated cells were observed at different times (48, 72 and 120 h). TICs and GCs were observed under light microscope, showing different morphological characteristics. TICs have a fibroblast-like phenotype, a fusiform or triangular shape, while GCs are polygonal or cuboidal, as previously described (Tian et al. 2015).

No morphological signs of apoptosis have been observed neither at 72 nor at 120 hours (Figure 1).

### Molecular characterization

To further investigate GCs and TICs populations, cells were lysed in lysis buffer for RNA extraction.

The two cell populations display a different molecular profile, which helps in recognizing their mutual differences. Namely, several markers differ significantly between the two cell populations, as reported in the literature (Hatzirodos et al. 2015).

We investigated the expression of FSH receptor (FSH-R), 17α-hydroxylase (CYP17A1), 3β-Hydroxysteroid dehydrogenase (3βHSD) genes by real time-PCR.

As expected, the expression of FSH-R was mainly observed in GCs, while in TICs its expression was almost undetectable. On the contrary, expression of CYP17A1 and 3βHSD, two markers of theca endocrine cells, was found mostly in TICs (Figure 2). Statistically, distribution was highly significant on average.

This selective compartmentalization of gene expression epitomizes the differences between the two cell populations. Noticeably, these specific patterns of gene expression reflect a very basic distinction in biological function and physiological properties among the two groups.

TICs are involved in the androgen synthesis and the production of androstenedione and testosterone. In turn, both these androgens are the very basic substrates necessary to GCs to synthesize 17-beta-estradiol and other estrogens. GCs respond to FSH stimulation with an increased expression of aromatase and a consequent increase of estradiol.

It is noteworthy that only a few GCs express CYP17A1 and 3βHSD, whereas only a small proportion of TICs show detectable levels of FSH-R mRNA.

The method we propose, although being similar to that described by Liu (Liu et al. 2017), presents some

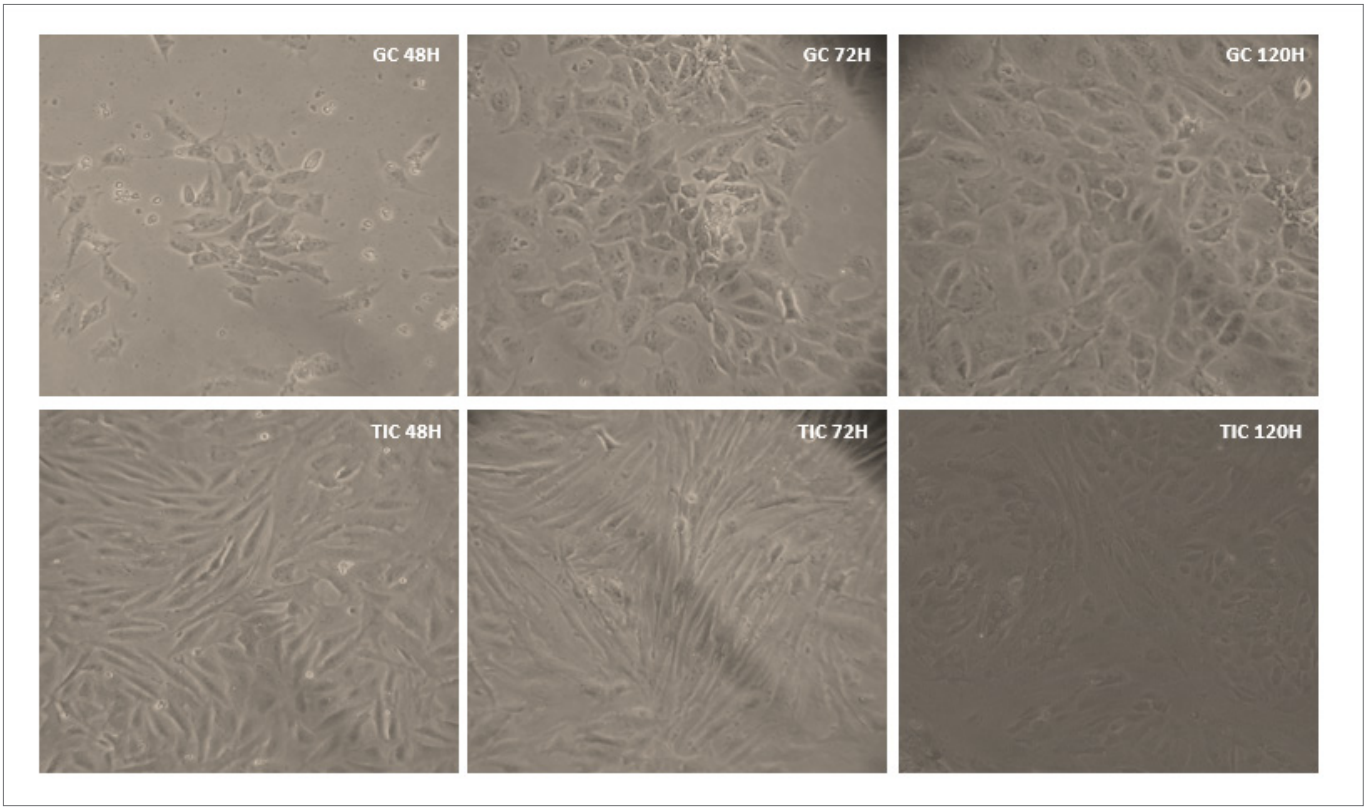


**Figure 1:** Morphological characteristics of GCs and TICs, light microscope. Magnification: 40x.

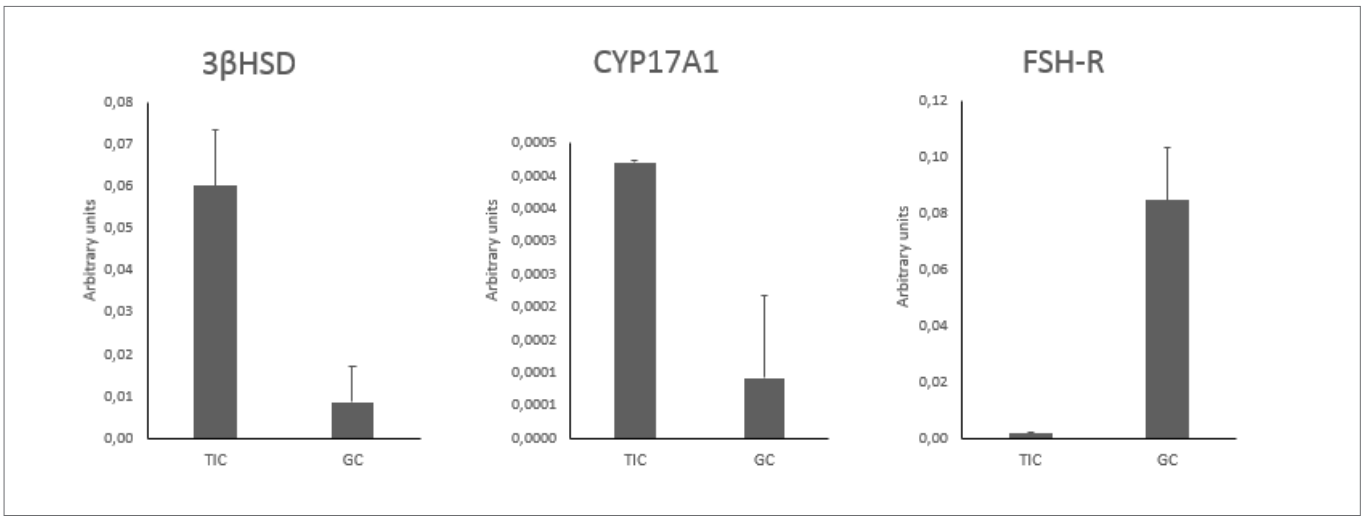


**Figure 2:** Differential genes expression in GCs and TICs. Values are normalized versus Actin expression. These differences reached statistical significance in all the studied markers. 3βHSD: P = 0.023; CYP17A1: P = 0.049; FSH-R: p = 0.020. Results are the mean ± s.e.m. of 3 independent experiments.

critical differences. To avoid the mix between GCs and TICs, mostly depending on the compresence of small follicles, we isolated preantral follicles with a 25 gauge-needles, under the stereomicroscope, and then we discarded them. This approach helps in eliminating granulosa cells from preantral follicles that otherwise might pollute theca cells. Consequently, we were able to recover both TICs and GCs with a high degree of purity.

Further studies are warranted to confirm the suitability of the proposed method and to improve isolation techniques for TCs and GCs.

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# Science as Magic

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**Abstract**

We draw an analogy between illusionism and scientific research. Based on the conceptual distinction between "external" and "internal life" often used in magic, we discuss how these two worlds also coexist in science, one of them being hardly accessible to both scientists and spectators. The task of the scientist is situated in the context of the spectator of a magic effect, whereas the inner workings of nature are compared to the secret maneuvers of the magician. Such a split and subsequent clash of worlds enables the outcome of the magic trick to produce the so-called "illusion of impossibility", whose consequences we map to the process of scientific discovery, invention and understanding. We illustrate our proposal with three paradigmatic examples from the scientific and magic literature, and end by discussing the limitations of the analogy and its implications for improving the practice of science.

**Keywords:** *magic, science, illusion of impossibility, cognitive biases, ecological research*

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*"The first principle is that you must not fool yourself, and you're the easiest person to fool."*
Richard Feynman

## Introduction

One of Heraclitus' fragments reads: "Nature loves to hide" (Hadot 2006). This may simply reflect that flowers disappear from trees until spring is back, but at the same time contains the insight that reality is somehow concealed under the appearances, which is what we have access to. Nature seems to have her secrets and keep them. So do magicians. In their performances, we are aware that something important is concealed, at the same time that we often fail to know what that is. And yet, we want to know. Curiosity leads to amusement and amazement, even triggering bewilderment. As the contrast between effect and trick pervades the world of magic, so does the tension between phenomenon and mechanism engross the minds of scientists (especially upon forgoing Goethean science and Husserlian phenomenology). We struggle to avoid appearances and illusion (Rosset 1976, Barfield 1988). Astonished by the spectacle of nature, scientists ask: "what's the trick?!"

Here we draw an analogy between magic and science. We situate the task of the scientist in the context of the spectator of a magic effect. By means of this analogy, one can then emphasize certain aspects of the scientific practice that are seldom explicitly considered, and then turn those challenges into opportunities for science. We argue that extrapolating from illusionism into the process of scientific discovery can improve our study of the inner workings of nature.

What is magic? For our purposes here, let us define magic or illusionism (we use both terms as synonyms) as the art to provoke in the spectator the so-called "illusion of impossibility". This is an illusion that consists of a cognitive dissonance that results from the contradic-

tion between the expectations created by the magician during the presentation of the effect and what the spectator perceives and experiences during the final climax. During a magic show, several effects are usually performed, the structure of which consists of a presentation stage followed by one or several climaxes. At the end of an "impossible" trick, spectators react with various emotions, often a brief surprise followed by admiration, enchantment, and sometimes unease (Camí et al. 2020).

In every magic effect, two different worlds coexist. The first world is what the Spanish magician Arturo de Ascanio called its "external life", which consists of what the audience experiences during the presentation of the effect. The second world is the so-called "internal life", which includes everything that the magician secretly manipulates towards the final climax (Etcheverry 2000). This concept of double or split reality is fundamental to understand how magicians interact with their audience: "To achieve the illusion of impossibility it is necessary for the magician to coherently combine the obvious and patent actions of the "external life", with the concealments, secret maneuvers and the use of various gimmicks and gadgets, that live only in the "internal life" (Camí et al. 2020). This concept of double reality is also central to understand the analogy we are proposing here between illusionism and scientific research.

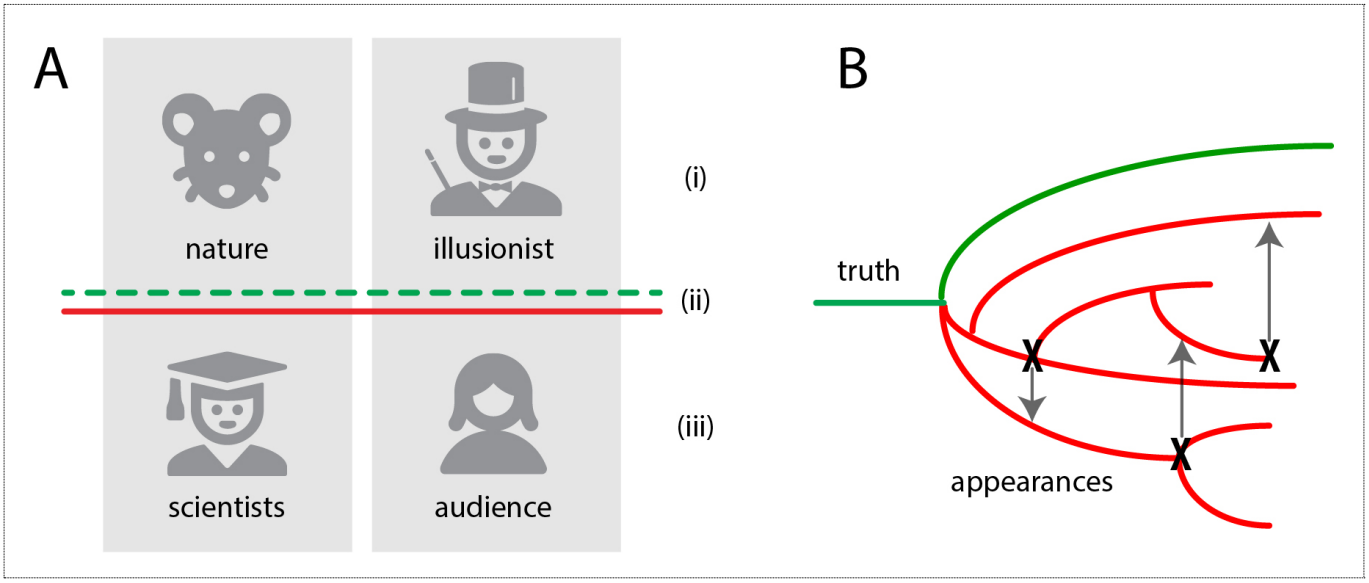


**Figure 1:** The Science as Magic analogy. (A) Magicians are to their audience what nature is to scientists: (i) both nature and illusionists keep their secrets in a way that (ii) their "internal life" is virtually impenetrable from the "external life" of the spectator/scientist, (iii) who are both astonished and also eager to know the trick. (B) The split between internal and external lives eventually causes the scientist to reject hypotheses, reformulate theories, and even experience a sort of "illusion of impossibility" that may lead to paradigm shifts, in an endless quest for higher quality ignorance.

## 1. The analogy

In a word, our "Science as Magic" analogy (or SAMA) goes as follows: the magician is to the spectator what nature is to the scientist (Figure 1A). We propose that (i) magicians conceive and carry out their magic effects akin to how nature works, while (ii) spectators of a magic trick fall into a similar cognitive space to that occupied by scientists in their research, so that (iii) the consequences of the "illusion of impossibility" as perceived by the spectators of a magic show are comparable to those provoked by the mysteries and secrets that scientists try to unravel (Figure 1B). Let us unfold these analogies and supplement them with concrete examples.

## 1.1 Magicians and nature

If no one looks at the magician, there is no magic; if no one looks at nature, there is no science. Magic needs to be performed. In the same way, there is no nature at an instant. Both magic and science are processes. Rather than an appeal to the supernatural, magic can be conceived as the identification of an object of study (Pujol 2015). This is precisely what nature provides. The illusion of impossibility at the outcome of a magic effect is not at odds with the plausibility of the presentation of the effect. As we understand them, magic and science are agnostic to the existence of miracles.

Contrary to what it might seem, magicians never really improvise and, in the face of any unforeseen event, they always manage several exits to save the effect. Magicians conceive, structure and present magic effects with the goal to attain the best possible outcome, never leaving anything of what they say or do to chance. If the circumstances demand so, such as in risky stages, magicians always have ways out and alternative plans that spectators hardly ever notice (Ortiz 1995). Similarly, and acknowledging the difference in timescales, through evolution nature has progressively refined her workings (let us not subscribe to mere mechanism nor to strict finalism). Nature has multiple strategies to course-correct, although we often remain unaware of them. Both in nature and in magic (be it a mouse in a lab or a prestidigitator in a theater), processes take place in real-time and in closed-loop, quickly adapting to the unforeseen.

Magicians are peculiar artists: they make hard things look as easy as possible. So does nature. In the realm of the inert, trajectories comply with the least action principle. In living organisms, optimal is often not good-enough (Loeb 2012). Clever heuristics confer adaptive behavior and improve fitness (Gigerenzer 2007). Interestingly, magic tricks can and do go wrong too. Nature is also capable of error (scientists actually take advantage of it). The study of pathology, for example, illuminates the physiology of the normal (Canguilhem 1991). The study of monsters can reveal a great deal of the structure and function of normal life forms (Alberch 1989). Despite the multiple checkpoints that nature affords (development being a paradigmatic example), nature can abort upon error, but the magician's show must go on.

A magic effect always lives in two worlds. As we have mentioned, magicians present their effects having two parallel worlds in mind (and under control). In the world corresponding to the "external life", sustained by the narrative and non-verbal communication, magicians propose a plot with its own logic and present it with naturality, consistency, timing and rhythm (Etcheverry 2000). All with the sole purpose of avoiding the appearance of any contrasting hints that might drive the audience away from the plot that the magician wants them to follow during the presentation of the effect. Every single act must be thus justified, with the only goal to achieve the "impossible" outcome. Throughout the exposition, the elements of the "external life" are combined with those concealments of the "internal life" in a perfect choreography that makes the secret behind the trick impenetrable for the audience.

In our analogy, we propose that nature does indeed present itself to us compounding two different concurrent realities: one that includes observable effects (always theory-laden, though) and another with supposedly impenetrable content. Baseball players are magicians at catching very difficult balls; they do not compute difficult mathematical equations but run so as to maintain the target along a linear optical trajectory, namely, with optical speed constancy (McBeath et al. 1995). So do dogs when catching Frisbees (Shaffer, 2004). The clash between these split worlds is particularly relevant in the life and mind sciences, since organismic behavior is both intrinsically prescribed by biological needs and also extrinsically describable by mathematical principles, disclosing the tension between scientist-centric and animal-centric perspectives and interests (Gomez-Marin 2019).

As magicians deliberately manipulate certain aspects of the external life so as to achieve the best possible outcome, it might also be that our experimental observations of nature should not be necessarily interpreted in a transparent fashion. Not even when those observations and interpretations are reproducible, as reproducibility does not exclude the impact of the observer's errors and biases (Pashler & Wagenmakers 2010, Staddon 2017, Albright 2017). As in the presentation of a magic trick, what we observe in nature may be modulated by another aspect of reality that is impenetrable to the scientist. One way to penetrate the secret of nature, as in magic, is to pay attention to the contrasting elements, those that do not fit well with our narrative hypotheses.

Negative results, pre-registered experiments (Simons & Holcombe 2014, Simons et al. 2014), outliers, among others, could be doors to the inner workings of nature and, nevertheless, are generally discarded. The invisible world manifests when the visible world fails to close.

Magicians do not perform for the "average spectator". Neither does nature. Magicians pursue a 100% efficacy in their magic outcomes. A statistically significant success on the audience members is worse than suboptimal and unthinkable for them. Magicians are also aware that spectators react with great inter-individual variability (Gea 2018). In order to minimize the potential risks of this diversity, magicians segment the presentation of their effects according to a particular type of audience (as we will see later), and have context into account as a constitutive element of their job.

In our understanding of natural processes, the demands that magicians impose themselves set to us, scientists, a high bar. Making the comparison, we wonder about the acceptability of many scientific results reaching slightly above chance, the reasonability of statistical conventions about significance, or the scarce science done in ecological context. Natural phenomena are differentially affected across populations and contexts (Bar 2004, Blanchard-Fields et al. 2008, Nikolic 2010, Carandini & Heeger, 2012, Louie et al. 2013, Gomis-Pont et al. 2020). For instance, a new medicine may not work the same way in children and adults, or men and women. The obvious is often not necessarily trivial. Moreover, the laboratory is not a substitute for the world; it is just another, often very different, arena (Matusz et al. 2019). The power of reductionism can become a huge limiting factor of the knowledge that we have in reach.

## Example 1. "Broken mice"

In several of his well-known effects, the great Italo-Argentine magician Tony Slydini constantly raised and lowered his hands near the edge of the table. Once the spectators got used to this type of movement, they stopped paying attention and thus, the magician could make anything disappear simply by dropping it onto his lap before the surprised and oblivious audience.

Coined by Ascanio (Etcheverry 2000), "conditioned naturalness" is a concept that refers to a kind of very fast conditioning in which one seeks to normalize, always by repetition, something that in any other context would attract attention. Slydini's concealment moves may at first seem strange, unnatural, and even unreal, but before long the audience became familiar with them, embedding them in the natural logic, in the perceived reality of the game and ceased to be aware of them. Slydini had effectively conditioned their naturalness, managing to reduce the contrast of unnatural manipulations. As scientists, like a magician's audience, we learn by repetition and overexposure to naturalize artificial experimental approaches that, at best, offer us a vision (disciplined with abstractions and technological prostheses) of reality that is incomplete (Kayser et al. 2004). A paradigmatic example is offered by the use of laboratory animals.

Scientists know that wild-type laboratory animals are not really wild. Nevertheless, we use them for the many practical advantages they offer. We then publish our studies under the premise, too often implicit, that what we find in the lab applies outside its doors and walls. The artificial has become "natural enough". Nature in the lab has become the rule. We have just got used to it.

In mice, the mammalian organism model *par excellence* in biomedical research, this situation can be particularly crucial. Most of the animals used for research come from a handful of providers, which create a peculiar selective environment where mice live in captivity for generations without predators. Moreover, the young ones are selected for fast reproductive output, sacrificing them before they reach an older age. What could go wrong?

It is known that mice have very long telomeres. The question is whether this is a characteristic of the natural world or one induced by the artificial conditions in which we study nature to decipher its secrets. Work from the laboratory of Carol Greider (Nobel laureate, and the co-discoverer of enzyme telomerase) actually showed that wild-derived inbred mouse strains have short telomeres (Hemann & Greider 2000). Reared for decades, inbred mice used in laboratory studies have telomeres spanning from 30 to 150kb, whereas the telomeres of those "wild" mice tested in Greider's lab were less than 20kb long. Despite no correlation being found between telomere length and lifespan in mice, such a discovery lays out intriguing implications for biology writ large under the so-called "reserve-capacity hypothesis" (Weinstein & Ciszek 2002), which establishes a trade-off between tumor suppression and tissue repair. Leaving aside the

fascinating theoretical implications that would bridge evolutionary and molecular biology as pioneered by Weinstein, the concerning practical consequences are that this feature of laboratory mice would make most of the basic results and biomedical applications derived from the study of senescence and tumor formation unreliable, if not dangerous, as one would underestimate tissue damage and overestimate cancer risk in those "mouse models" of human disease (Weinstein & Ciszek 2002). In sum, the answer to the question as to whether normal mice have long telomeres depends on what one means by normal and what one means by mice. As it turns out, for the bulk of the scientific community normal is actually not necessarily natural. And yet, the difference matters as it can profoundly fool us (Figure 2A).

## 1.2 The illusion of impossibility and the intelligibility of nature

We strive to know the secret of things. The experience that an "impossible" outcome induces on the spectators of a magic trick (independently of the particular cocktail of emotional reactions) compels many of us to ask "how does the magician do it?" Note that spectators willingly attend the show knowing that the artist is going to use tricks in order to carry out the magic effects. In a similar way, the scientific community, astonished by virtually everything that takes place around us, feels the urge to unravel how nature works. As *Homo sapiens*, we have a drive to expand our knowledge (and domination) on nature.

In magic, the same end can be achieved with different means. The world of magic dramatically teaches us that one can achieve the same "impossible" outcome, with the same experience for the audience, but via very different methods and materials (Tarbell 1999). In other words, to reach the same goal, both the magician and nature can use pathways that involve very different systems, materials, and complexity. This is actually how some magicians are able to fool other magicians. In our understanding of nature, knowing its products is not enough; one must figure out the processes that gave rise to them. In evolution and neuroscience, it is well-known that different neural substrates can produce the same behavior and that different behaviors can be produced by the same neural substrates (Lorenz 1974, Sakurai & Katz 2017).

A magical effect is truffled with false clues that make it difficult for us to figure out the secret (Tamariz 2011). Both in magic and in science, we are too often fooled along the way, since things are always less obvious than they appear to be. Spectators have a very difficult time to discover the magician's secrets. Similarly, when studied by scientists, nature is much less transparent than what we think. During the presentation of effects, magicians may use false clues so as to break down our inference on causality relations. In addition, they structure the content and presentation of the effects to minimize that spectators revisit what has really happened (Camì et al. 2020).

Analogously, our observations and inferences about nature are not free from the same obstacles and traps. In the same manner that magic audiences cannot perceive anything without their own heuristics, scientists too fail to face natural phenomena without imposing their own preconceptions, which are based not only on the data of their experiments but also on the context of their hypotheses and previous knowledge. One could argue that both Golgi and Cajal looked through the same microscope at the same histological preparations (although Cajal improved the method), and so they both could see dendritic spines. However, while Cajal thought they were signal, Golgi was convinced they were noise (Yuste 2015). The challenge is to notice all these worlds hidden in plain sight.

Eureka moments in magic can anchor audiences to the wrong solutions. And yet, we have and cherish eureka moments. Despite all the obstacles that the spectator has in the way to figure out what is going on, the impulse to discover what has happened can cause an "aha! moment" that shall be taken as an explanation of the witnessed phenomena (Ortiz 1995). However, very often in magic the spectator may wrongly speculate about the underlying solution. Even worse, after the "aha! moment" the chances are that one abandons reasoning on alternative solutions, the so-called Einstellung effect (Bilalic et al. 2010). In other words, when one believes to have reached a solution, one is more handicapped to think of alternative explanations. We claim that in science one comes across the same problems. While searching for answers to natural phenomena, it is more than possible that we get stuck in the first answers we find which, even if reproducible, may not be the unique or the main solutions to the conundrum. In fact, and despite grand claims for "disruptive research" or "scientific excellence", out-of-the-box thinking is actually discouraged. We all know instances of how such discouragement is materialized (funding environment,

publishing games, career building). The scientist is also collective made.

## Example 2. "Soups and sparks"

The great Spanish magician Juan Tamariz developed the theory of "false clues" (Tamariz 2011). He thought that, in order to prevent the audience from "rewinding" and trying to assess the logical steps of the magic trick, it would be much more effective if, along the way, the magician created false expectations, perhaps by subtly suggesting solutions to the spectator, that would end up being proved wrong. Taking the audience away from the real method behind the magical effect (which is actually a side-effect of the use of false clues) would enhance the illusion of impossibility at the end of the trick. But, most importantly, would make it impossible for the spectators to reconstruct the logic and thus guess how the trick is done (which, together with creating the "illusion of impossibility", is a great obsession for magicians). False clues would prevent the audience from reaching premature conclusions about the method behind the magic trick. This is important because, whether their deduction be wrong or not, an "aha! moment" would ruin the magical experience; the spectators, believing they have discovered the trick, would cease to be impressed (Ortiz 2015).

Once an idea becomes reasonable in our minds, it is very difficult to consider other alternatives, even if they are actually more viable. It is, again, the most perverse consequence of the afore mentioned Einstellung effect (Bilalic et al. 2010). A sensation of truth is apparently all that matters to generate high confidence in it, as well as positive emotions and increased memorability (Danek et al. 2013). This is as true in magic as it is in science. In fact, in our experiments with nature, false clues do also abound. Although it is not generally possible to prove that a hypothesis is correct (authentication is no proof), we still design most of our experiments and write our grants as if it were; the rebuttal of our starting hypotheses or other alternative viewpoints are often not even considered. But even when reproducible and somewhat backed up by empirical evidence, our working hypotheses can, as false clues, lead us uncritically towards wrong conclusions (Figure 2B). Let us see an example in the field of neuroscience.

Towards the end of the 1930s, the nature of interneuronal communication haunted neuroscientists. Two schools of thought steered the search: one (the most pharmacological one, led by Henry Dale) proposed that synaptic transmission was mediated by messengers of a chemical nature; the other (the most physiological one, led by John Eccles) claimed that communication was direct through a continuous flow of electric charges. The so-called war of the soups and the sparks went on with apparent successes taking place on both fronts.

Eccles showed that the cardiac pacemaker of the cat had a long latency of about 0.1 seconds, and a slow time course of seconds. Led by this "false clue" (stemming in this case from his own reasoning, but in other cases a product of the scientific consensus about the workings of nature), he wrongly concluded that these slow dynamics were the signature of all chemical transmission. Hence, he deduced, synaptic excitation in the central nervous system (with its low latency and fast rate) was too rapid for a chemical process. The electric hypothesis seemed to gain ground. In 1944, an encounter with Karl Popper caused Eccles to reformulate his questions and to radically change his experimental approach (Todman 2008). Then, using as a model an inhibitory synapse, Eccles postulated that, if the chemical hypothesis was correct, the membrane potential of the postsynaptic cell would become more negative when activating the presynaptic neuron. That should not occur if the nature of the communication was electrical. The experiments showed the negative postsynaptic potential and the rest is history (Cobb 2020). The greatest advocate of the electrical hypothesis had just shown that neural communication was chemical in nature. Underperforming big ideas can indeed become entrenched in a community (Joyner et al. 2016).

## 1.3 Magic spectators and scientists

One of our main tenets is that the scientist is not the magician of nature but its spectator (Figure 1A). We are simultaneously astonished and fooled (Figure 1B).

We love secrets, we simply don't like being fooled or not knowing them (regarding the critique of the logic of "model organisms" in laboratories as general representatives of natural truths, note the irony in the ease with which we tend to speak of "humans" in general). The audience of a magic show (like scientists) know that magic (like nature) has its secrets. As an audience we are naturally impelled to discover what's behind the trick. Likewise, as scientists, we feel the urge to fi-

gure out the mechanisms that hide behind each natural phenomenon. The problem is that we are all really easy to fool. But not all spectators are alike, and neither are scientists. Magic is dependent on cultural contexts, previous knowledge and cognitive development (Camí et al. 2020). So is behavior (Gomez-Marin & Ghazanfar 2019). In drawing these analogies, we would like to emphasize only two broad classes of spectators: kids and adults. As it turns out, each of them requires a different modality of magic effects.

Kids require a specific kind of magic that fits their own developmental conditions, and which is distinct from that which conventionally works in adults. Due to their unfolding cognitive processes, children tend to concentrate more on details without great abstractions or a great deal of extra assumptions. This can be a problem during a magic trick conceived to work in adults. For kids, signal can become noise (thus, not showing interest in the trick), and noise can become signal (thus actually discovering the trick). This can easily ruin a professionally performed magic show (see Example 3). Thus magicians tune their effects and the way they present them accordingly. In the analogy with science, we can think of young scientists whose naive and uninhibited curiosity prevents them from prematurely discarding little details that may turn out to be crucial. Without needing to be a genius, their lateral thinking, willingness to try new things, and indifference to ridicule may put them in a privileged position to carry out game-changing discoveries.

The limitations of magic for adults when done in kids actually demonstrate the opportunities available to break into the supposedly impenetrability of the "internal life" of the effect. In science these opportunities also exist, for instance in outlier data, in discarded information, failed experiments, alternative hypotheses, or negative results. In some of such discards one may find the entry point to a wealth of knowledge, as in the case of the so-called "junk DNA" (Pennisi 2012). Adults, but not kids, generally over-determine what they see. As magic for kids remains a challenging endeavor, so is a science of minority reports beyond the community sanctioned interests and habits.

The great majority of magic is thought for adults, namely, grown up people whose cognition follows well-trodden cognitive biases. For instance, magicians have learned to manipulate instinctive decisions by exploiting well established heuristics and cognitive biases characteristic of adults. In fact, magic for adults is the "safest magic", since it comprises the great bulk of efforts, means, history and magic theories. When it comes to science, we can think of this bulk of adult spectators as the majority of professional scientists; a majority that, with time, may over-interpret what they observe, and whose critical thinking may progressively decay, as certain recent crises attest (Head et al. 2015, Ioannidis 2005, Munafò et al. 2017).

## Example 3. "Genetic scissors"

About 30 years ago, the professional magician David Williamson invited a 6-year-old boy called Murray to participate in a magic trick during one of his prime-time TV shows. The game, which the magician had rehearsed for months, was based on a classic magic trick involving the use of a carefully crafted special set of cards. What could go wrong? Williamson started laying out the playing cards on the table, claiming that there were three. But Murray stopped him at that instant by pointing out that he could see a fourth card stacked to one of the others (Williamson 2011). The impenetrability of the internal life had been irremediably exposed. The young spectator had defeated the magician. That night was a turning point in Williamson's career; he experienced in his own flesh that there are different types of audiences; different views, such as Murray's, who could see what hundreds of thousands of other people before, mostly adults, did not see (Olson et al. 2015). Magic does not work the same in children.

We see mostly what we expect to see (Figure 2C). Our experiences are shaped by our expectations, which in turn are shaped by evolution as well as by our culture. They also change with age. Naturally uninhibited, children give more importance to details that are considered superfluous information by adults. Unfortunately, curiosity and creativity tend to fade as we grow up.

Just like Murray's fresh look at Williamson's card trick, scientific breakthroughs often emerge from completely unpredictable origins. As scientists, we tend to design our research projects based on the current scientific context and fads. However, it was sheer curiosity what drove a young Francisco Mojica to persevere on the margins of science, without a grant, and with his main papers rejected in top tier journals for years, in his quest to understand a strange microbial DNA repeat sequence that would lead to his discovery of CRI-

SPR (Mojica et al. 2005, 2013). His contribution was a foundational one to its recognition as an adaptive immune system and its biological characterization, that would end up being fundamental to its repurposing for genome engineering, thus transforming biomedical research in unprecedented ways (Lander 2016). As Lander points out: "It is instructive that so many of the Heroes of CRISPR did their seminal work near the very start of their scientific careers (...). With youth often comes a willingness to take risks —on uncharted directions and seemingly obscure questions— and a drive to succeed." How many discoveries await until we nurture a way of doing science in tune with the limitless curiosity that leads a child to discover that a hardly noticeable card stacked under another is the difference between illusion and reality?

Minority reports can have major consequences. Note that during a magic show everybody applauds even if not so enthusiastic about the magic effect. There is a social component that is even stronger during standing ovations (some jumped from their chairs enthralled, others are forced to do so since they do not want to be left sitting down while the rest is up and clapping). In science, consensus by our peers is a valuable self-correcting mechanism. However, paraphrasing Giordano Bruno, truth does not change because it is, or it is not, believed by a majority of the people, even experts (Sackett 2000). These and other important aspects of the sociology of science need to be dealt with (Lazebnik 2018).

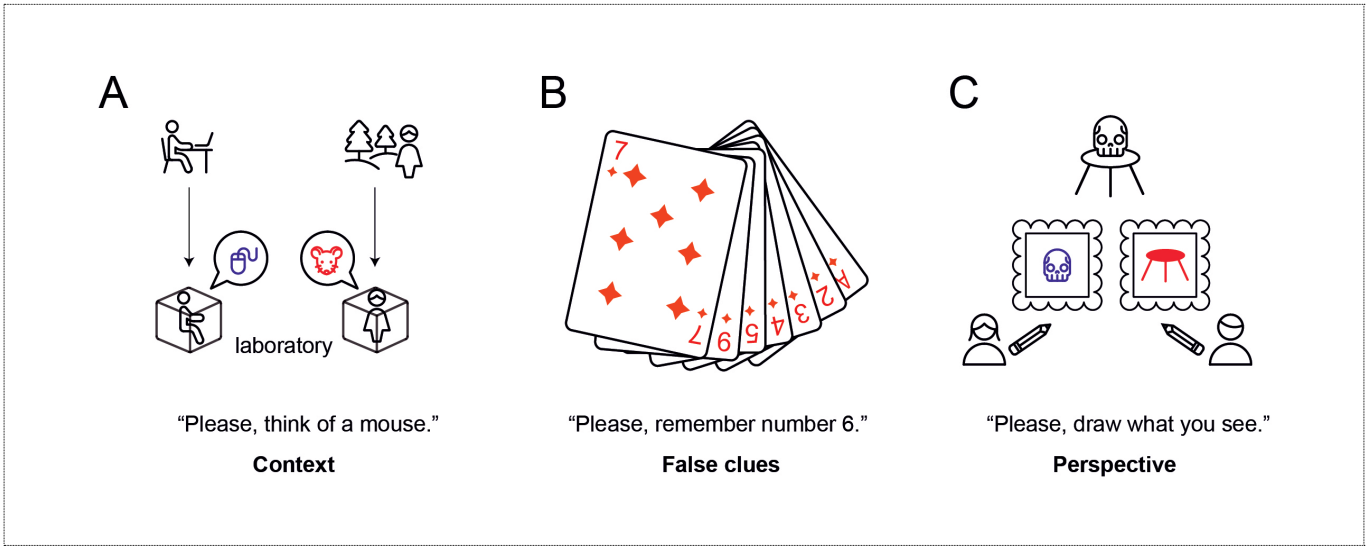


**Figure 2:** Challenges and opportunities that magic proffers to science. (A) Context is constitutive. While magic succeeds in the real world, reductionist laboratory science insists in getting rid of context, ultimately trumping replicability and generalizability. (B) False clues abound. Magicians purposefully lay them in order to torpedo our post-hoc logical reconstruction of the trick (panel adapted from Edward Marlo effect, Genii Magazine Sept 2008). Despite the fact that science is a self-correcting enterprise, scientists have a hard time realizing their blind spots, false paths, and dead ends. (C) Perspective matters (panel inspired by Edward Steed's cartoon). Having performed in front of diverse audiences, magicians know that what we see depends on our interests, heuristics and cognitive biases. Thus there is magic for adults and magic for kids, due to their different cognitive developmental stages. However, scientists' quest for objectivity and tendency for uniformity in their thinking can defeat the purpose.

## Concluding remarks

"Science as Magic" is an analogy that presents the scientific quest through the lens of the processes that take place during a magic effect. This is not to be confused with "how magic became science" (Williams 2020), the "science of magic" (Macknik et al 2008; Kuhn et al. 2008) or "magic for science" (Lamont et al. 2010, Camí et al. 2020).

Analogies and metaphors are essential to language and reasoning (Lakoff & Johnson 1980). They allow us to understand one thing or concept by means of another. For instance, when we say that "time is money", we borrow meaning from the structural and functional properties of "money" in order to better grasp those of "time". Here we have highlighted the coherent structure that both magic and science share. In fact, the very existence and necessity of analogies for thinking challenges a theory of mind that assumes that rationality is conscious, dispassionate, linear, logical, disembodied and universal. As magic demonstrates, most of what we perceive or decide is entangled with our emotions, may not take place logically or linearly, strongly depends on the particular context where it takes place and, finally, on ontogenetic factors and cultural background.

Magic actually works thanks to our many cognitive blind spots. The effectiveness of magicians is due to a large body of reproducible techniques and the use of particular materials and methods that have been developed empirically for centuries. These involve many scientific disciplines such as mechanics, electronics, mathematics and, above all, the cognitive sciences. In fact, the efficacy of magic effects is entangled with the magician's capacity to interfere with the attention, perception, memories, decisions, and other cognitive processes of the spectator (Camí et al. 2020). As illustrated by Millikan's example on the measurements of the charge of the electron, it is so easy to fool ourselves (Feynman 1974). Thus, the more we are aware of those biases, the better science we should be able to practice. Any theory of nature is inseparable from a theory of knowledge.

## Limitations of the "Science as Magic" analogy

Our analogy, of course, breaks down when overstretched. First, note that the spectator, as opposed to the scientist, does not enjoy the possibility of repetition. And if the magician repeats a certain movement or trick, it certainly is in the service of deception (such as in "conditioned naturalness" or upon "false clues" as discussed above). Second, spectators just watch with their eyes, while scientists use all sort of instruments and abstract symbolic formalisms. Third, the scientist, contrary to the spectator, can perturb the system in order to establish counterfactuals. This is actually the essence of experimental science: to combine observation with manipulation so as to upgrade correlation to causation (however, intervening in their system of study, scientists may also inadvertently affect certain aspects of its internal life, especially if the system is complex, quantum, or a simply living organism). Fourth, scientists can and actually do design their experiments, whereas spectators are just presented with a very carefully designed show from the part of the magician. When spectators are called to participate, they often do not influence what is going to happen (everything is under the magician's control). Fifth, although there is no magic without at least one spectator, there can be nature without science (but probably not the other way around). Finally, magicians bring the spectators to their theater, while we, scientists, rather than meeting nature at her place, have got used to bringing her to our laboratories.

## Challenges and opportunities

If we now concentrate on the differences between magicians and scientists, rather than in the similarities between spectators and scientists, we can better appreciate the huge feats that magicians achieve. When applied to science, such challenges become opportunities. Magicians really have skin in the game. First, note that the magician does not target the average spectator, but each and every individual in the audience. A "statistically significant trick" is nothing but a failure. Second, magicians perform *impromptu* magic and succeed in the "real world", while scientists still struggle (Matusz et al. 2018). The street is not a laboratory, and spectators are not inbred mice reared in the house. Quite the contrary to most laboratory practices, rather than pruning context away, magicians deliberately provide it. To put it metaphorically, the absence of a dressing code does not imply that those attending the event will come naked. In fact, each one will bring their own garment. Third, magicians execute very refined protocols (the experimen-

tal task, for a scientist) that actually work in real time and in closed loop. In addition, they have a "plan B" and "plan C" for virtually any situation. Robustness is not incompatible with the ability to improvise. Finally, the magician's work is subject-centric and dual in terms of worldviews; the magic effect is effective not only because of the trick they perform hidden in their "internal life", but also because the magic effect overlaps with the spectator's "meaningful environment" (the so-called *Umwelt*). This last point is actually crucial for the life and mind sciences, and for scientific thinking in general. When stuck in a worldview, we can only study those things that fit it, or gamble (Lahti 2015). But when the things we study have their own worldview too (humans, but also mice, flies and even worms), it is necessary that we are willing to commute from third to first person experiences (Gomez-Marin 2019b).

In sum, magicians thrive with real individuals in the real world, conditions that the laboratory-bound, reductionist, and die-hard objective approaches to science fail to deal with.

## Outlook

We often conflate what is obvious with what is trivial. But the more obvious a trick is, the more deceptive it can become (the notion that the earth is flat, for instance). One thing is not to know how something happened, and quite another is to believe that what has happened cannot be. Science is the belief in the ignorance of experts. Magic is the art of honest deception (in a way, so is cinema). Excess of credulity is always problematic, but so is its lack. Skepticism is a fundamental element of the magic experience and also of science. So is the enchanted mind. Note that magic spectators are fooled despite knowing in advance that they will be fooled. Scientists should also acknowledge that they will remain ignorant despite their increasing knowledge of the natural world (Firestein 2012). For a magician to suggest or pretend that magic is real is comparable to the scientist's assertion that we now know the truth of the matter. To let the audience know that magic is honest deception is equivalent to the conscious ignorance that preludes every real scientific advance. The will to step into the unknown and to face the mystery are indistinguishable. Granting purpose to nature, we could say that she does not want to fool us as much as to shake us in wonderment. Nature is also that which both magicians and scientists share, both working to simultaneously enchant and disenchant. At the end of the day, the world of magic and the magic quality of our world may not be so far apart as they seem.

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# Chronicle of a Pandemic Foretold

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The emergence and spread of the COVID-19 pandemic has raised many questions and doubts, ranging from the true "origins" of the virus (SARS-CoV-2) to problems related to clinical management of the disease. Here we discuss serious concerns that have emerged about the health policy measures adopted in Italy.

In order to properly assess these issues in perspective, we provide a detailed documented chronicle of the events as they came to light.

## Background

27th April 2018. During a conference organized by the Bill Gates Foundation with the prestigious New England Journal of Medicine and the Massachusetts Medical Society, Gates claimed that it was necessary to prepare for an imminent flu pandemic: "The world needs to prepare for pandemics in the same serious way it prepares for war [...]. This preparation includes staging simulations, war games and preparedness exercises so that we can better understand how diseases will spread and how to deal with responses such as quarantine and communications to minimize panic" [1]. Nothing new. Alarm about a future pandemic had already been raised long before the emergence of COVID-19, and at least since the outbreak of the SARS epidemics [2].

December 2019. On an uncertain day, the US Secret Service alerted its National Service counterpart about what was going on in Wuhan province, China. Fox News broadcast the information. In Italy the news was issued by Adn-Kronos (*Coronavirus, Fox News: 'Intelligence reports warned the Italian Government of the risks'*) [3]. The first COVID-19 patient appears to have been admitted to a Wuhan hospital on 8th December with a "mysterious disease". For seven weeks after that date, about 30,000 people per day left Wuhan for the rest of the world.

Early December 2019. Dr. Li Wenliang, an ophthalmologist in Wuhan hospital, recorded an unusual number of cases of atypical pneumonia among his patients. He posited a correlation with a virus-based epidemic associated with conjunctivitis, similar to SARS (severe acute respiratory syndrome). He mentioned this in chats with colleagues and patients. As often happens in communist regimes, he was consequently dismissed, imprisoned for a while and later reinstated. He died on 6th February after returning to his workplace to fight COVID-19. About 40 other Chinese doctors suffered the same fate: first marginalized and "silenced", then "rehabilitated" [4].

27th December 2019. Zang Jixian, a doctor in Hubei Provincial Hospital, reported to Wuhan Health Autho-

rities that a virus belonging to the SARS family was causing the infection. More than 180 patients were already infected by then, according to a South China Morning Post report [5]. This is the first reconstruction to put the Chinese authorities in difficulty [6].

## The Chronicle

31st December 2019. China formally notifies the WHO (World Health Organization) of the existence of a mysterious new influenza virus.

1st January 2020. Chinese authorities confirm the first victim of coronavirus.

7th January 2020. China communicates some clinical/biological traits of the virus to the WHO and takes the first measures to contain the disease.

12th January 2020. The WHO declares that there is no scientific evidence of human-to-human transmission of the virus [7].

13th January 2020. The first coronavirus victim outside China (a woman in Thailand).

15th January 2020. The first case is identified in the USA (an American citizen from Wuhan). An analysis by the Johns Hopkins University highlights the spread of the epidemic since November 2019 [8].

16th January 2020. First public report of the Italian National Health Institute on the spread of COVID-19 recommending to "strengthen standard measures of prevention and control of infections, especially in emergency rooms and emergency medicine departments". "Between 31st December 2019 and 12th January 2020, the Chinese Health Authorities identified 41 cases of pneumonia in the city of Wuhan caused by a new coronavirus (2019-nCoV). In January 2020, Thai and Japanese Health Authorities also reported two cases of 2019-nCoV infection in people from Wuhan City. Based on the information currently available, *WHO does not recommend any restrictions on travel or commercial routes* and the ECDC [European Council of Disease Control] considers the risk of introduction of the virus into Europe to be "low"" [9].

20th January 2020. An official speech about the virus by President Xi Jinping, broadcast by many international mass media, reckons that the infection travels from human to human, as confirmed by the National Health Commission of Beijing. On 20th January, Chinese scientists describe the genomic structure of the virus in detail (the virus was isolated on 7th January and the sequence was made public on 9th January 2020) and its similarities to SARS, as reported in an article published by *Nature* [10].

Significantly, the first officially ascertained case dates back to 1st January, and five more patients have been studied. Considering the amount of work this involves (the responses of numerous animal species to the virus were also highlighted in the study), we wonder how this research could have been conducted, completed, written and sent in less than 19 days. Such work generally takes much longer (the data must also be verified and replicated) and it is truly amazing that no one pointed out this discrepancy: how could the Chinese laboratories – even considering the understandable "urgency" and political pressure – have produced that result in less than three weeks? Actually, the entire sequence of events is highly suspect. The first official case is identified on 12th December 2019; the next day the Wuhan Animal Market is closed; 6 days later the virus has been isolated (!) and after two more days its sequence is published [11], after testing the virus on a battery of cells from different animals (including humans), and identifying the receptors to which the virus binds! Moreover, on 16th January, swabs and kits for RT-PCR analysis are distributed throughout the state of Hubei. Between 19th and 21st January, all Chinese provinces and regions are supplied with them. It is even more surprising that these kits were already supplied in advance to the WHO as of 12th January. Many of these procedures are compatible with the times reported, but not all of them. The suspicion is that the virus *was known and had already been studied before 9th January, when China officially sent data on the virus to the WHO*, as described in the joint WHO-China report (24th February) [12].

These questions fuel doubts about the Wuhan laboratory and raise new questions about the reliability of Chinese sources and reports. In particular:

1) *Contamination*. The Wuhan laboratory has been the subject of several surveys, as it was suspected to be responsible for accidental contamination. An article from Nature in 2017 details these risks [13]. The laboratory was planned in 2003, and built in 2004 with the direct participation/support of France (which boasts some of the best skills in the world in this sector). France also trained several young Chinese scientists in a laboratory in Lyon. In recent years, there have been accidental di-

spersals of SARS viruses from Beijing biosafety level 4 laboratory (China has two BSL-4s). China is currently planning two more BSL-4s with the pretext of studying SARS, claiming to have a large number of monkeys to test on. However, as international observers note, "We are not convinced of the need for more than one BSL-4 in mainland China". Ebright suspects that the expansion is a reaction to the networks in the United States and Europe, which he says are also unwarranted. "These facilities are inherently dual use", he says. The prospect of ramping up opportunities to inject monkeys with pathogens also worries, rather than excites, him: "They can run, they can scratch, and they can bite" [14]. The real boulder is in fact the lack of identification of the intermediate link of the virus, the one via which the virus passed from bat to man, "jumping" species (spillover). This is what renders plausible the hypothesis that the virus escaped from the Wuhan laboratory to infect humans directly. This is a critical question. If there is no evidence to explain the (hypothetical) spillover, other explanations remain in place, including accidental release of the virus from the Wuhan laboratory. Indeed, zoonotic spillover should not be given undue credit, because the epidemic curve is consistent with substantial human-to-human transmission [15]. Obviously, this possibility raises a number of embarrassing concerns.

2) *Viral manipulation: was COVID-19 "manufactured"?* This question arose because it is not clear how the virus, normally hosted by bats, could pass to humans without first adapting to an intermediate host. The question has not yet been answered. A study (hastily) published in *Nature Medicine*, excluded any intentional manipulation but left many hypotheses open: "Although the evidence shows that SARS-CoV-2 is not a purposefully manipulated virus, it is currently impossible to prove or disprove the other theories of its origin described here" [16]. It is however indisputable that COVID-19 comes from SARS (which was studied in Wuhan); indeed, the official name incorporates this derivation. It is also strange that "the overall molecular structure of this virus is distinct from that of known coronaviruses but most closely resembles viruses found in bats and pangolins that have been little studied and never known to cause humans any harm" [17]. Furthermore, Chinese scholars write: "According to researchers from Nanki University in Tianjin China, COVID-19 contains a strange HIV-like mutation that may make it more contagious and give it properties not found in other coronaviruses. The Chinese study builds on earlier research in India that concluded that the disease was unlikely to have originated in nature. This comes amid speculation that COVID-19 originated in a Chinese research lab located in Wuhan. While these theories remain unconfirmed, they should not be dismissed as conspiracies" [18]. Indeed, previous Hantavirus outbreaks have been associated with laboratory rats in Yunnan (China) [19], while genetic modifications have purportedly been performed on different strains of coronavirus. Namely, the receptor-binding capacity of coronaviruses has been investigated by combining a human immunodeficiency virus-based pseudovirus system with cell lines expressing the ACE2 molecules of humans and animals [20], thus enabling the coronavirus to enter human cells while native viral proteins are unable to do so. Indeed, it is quite unlikely that a virus acquire such unique insertions naturally in a short time. This structural change may also have increased the range of host cells that 2019-nCoV can infect. A hypothesis of this type was recently supported by Nobel laureate Luc Montagnier and bio-mathematician Jean-Claude Perez, who suggested that the new COVID-19 could be the fruit of inexperience in an attempt to produce an anti-HIV antibody [21]. Several scientists have rejected this statement with outrage; however, since the claim comes from the very scientist who discovered and isolated the HIV virus, the hypothesis deserves to be held in high regard.

3) *Distorted chronology*. The temporal reconstruction of the events is manifestly distorted, as stated by Dr Li Wenliang, who already in a chat of 31st December 2019 spoke of "six patients with identified viruses belonging to a SARS-like subtype, of the coronavirus type" [22], suffering from conjunctivitis and atypical pneumonia. This admission documents how a pre-characterization of the virus had already been done before 20th January. Dr Li recommended that friends and relatives take precautions. Two days later he was interrogated by the police, warned by the authorities not to spread alarm and removed from the hospital, only to return a few days later. A similar fate has befallen other doctors. In fact, according to investigations by the Johns Hopkins University, recently confirmed by the South China Morning Post [23], the first cases of SARS-CoV-2 date back to early November 2019 (3rd-17th November).

21st January 2020. China decides to isolate the Wuhan Region with blockade of trains and planes.

22nd January 2020. The victims of COVID-19 in China are officially 17. Meeting of the operational Task Force of the Chinese Ministry of Health.

23rd January 2020. Two Chinese tourists disembark at Malpensa Airport in Milan. They arrive in Rome on 29th January, where they are stopped and identified as infected with COVID-19. The WHO, for its part, organizes a meeting to decree an emergency, however, China blocks that step. WHO General Director, Tedros Adhanom Ghebreyesus, who was accused of "hiding" three cholera epidemics when he was Minister of Health in Ethiopia [24], flies to Beijing to discuss the matter with the Chinese President. On his return, he praises China for its efforts in health management and declares that there is no reason to "interfere" with the free movement of persons and goods by freezing transport to and from China.

24th January 2020. It is revealed that the first COVID-19 patient in China was hospitalized in Wuhan on 8th December 2019, recorded as having a "mysterious disease". From that moment on, for seven weeks, about 30,000 persons per day left Wuhan for the rest of the world. A suspected case of COVID-19, a musician who had returned from Wuhan, is identified in Parma in January [25]. In the same days, the first three infected persons are discovered in France, one coming from Wuhan [26]. The escalation of cases is worrying. In the face of these signals, the Western media shows skepticism and tends to minimize the "accidents". Italian newspapers prefer to "constrain" any form of "alarmism". According to Il Foglio, "Concern and caution are one thing, but the alarmism that has been raging in the last few days is proliferating faster than the virus and risks doing serious damage. The concern is also about China: the regime tries to appear responsible but according to various experts the precautions it has taken so far have been too weak and too late" [27]. Faced with this news, it is disarming to read in the *Giornale della Protezione Civile* on 24th January: "Italy has a plan against the coronavirus, but so far no alarm" [28].

25th January. China declares a 60% increase in infections in two days [29]. The Americans evacuate from China [30].

27th January. Israeli analysts link the coronavirus emergency to the Wuhan laboratory, suggesting that an "engineered" virus could have escaped, by mistake or through incompetence, and infected the town's animal market [31].

28th January. A case of COVID-19 infection is identified in Germany in a person who has never been to China [32].

30th January. The WHO declares COVID-19 a "Global Health Emergency" [33]. The first two cases are identified in Italy.

31st January. The Italian Council of Ministers decrees a state of emergency for six months. The Emilia Romagna Region organizes a "task force" and decides to set up two "rooms for restrictive isolation per Province, reserved for serious and stable patients", one with respiratory support. It also decides "not to isolate symptomatic patients from Southeast Asia, except those from the Wuhan area" [34].

2nd February. The Chinese community of Bologna has been procuring medical supplies to send to Wuhan for two weeks (10 thousand masks, 550 coveralls and 10 thousand pairs of gloves) [35].

15th February. A Chinese returning from Wuhan with COVID-19 dies in France [36].

22nd February. The Italian Council of Ministers launches the first real decree to counter the transmission of the coronavirus.

16-24th February. Although the WHO has declared a "global health emergency" as of 30th January, only now (16th February) does a WHO delegation travel to China, where it remains for nine days to conduct an extensive investigation [37]. Its report highlights that sporadic cases of abnormal pneumonia, suspected to be caused by a SARS-like virus, had been reported since October 2019. The second critical point concerns the origin. Since the natural reservoir of the virus is bats, the intermediate host through which the virus reached humans has not been identified. Failure to find the missing link leaves open the possibility that the virus escaped (accidentally) from the Wuhan BSL-4 laboratory. The report also highlights that health workers are particularly exposed to the infection (2055 health care workers are reported to be infected in 476 hospitals in China) and require special and urgent precautions. Indeed, on 20th February, China implements additional measures to protect physicians. The WHO report is in the hands of the Italian government as early as 25th February, yet no measures are taken to protect health professionals. Overall, however, the WHO report is contradictory:

a) Traditional belief in the benefits and miraculous virtues of certain foods of animal origin has nourished unhealthy and dangerous dietary habits among Chinese

citizens, such as "jinbu" or intake of meat from live animals or otherwise without prior sanitation. Numerous reports indicate that the experimental animals used in Wuhan's BSL-4 laboratory are often "resold" to the local market for extra earnings, instead of being properly disposed of by cremation, as the law requires. One Beijing researcher, now in jail, made the equivalent of a million dollars selling monkeys and rats on the live animal market, "whence they likely wound up in someone's stomach" [38]. The report does not suggest any recommendation regarding this critical question.

b) The report lavishes embarrassing flattery on the Chinese regime for the effectiveness and promptness of measures that nipped the epidemic in the bud, making it unnecessary to issue a general alarm. On the other hand, however, the report cannot fail to detect that the virus is "highly contagious, can spread quickly, and must be considered capable of causing enormous health, economic and societal impacts in any setting". The report does not stigmatize the fact that the Chinese authorities deliberately decided not to count asymptomatic patients among the infected: this underestimation has enormous consequences in terms of epidemiological evaluation. In fact, as early as February, data silenced by the Chinese government clearly indicated that more than a third of positive patients are asymptomatic and vehicles of infection [39]. Keeping this information secret objectively favored worldwide spread of the virus and weakened any strategies for containing the epidemic, since by definition and at the indication of the WHO, asymptomatic cases were not traced or tested. This indication was followed by the *Italian Consiglio Superiore della Sanità* on 26th February [40]. According to an analysis reported by Science [41], asymptomatic cases caused 79% of clinically detectable cases. The report finally borders on paradox when it considers other countries unable to take the measures adopted by China with equal efficacy and determination: "Much of the global community is not yet ready, in mindset and materially, to implement the measures that have been employed to contain COVID-19 in China. These are the only measures that are currently proven to interrupt or minimize transmission chains in humans". Finally, the report asks other countries to activate the "Emergency plan", immediately at maximum level: "Prepare to immediately activate the highest level of emergency response mechanisms to trigger the all-of-government and all-of society approach that is essential for early containment of a COVID-19 outbreak".

In the same period in Italy, government officials tried to minimize the infection. Towards the end of February 2020 certain politicians declared that there was no cause for concern, since the disease in question was "little more than a flu". The Mayor of Florence Maurizio Nardella organized a dinner with many guests in a Chinese restaurant to dispel "legends" about the risk of spreading the disease. Slogans in the month of February were "Embrace a Chinese" and "Reopen Milan" (27th February, "Coronavirus, Sala calls Conte: "Let's reopen Milan as soon as possible") [42]. Heedless of the serious danger posed by the unfortunate situation already existing in Italy, those who called for urgent measures to contain the contagion were dubbed "fascists" or "racists" by exponents and fans of the current parliamentary majority.

23rd February, 2020. The Italian Government issues the first containment measures, without giving any details [43].

11th March 2020. The WHO declares the status of pandemic, when coronavirus cases have reached 287,000 worldwide, on five continents [44].

## Data uncertainties: diagnostic unreliability and the limits of statistical models

From the outset, the management of the ongoing epidemic has been characterized by four orders of uncertainty pertaining to the reliability of the data. This is of no secondary importance, because the narrative of the mass media, government decisions and opposition responses were based on data, which was on the whole absolutely unreliable. Doubts regarding the available information include:

1) *Data from China*. We know today that the first cases of COVID-19 were recorded in early November 2019. The Chinese authorities began to study and isolate the virus long before 9th January 2020, when they sent their preliminary report to the WHO. Uncertainty also remains regarding the true death rate and the incidence of COVID-19 [45].

2) *Italian data*. In the absence of a population-sample-based screening program (which can then be extrapolated to the general population) or a systematic

investigation in the most affected region (Lombardy), assessment of the spread of the epidemic (number of people infected regardless of their clinical status) was absolutely inadequate. This not only led to underestimation of the extent of the infection, but also to gross calculation errors, especially regarding the lethality of COVID-19. Lethality is obtained by calculating the ratio of the number of casualties to the number of positive cases. If the denominator increases, the value of lethality decreases. Current estimates suggest that the number of infected people greatly exceeds the number of positive tests, e.g. by a factor of 35 [46]. Moreover, there is growing evidence that the death figures reported daily by Italian officials may be grossly underestimated [47].

3) *Technical limits*. Technical limits concern how the data was collected, the inclusion criteria (especially with regard to cases of death) and the reliability of the analytical determinations (buffers, virus genomics etc.). To limit the example to China, out of 76,314 cases reported in an extensive review, 22.4% were classified as "suspect cases", 14.6% as "clinically diagnosed" and 1.2% as "asymptomatic" [48]. This means that 37% of the cases reported in Chinese statistics so far were only diagnosed on a clinical basis ("suspect cases" according to the WHO definition). Yet mainstream information to the world population presented them all as "firmly established". Antibody testing, which should have been considered fundamental for confirming or refuting acute infection, was ignored until the end of April. This technique was available from the beginning, but was not used until much later. Confirmation of cases was mainly based on nasopharyngeal swabs and gene amplification by RT-PCR, a non-validated, non-standardized technique that seems to give many false positives and false negatives [49]. Another concern about PCR-based tests is that there has not been enough time to assess their sensitivity and specificity. Based on personal communications with colleagues, a significant proportion of patients who meet clinical and chest CT diagnostic criteria for COVID-19, including many hospitalized patients, tested negative for viral RNA [50]. Other common respiratory etiologies, such as influenza, were excluded but remain "suspect" cases that may be false negative to PCR [51]. In some patients, the virus may be present in lower respiratory secretions but absent in the upper respiratory tract. With current tests, it is therefore difficult to obtain a meaningful assessment of the percentage of symptomatic infected patients [52, 53]. There is also a huge problem relating to the attribution of causes of death. First, only a few autopsies have been performed, and this makes it impossible to correctly ascribe the cause of death to the virus and to understand the pathogenic mechanisms involved. Secondly, because the lethality risk data is artificially overestimated when pre-existing pathologies – often more serious than the COVID-19 infection – are discarded. In Italy, the "all in" criterion was used, whereas elsewhere, as in Germany, the more rational approach of assessing the predominant "causative" role of the virus was applied. This discrepancy could explain the hugely different lethality rate between Italy and Germany at the end of March 2020 (11.40% versus 0.9%). Then if we want to evaluate mortality (as distinct from lethality), we have to compare all deaths recorded in a certain region with those recorded in previous years to verify whether the epidemic caused (and to what extent) more deaths than expected.

4) *Ambiguous and confused communication*. Finally, these elements influenced the communication model adopted by the government, generally characterized by an absence of officialdom (interviews in the context of entertainment programs, TV lounges and talk-shows), based on contradictory announcements (statements not followed by concrete acts or worse still denied shorty after), improvised (communications of closures and lockdowns not associated with the necessary containment measures) and based on alarmism rather than on the need to inform and equip the public. Thus, the habit of communicating the "war bulletin" every day at a given hour, together with the uninterrupted procession of experts, each eager to give his own version, aroused concern far beyond what was necessary and desirable, as well as anxiety and confusion.

## Failure to act and political inadequacy

a) *The World Health Organization* (WHO). The behavior of the WHO appears to have been based on a serene temporal reconstruction, discreditable to say the least and showing no hint of self-criticism or self-reproach. The WHO has shown culpable omissions, delays and inadequacies:

1) The WHO did not monitor the Wuhan laboratory, despite the fact that its activities had long been targeted by the scientific community.

2) It did not promptly call a state of alert, necessary on isolation of the new viral strain (9th January 2020).

3) It issued the pandemic alert about 40 days late (15th January), when besides the Wuhan outbreak, full-blown cases of COVID-19 were already known in Thailand, Japan and the United States. By 20th January, it was clear that the infection was transmitted between humans via the respiratory route. Yet in its press releases and the final report downstream of the control visit to China (24th February), the WHO persisted in declaring the situation to be under control and in praising China for the measures implemented. At the same time, the WHO firmly stated that blocking flights to and from China "was an error" [54]. This position was even reinforced by the Director-General, Tedros Adhanom Ghebreyesus, according to whom "these limitations risk increasing fear and discrimination and have poor public health results" [55].

4) In the meantime, the WHO was ignoring alarms from Japan, Korea and Taiwan. All too ready with praise for China, incredibly the WHO did not indicate the efficient protocols implemented by Korea and Taiwan as a model to stem the epidemic. Even more incredible, it deliberately ignored the alarm launched by Taiwan in early December about the possible outbreak of an epidemic from China [56].

In summary, there is no doubt that relations between China and the WHO are not transparent. As already happened in 2003 for SARS, China has clear responsibilities regarding emergence of the epidemic, its worldwide spread and other countries' delay in dealing with it.

b) *The Italian Government*

The Italian government continues to show hesitations and inadequacies that must be clearly denounced.

First of all, it shows a general lack of awareness of the epoch-making problem of dangerous epidemics. At the centenary of what is known as the Spanish flu (1918-1920), re-examined in several recent books and reports, its absence of understanding of the danger of periodic flu-based epidemics is incomprehensible [2, 57, 58]. The Lancet has appropriately stigmatized this incompetence on the part of political authorities by speaking openly of "trained incapacity" [59]. The Italian government is no exception: like other States it demonstrated that it had failed to learn from previous experience and failed to heed the recommendations of the scientific community, which has been expecting a new flu pandemic since the turn of the century [60]. It is embarrassing, to say the least, that the Italian government was late in activating the National Plan of Preparedness and Response to a Flu Epidemic (updated in 2016) [61], despite widespread alarm. The objective of the Plan, articulated in six activation phases, is to prepare the country to deal with an epidemic/pandemic threat. The plan was conceived as a resource that could be brought into action, even in the absence of alarm issued by the WHO. The plan remained inactive, despite outbreaks of atypical viral pneumonia in many areas of Lombardy, Veneto and Emilia Romagna in December 2019 [62]. Furthermore, as early as 28th December, 40 abnormal cases of viral pneumonia were reported in Piacenza hospital [63]. A subsequent study by the University of Milan pinpointed onset of the epidemic in Italy between October and November, well before the first confirmed case at Codogno [64]. These reports prompted the Ministry of Health to issue a specific decree on 5th January, warning about the possibility of an unusual "flu epidemic", and requesting special attention to viral pneumonia coming from China [65]. The alert, reiterated on 12th January, continued to link virus and epidemic, but paradoxically highlighted that based on information received from China, the WHO was "reassured by the quality of the ongoing investigations [in China] and response measures implemented in Wuhan." Finally, on 16th January, we witness a turnaround, a real manipulation of the truth, in a document that goes back to talking about a possible epidemic but the link with China is deleted and replaced with "Japan", the source of the epidemic ("Japan (ex-China)") [66]! To sum up, the Plan for the management of pandemics was ignored, then activated late and incompletely, as pointed out by the Italian press [67].

## Concluding (preliminary) remarks

In September 2019, the Johns Hopkins Center for Health Security issued a long document on the dangers of a forthcoming flu pandemic, made increasingly probable by globalization phenomena and unsafe handling of animals and the meat market, which significantly increase the risk of transmission of zoonotic disease to humans [68]. We had a "taste" of these dangers with the epidemic of bovine spongiform encephalopathy (BSE, also known as mad cow disease) in 2001, and later with

the SARS, MERS and other avian epidemics since 2003. The current epidemic is not exactly an "unexpected" event that took the authorities by surprise. Scientists and technicians of the state administration knew perfectly well that it could happen, and in a sense, they considered it an imminent possibility. On 11th March 2019, *La Repubblica*, a major Italian newspaper, wrote: "It is not a question of whether but of when (the pandemic) will arrive. The Global Influenza Strategy is the newly launched program to address risk. It has two objectives: investing in research and improving surveillance and intervention systems" [69].

The COVID epidemic has highlighted two other fundamental limits of healthcare organization in Italy:

1. *Inadequate support to scientific research*. For years, Italian scientific research has received inadequate funding compared to other countries in the European Union, despite its high level and productivity (three times that of Germany on a quarter of the funding). Four months after the outbreak of COVID-19 and even now, few resources (less than 20 million euro) have been allocated for the scientific emergency related to COVID-19.

2. *Shortfalls in Italian healthcare architecture*. In recent years the concept of medicine inherited from the great Greco-Roman and Christian tradition has been demeaned. Hospitals, once known as *Hôtels de Dieu*, have become Business Companies for Health (*Azienda Sanitaria*), where decisions are made and evaluated on the basis of financial and management efficiency criteria. This destroyed territorial (precinct) medicine, and created multi-specialist centers of attraction, conceived as all-inclusive healthcare terminals for entire macro-regions. Territorial medical units enable better care and management of patients in the home setting. They also make technical and financial resources available for diversifying health services and their quality. In particular, the need for more intensive care units had been known since 2012, when the Monti government cut about 2/3 of the beds. Sadly, nothing was done to remedy this shortage and even in the first days of March nothing happened immediately. Another aspect is the gradual disappearance, since the 90s, of the term "prevention" from health managers' vocabulary. The concept of prevention flourished in the 70s and 80s to address complex issues such as pathologies caused by environmental pollution, occupational exposure, as well as degenerative (cancer) and metabolic (obesity, diabetes) diseases. Sacrificing the paradigm of prevention meant underestimating the risks of foreseeable new pandemics.

However, the COVID-19 outbreak offers an opportunity to rethink the health model of recent decades, including the role played (or that should have been played) by certain international organizations.

1. *The European Union*. The EU made disparate belated decisions, and indeed shone for the lack of coherent measures. No meeting was organized to specifically address the SARS-CoV-2 emergency, and the European Commission's constituent program was largely overlooked, particularly in its capacity to cope with "emerging global threats" [70].

2. *Finally the WHO*. The WHO is guilty of remaining silent and underestimating the danger, as well as covering China's errors and delays. China's behavior has been ambiguous: we still do not know with certainty when the epidemic broke out, how many deaths it caused and where the virus originated. Certainly Chinese research in the field of transgenesis and molecular biology has long been the subject of attention due to its ethical and safety implications. Over the years, Chinese researchers have too easily circumvented safety rules and ethical principles, in many cases incurring criticism and criminal convictions, as in the case of Dr He [71]. The risks associated with the possibility of pandemics related to inappropriate gene manipulation of viral and bacterial strains have been the subject of many studies [72]. For too long, "preparedness strategies for public-health emergencies have been neglected, and communities remain ill equipped to face a sudden epidemic, let alone a global pandemic. Perhaps the looming specter of a potentially devastating pandemic will kill off this false sense of security, and concentrate the minds and budgets of both governments and research communities towards preventing another superbug scourge" [73].

The real possibility that this scenario could materialize calls for application of the precautionary principle, in the spirit of the Cartagena Convention (1992). It is still not too late to propose a moratorium on projects of transgenesis and genetic modification of viruses and potentially pathogenic bacteria.

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# Thinking Beyond the "Epidemic of Epidemics"

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One of the major challenges of any historical science is the role of forecasting. Biology is a historical science, because organisms can only be understood in a temporal, phylogenetic and ontogenetic, perspective. In particular, the time of biology, and therefore of correlated sciences (ecology etc.), is a time of change in the "space of possibilities" (of "phases" as we say in physics, of ecosystems and species in biology), punctuated by rare events - evolutionary novelties, speciation. In physics, the space (of phases) is fixed as "a priori" of knowledge, as "condition of possibility" for "writing equations", explain Newton and Kant: it a priori contains all possible trajectories - unpredictability is within these trajectories (the randomness of a die concerns six possibilities, no more, no less). In biology, to physical randomness is added the unpredictability of changes of the space of possibilities and of rare events, to which one cannot even assign probability values, see (Longo, 2020). The historian of human affairs will recognize there elements of his theorization, although, of course, symbolic culture, in all its forms, imposes an important change in the tools of analysis.

Faced with the intrinsic unpredictability of the history of the living, should we remain silent? No, science is not (or not only) the analytical game of "experience/ observation, theory, prediction, verification/ falsification", but first of all a construction of objectivity, or even objects of knowledge, through difficult operations of "carving-out" (separating/distinguishing) and "qualification" of reality. This is how Darwinian theory offers us a remarkable historical theory of the living, of "species", a notion that is constantly being re-carved-out and re-qualified. This theory does not allow us to predict, but, by making us understand, it allows us to act, if we assume the risk of relying on the best available knowledge. We then decide to measure "biodiversity", an admittedly arbitrary partition of species and life forms, which is always open to discussion and revision; to assess the impact of man on an ecosystem, a difficult qualification of the consequences of activities that are sometimes centuries old. We can also give ourselves a measure of the notion of 'epidemic' and draw the historical diagram shown here ("The Evolution of the number of epidemics of infectious diseases in the world from 1950 to 2010").

Partial and revisable knowledge: is it enough to act? Yes, it is. Since the 1990s, many epidemiologists have been warning us: the notion of "epidemic of epidemics" dates back to 1993 and this 2015 diagram shows it to us (Fig.1). The reasons are well described in (Morand and Figuié, 2015): some cases may be due to a synthetic biology that claims to be all-powerful and let us believe that we can fully control living organisms by modifying ("editing") their DNA/RNA, but more than 70% of these emerging infectious diseases come from animals, at new interfaces with the environment. Deforestation for agricultural settlement accompanied by intensive livestock farming enables the passage of bacteria and viruses from wild animals to livestock and then to humans. None of these cases and microorganisms were indivi-

dually predictable, and none will be in the future: they and their causes are known a posteriori.

The self-serving denial of the history of life, of the evolutionary construction of ecosystems, of their specificity and diversity, is the main cause of the activities that destroy them. Often this denial finds its justification in a new scientism, which erases science. On the one hand, the spontaneity of the man/economy/nature dynamics would choose the best possible path - a misuse of the mathematics for the equilibrium physics of the 19th century. On the other hand, nature itself would be an adjustable, even programmable machine, with disposable material and biological "resources". A new awareness and a science of these phenomena is being built, change is possible: the knowledge of a history, as in the diagram above, and a vision of organisms in their autonomy and their dependence on the ecosystem make it possible to act.

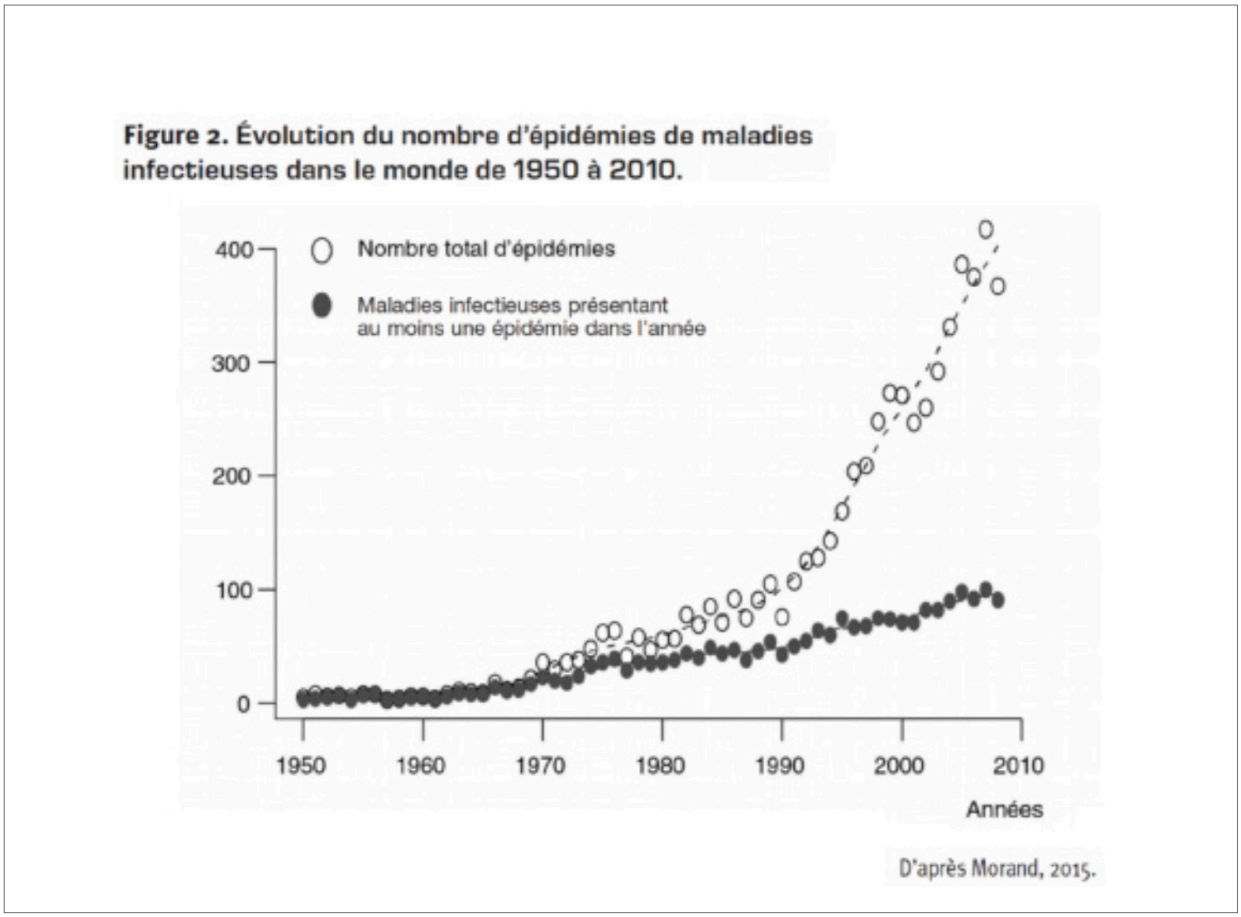


**Figure 1**

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# COVID-19 Calls for Mathematics, Part 1: Neuraminidase Inhibitors, Chloroquine and Hydroxychloroquine

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**Abstract**
Looking at the outbreak of SARS-CoV-2 and the global state of emergency imposed due to its pandemic spread, the necessity for antiviral drugs to be immediately available is a priority for the scientific community. Considering that research and implementation of new antiviral therapies or vaccines usually take a long time, the World Health Organization (WHO) has proposed to use in commerce drugs: in fact, repurposing drugs which are already accessible in large quantities is easier to fight against the virus, at least during the first emergency phase. In this article, we discuss various mathematical models which simulate the action of antiviral drug therapies, such as neuraminidase inhibitors (NIs), for the treatment of H1N1 Influenza A virus, by using data collected through in vitro and in vivo experiments. This constitutes a paradigmatic case of study for paving the way to a systematic investigation of the effects of chloroquine and hydroxychloroquine as therapeutics in the treatment of SARS-CoV-2.

**Keywords:** SARS-CoV-2, Influenza A virus, basic reproduction number, neuraminidase inhibitors, interferon-mediated immune response, target cell-limited model, antiviral treatments, chloroquine and hydroxychloroquine, numerical simulations, experimental data

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## 1. Mathematical modeling of influenza: a virus dynamics

Given the current lack of a vaccine for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the virus causing COVID-19, it is worthwhile evaluating potential prophylactic or therapeutic effects of drugs which are clinically approved for other indications. Chloroquine, and its derivative hydroxychloroquine (HCQ), have been already used with good results for the treatment of malaria and also in some autoimmune diseases, with the most common side effect being eye damage after heavy dosage and long-term administration [5]. The precise mechanisms through which chloroquine may act to attenuate SARS-CoV-2 infection are of considerable interest, as this information could be valuable for identifying new treatments, while waiting for a vaccine.

Chloroquine is a weak base which becomes entrapped in membrane-enclosed low pH organelles, interfering with their acidification [17]. Chloroquine accumulates in the digestive vacuole of infected cells, in which the treatment leads to an increase of lysosomal pH. Speculation on chloroquine-induced antiviral effects hence include inhibition of pH-dependent viral fusion/replication and prevention of receptor binding by viral envelope glycoproteins; chloroquine may also inhibit virions assembly in endoplasmic reticulum Golgi intermediate compartment (ERGIC). Moreover, due to its potentiality in diminishing the expression of pro-inflammatory factors and receptors, such as cytokines, chloroquine has already been proposed in 2003 as treatment for SARS [17], which is primarily responsible for coronavirus-associated mortality.

It has been demonstrated that chloroquine is a broad-spectrum inhibitor of nanoparticle endocytosis by resident macrophages, since chloroquine decreases the accumulation of some synthetic nanoparticles in cell lines [23, 16]. Indeed, mechanistic studies have revealed that chloroquine reduces the expression of phosphatidylinositol binding clathrin assembly protein (PICALM), which is a cargo-selecting clathrin adaptor sensing and driving membrane curvature, thereby regulating the rate of endocytosis [14]. If PICALM runs out, then the clathrin-mediated endocytosis, which is a predominant pathway for synthetic nanoparticle internalization, is inhibited. Furthermore, chloroquine is known to prevent lysosome acidification, which is likely to interfere with upstream endocytic trafficking, causing a 'traffic jam' scenario that blocks effective transport of cargo to and from the cell membrane [23, 16].

It has been shown that SARS-CoV-2 falls within the same size range (60-140 nm) and shape (spherical) as commonly studied synthetic nanoparticles [24], which are typically sensible to chloroquine action [16, 9]. Therefore, one of the mechanisms responsible for chloroquine-mediated effects against SARS-CoV-2 could be a general decrease in the ability of cells to perform endocytosis. Moreover, previous studies for SARS-CoV-1 infection [10, 22] are possibly useful also for SARS-CoV-2, since these two viruses might employ similar angiotensin-converting enzyme 2 (ACE2) mediated mechanisms of cell entry. Even if chloroquine mechanism of lysosome acidification is likely to interfere with

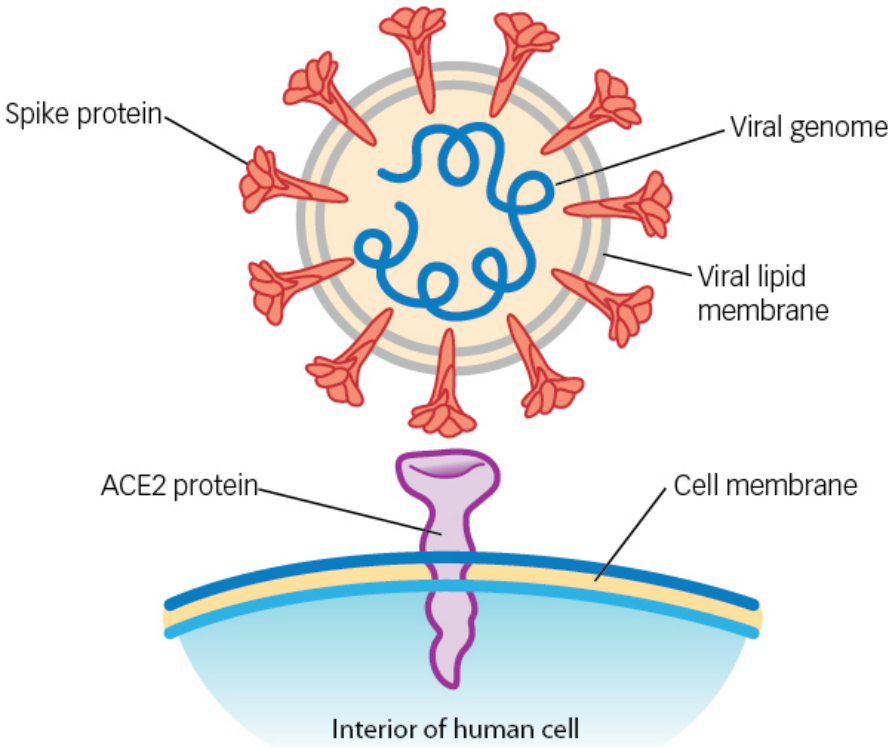


**Figure 1:** SARS-CoV-2 enters human cells using spike proteins as a bridge between viral envelope and cell membrane – www.perioimplantadvisory.com/periodontics/oral-medicine-anesthetics-and-oral-systemic-connection/article/14173521/covid19-and-the-problem-with-dental-aerosols.

the action of membrane receptors, previous studies have revealed that chloroquine has therapeutic activity against SARS-CoV in cell culture but does not alter cell-surface levels of ACE2 [20]. Additionally, it has been proven that therapeutic doses of chloroquine do not substantially change the biosynthesis or glycosylation of the SARS-CoV spike glycoprotein [20], which is fundamental for virus entry into cells. On the contrary, this antiviral might have an important role in preventing the entry of virions in cells. Indeed, lysosomal acidification is responsible of a conformational change in the spike protein, bridging the viral envelope and the endosomal membrane together to enable fusion; thus, chloroquine-induced inhibition of endosomal acidification is likely to alter this fusion event, stalling the virus in endosomes (see Figure 1).

However, the use of chloroquine has some serious limitations, because part of its pharmacokinetics remains unknown, and its real efficacy is still not well defined [9, 19, 4].

As paradigmatic case of study, we discuss a mathematical model which illustrates the action of neuraminidase inhibitors (NIs), such as Zanamivir, for the treatment of H1N1 Influenza A virus, according to the results by Baccam et al. [1]. This model has been introduced to analyze the virus kinetics in the upper respiratory tracts of infected adults, by focusing on the importance of immune response to better fit with available experimental data. When we do not consider the application of antiviral treatments, the so-called *target cell-limited model with eclipse phase* is given by the following ordinary differential equations:

$$\begin{cases} \dfrac{dT}{dt} = -\beta TV \\ \dfrac{dI_1}{dt} = \beta TV - kI_1 \\ \dfrac{dI_2}{dt} = kI_1 - \delta I_2 \\ \dfrac{dV}{dt} = pI_2 - cV \end{cases} \tag{1}$$

where $T$ is the number of uninfected target cells, $I_1$ is the number of infected cells not yet producing virus (i.e. in the eclipse phase), $I_2$ is the number of infected cells actively producing virus, and $V$ denotes the infectious-viral titer expressed in TCID50/ml of nasal

| patient | $V_0$ TCID50/ml | $\beta$ $\mathrm{d}^{-1}(\mathrm{TCID50/ml})^{-1}$ | $k$ $\mathrm{d}^{-1}$ |
|---|---|---|---|
| 1 | $4.3 \times 10^{-2}$ | $4.9 \times 10^{-5}$ | 3.9 |
| 2 | $3.1 \times 10^{-7}$ | $1.1 \times 10^{-3}$ | 2.0 |
| 3 | $7.0 \times 10^{-1}$ | $1.7 \times 10^{-4}$ | 4.9 |
| 4 | 4.9 | $5.3 \times 10^{-6}$ | 4.0 |
| 5 | 1.7 | $2.7 \times 10^{-6}$ | 6.0 |
| 6 | 2.4 | $8.4 \times 10^{-6}$ | 4.4 |

| patient | $p$ $\mathrm{d}^{-1}$ | $c$ $\mathrm{d}^{-1}\mathrm{TCID50/ml}$ | $\delta$ $\mathrm{d}^{-1}$ | $R_0$ |
|---|---|---|---|---|
| 1 | 4.2 | $2.8 \times 10^{-2}$ | 4.3 | 30.4 |
| 2 | 10.9 | $2.1 \times 10^{-2}$ | 11.0 | 77.1 |
| 3 | 2.3 | $3.0 \times 10^{-3}$ | 2.2 | 40.3 |
| 4 | 3.8 | $1.3 \times 10^{-1}$ | 3.8 | 19.1 |
| 5 | 13.5 | $5.9 \times 10^{-1}$ | 13.5 | 3.5 |
| 6 | 3.8 | $7.1 \times 10^{-2}$ | 3.7 | 17.0 |

**Table 1:** Patient-specific best-fit parameter values for the target cell-limited model (1) as found in [1].

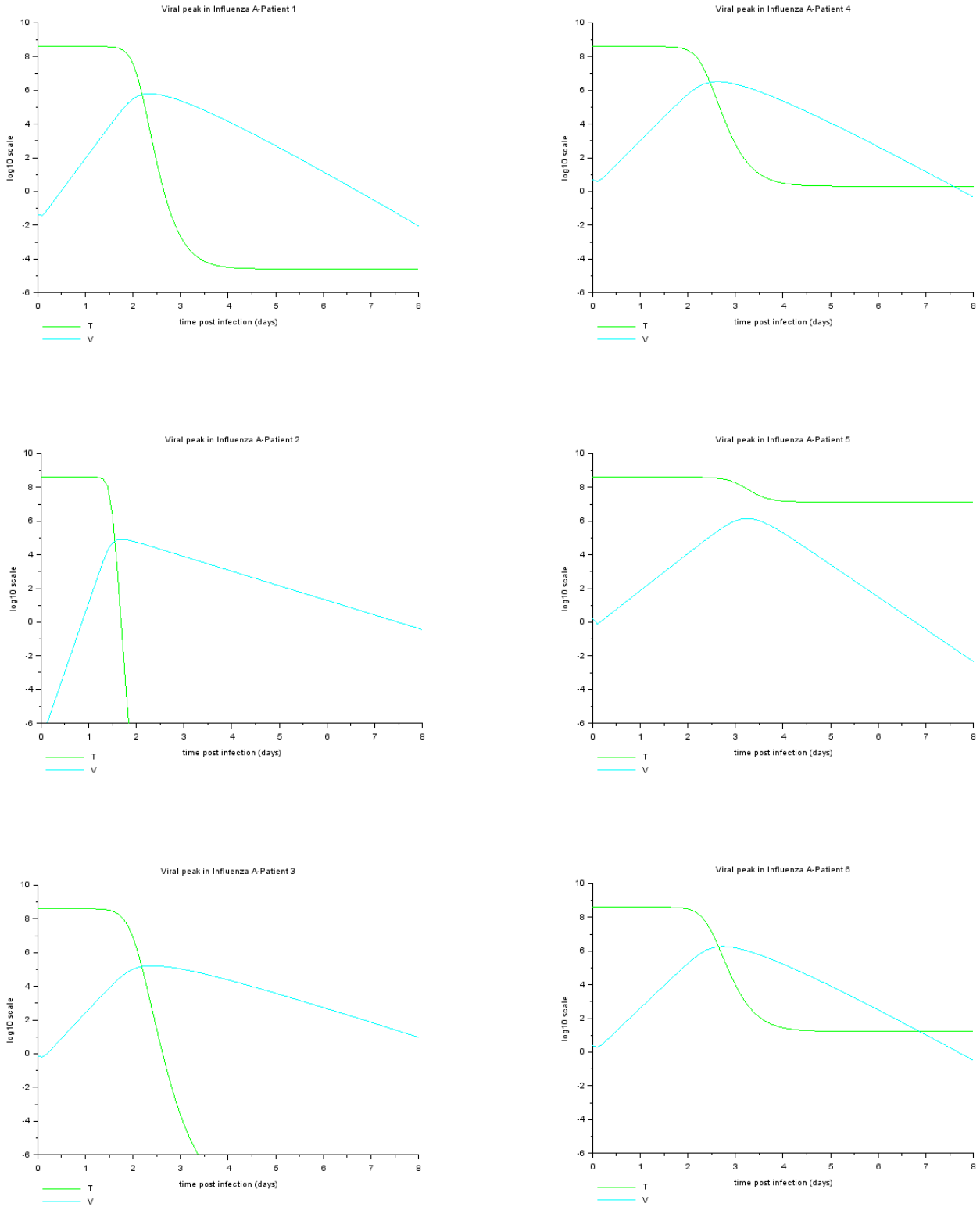


**Figure 2:** Viral titer in TCID50/ml of nasal wash and fraction of target cells remaining over the course of infection (8 days) in six different patients, corresponding to the experimental data in Table 1.

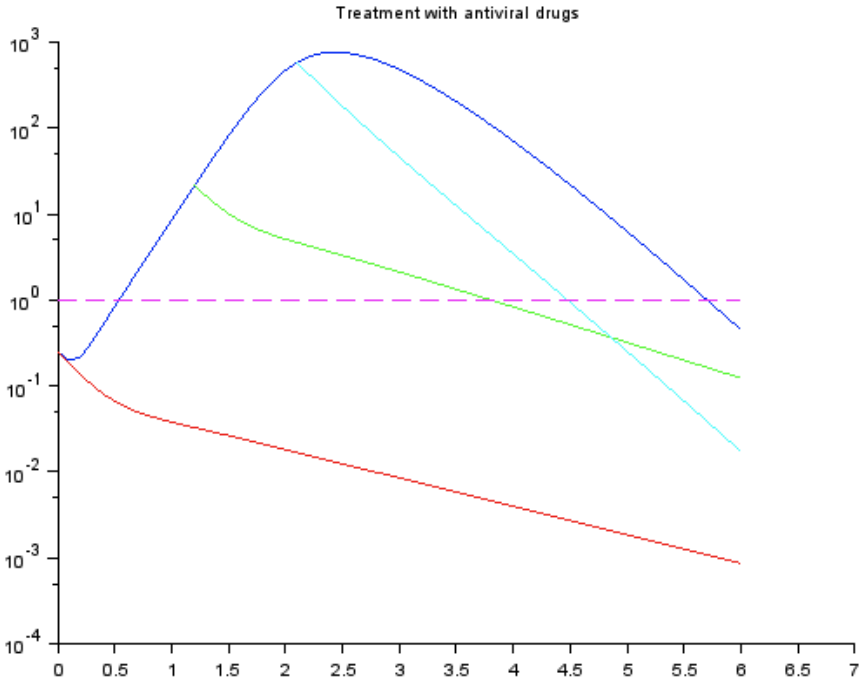


**Figure 3:** Course of Influenza A virus infection with and without the neuraminidase inhibitor Zanamivir administrated intranasally. The predicted virus titer dynamics from model (1) is shown for the placebo group (blue), the delayed-treatment group (light blue), the early-treatment group (green) and the preventive-treatment group (red). The horizontal magenta line marks the experimental limit of detection for the viral titer. We take the initial data for the green curve at time 1:2 of the placebo group and for the light blue curve at time 2.08 of the placebo group, together with 0.03 x $p$ as virus production rate (that is because we suppose an instantaneous change of $p$ value due to the antiviral administration with respect to previous placebo patients, and the system (1) is actually time-autonomous).

wash. The parameter $\beta$ represents the constant rate characterizing the infection of target cells, which become infected cells in the eclipse phase ($I_1$), successively transforming into cells which actively produce virus ($I_2$) with an average transition time $1/k$. In turn, these cells increase the viral titer ($V$) by releasing virions at an average rate $p$ (per cell), and die at a rate $\delta$ (per cell), thus $1/\delta$ is the average life span of a productively infected cell. Instead, free virus is cleared at a rate of $c$ per day.

We point out that separation of the infected cells into two populations increases the realism of the model, because delays in the production of virus after the time of initial infection are part of the viral life cycle. Furthermore, to be more focused on the process of cells decrease by the virus infection, the model (1) neglects target cells proliferation and natural death, since the infection typically exhibits a shorter timescale.

For the model (1), it is possible to compute the *basic reproduction number* $R_0$ as the average number of second-generation infections produced by a single infected cell initially placed in a population of entirely susceptible cells [1, 2, 4], namely

$$R_0 = \frac{p\,\beta\,T_0}{c\,\delta}\,, \qquad (2)$$

where $T_0$ is the number of target cells available at the starting time of the infection. If $R_0 > 1$, then an infection can actually be established and it expands exponentially according to this value, whereas it rapidly disappears if $R_0 < 1$.

We propose different simulations performed by exploiting the parameters in [1] (see Table 1). The initial number of target cells is assumed to be $T_0 = 4 \times 10^8$, which is an estimate of the number of units in the upper respiratory tract, while the experimental data for $I_1$ and $I_2$ are initially fixed to zero. The geometric average of $R_0$ values for the six patients in Table 1 is 21.8, suggesting that an initial infection spreads rapidly and would be difficult to extinguish.

Figure 2 shows that, near the viral titer peak, a majority of target cells has been eliminated in most of the cases. While this would seem to exclude the possibility of the infection lasting as late as 6/8 days, these simulations also suggest that, despite the few remaining target cells past the viral peak, the model can indeed sustain

infection during the predicted days (refer to the graph related to the fifth patient, for example).

Now, we briefly consider the model (1) with the usage of an antiviral drug therapy (more details are provided in Section 2). The rather large values of $R_0$ in Table 1 indicate that antiviral treatments need to be supplied before or very early after the infection outbreak. Thus, we focus on the clinical application with neuraminidase inhibitors (NIs) such as Zanamivir as done in [1], which are administrated at three different viral stages, in order to report the effects of both prophylactic and antiviral treatment. Since NIs prevent new virions from budding off an infected cell, their use is incorporated into the model (1) by reducing the viral production rate ($p$), and thus the corresponding basic reproduction number (2). Comparing the results of these new simulations with experimental data in [8], reasonable agreement is obtained when a reduction of viral production $p$ of 97% is set [1].

Figure 3 shows the simulation outcomes according to this change in the values of $p$. The other parameters describing the infection without therapy are $V_0$ = 0.25 TCID50/ml, $\beta = 1.4\times10^{-2}d^{-1}(TCID50/ml)^{-1}$, $k = 3.2d^{-1}$, $\delta = 3.2d^{-1}$, $p = 2.7x10^{-5}$ $d^{-1}TCId50/ml$, $c$ =3.2 $d^{-1}$, and these values are hold constant except for $p$, which is then set to $0.03 \times p$ from the time of drug administration onwards. We compare four different trends in order to express changes in the viral titer: one describes the course of infection in the absence of NIs therapy, whilst the others include the usage of NIs at different stages, more precisely 0, 1.2 and 2.08 (days) after the beginning of infection (referring to the preventive-treatment group, the early-treatment group and the delayed-treatment group, respectively). In all cases, the virus is predicted to be cleared before an infection can become established, consistently with clinical results in [8]. Thus, treatment of Influenza A virus infection with NIs should reduce the period of symptomatic disease and, furthermore, prophylactic usage with a highly effective NI is predicted to prevent infection.

Another model considered in [1] takes account of the infected cells as a unique population, instead of dividing them into two different subpopulations $I_1$ and $I_2$, thus also reducing the number of experimental parameters. This representation does not contemplate the delay in viral production (eclipse phase), which actually makes the model (1) more realistic. We point out that the effects of immune response are not explicitly described in the simple model (1), but they are implicitly included through the death rate of infected cells ($\delta$) and the clearance rate of virus ($c$). Thus, the infection resolution is a direct consequence of the target cells limitation. However, clinical reports from immunocompromised humans who shed Influenza A virus for prolonged periods suggest that the immune response plays a crucial role in clearing the infection, or at least in preventing it from becoming chronic and potentially lethal. Hence, we also make reference to relevant modifications of the model (1) which include the innate immune response component [1], or both the innate and adaptive ones [6, 7, 15].

Another variation of the model (1) is proposed in [1] by implementing the following delay differential equation:

$$\frac{dF}{dt} = sI_2(t-\tau) - \alpha F\,, \qquad (3)$$

in order to incorporate the important role played for the Influenza A virus infection by the interferons (IFN), a group of signaling proteins released by the host cells in response to the presence of various viruses. Therefore, it is assumed that IFNs are secreted from virus-producing cells ($I_2$) at a rate $s$ (per cell), but starting $\tau$ time units after cells begin producing virus; moreover, this amount is proportional to that made collectively by infected cells, monocytes, macrophages and plasmacytoid dendritic cells. The constant parameter $\alpha$ represents the loss rate of IFNs, either by binding to cellular IFN receptors or through degradation.

This modification allows to explain the emergence in some patients of two virus titer peaks, which were absent in the simulations of the original model (see Figure 2). The bimodal virus titer curves are a phenomenon already observed in several studies [11, 12, 13], either using the average virus titer of patients involved or taking the individual value for each patient (in this last case, the bimodal virus titer was present in about half of the patients). In fact, the bimodal virus titer curves in Figure 4 have been obtained by numerically simulating the model in [15], which includes the innate response as the interaction between IFNs and the target cells, and the immune response through the action of *Natural Killer cells*, always activated by the IFNs. Indeed, because of the presence of IFNs, the uninfected

target cell count decreases, since the cells become refractory to infection, whereas the Natural Killer cells induce cytolysis of infected epithelial cells. In this case, the bimodal characteristic of the virus titer curves could be due to the IFNs dynamics, which peaks shortly after the first viral peak, and then decreases rapidly, so that the second viral titer peak can be explained by the loss of the IFN-induced antiviral effect.

Nevertheless, the model (1) together with equation (3) is affected by some limitations, especially for the larger number of parameters compared to the lower amount of experimental data available for human influenza infections. On the contrary, studies on animals usually provide more data. The availability of additional data for immunocompromised animals, as well as data for dead cells and immune response components, has allowed to make further progress in discriminating between different possible models for the infection dynamics [7, 15]. These studies conclude that both an innate and an adaptive immune response component is required to properly describe the infection dynamics and, therefore, to provide an adequate explanation of the observed data.

## 2. Numerical simulation of the effects of antiviral treatments

In that context, we consider the presence of antivirals, such as chloroquine or hydroxychloroquine.

Let ε be the effectiveness of administrated drug and, according to [4] (see also the supplementary information), assume that antivirals with an effectiveness $\varepsilon$ work in reducing the basic reproduction number $R_0$ computed in (2) by a factor (1 - $\varepsilon$). This hypothesis can be included into the model (1) by modifying either $\beta$ or $p$ to obtain the following ordinary differential equations:

$$\begin{cases} \dfrac{dT}{dt} = -(1-\varepsilon)\beta TV \\ \dfrac{dI_1}{dt} = (1-\varepsilon)\beta TV - kI_1 \\ \dfrac{dI_2}{dt} = kI_1 - \delta I_2 \\ \dfrac{dV}{dt} = pI_2 - cV \end{cases} \tag{4}$$

and

$$\begin{cases} \dfrac{dT}{dt} = -\beta TV \\ \dfrac{dI_1}{dt} = \beta TV - kI_1 \\ \dfrac{dI_2}{dt} = kI_1 - \delta I_2 \\ \dfrac{dV}{dt} = (1-\varepsilon)pI_2 - cV \end{cases} \tag{5}$$

In order to promote an effective choice between the models (4) and (5), we have numerically simulated both systems to compare their solutions to that of the original model (1). As a matter of fact, the presence of the reducing factor (1 - $\varepsilon$) has a more significant impact on the dynamics of system (5), as shown in Figure 7, since a decrease of the viral load peak can be clearly observed with respect to Figure 5 and Figure 6. On the contrary, reducing the infection rate $\beta$ by the factor (1 - $\varepsilon$) does not produce any relevant effect on the overall dynamics, and there is a complete coincidence of the numerical simulations of the models (1) and (4), as shown in Figure 5 and Figure 6, respectively. This particular behavior might be explained by the fact that the parameter $\beta$ appears inside the equations always multiplied by the variable $T$, which from an initial value $T_0$ of the order of $10^8$ (refer to Section 2) drops rapidly toward zero (in an extremely short time), thus making the effect of the infection rate $\beta$ negligible as soon as $T$ vanishes From a mathematical point of view, the initial datum $T_0$ plays the role of a *stiffness parameter* for the model (1) and its variants (4) and (5), leading to the appearance of *fast-slow dynamics* usual ly reported for multiscale nonlinear processes [18].

Then, we focus our attention on the effectiveness $\varepsilon$ of antivirals, by studying how this factor eventually changes with time. Following the hypothesis made by Gonçalves et al. [4], we assume that the effectiveness of a treatment, at some time $t$ after its administration has begun, is related to the plasma total drug concentration $C$ through the empirical law given by

$$\varepsilon(t) = \frac{C(t)}{C(t) + EC_{50}}, \tag{6}$$

where $EC_{50}$ is the drug concentration typically required to produce 50% of its maximal effect during a given time interval. This is an intrinsic PharmacoKinetics (PK) drug property, representing the drug's generic po

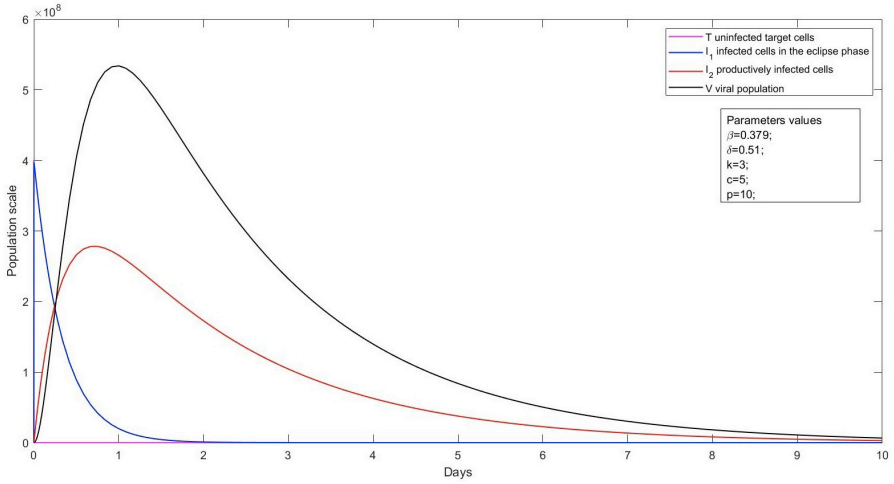


**Figure 5:** Target cell-limited model (1) without antiviral effects.

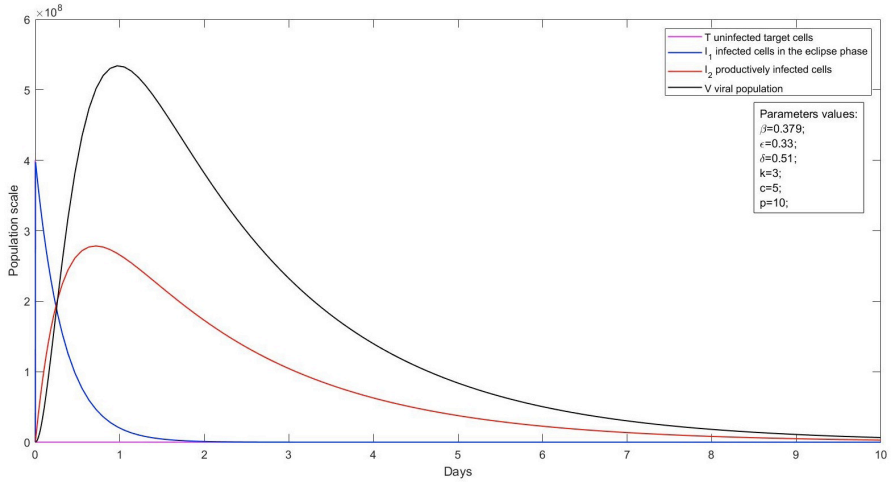


**Figure 6:** Target cell-limited model (4) with the reducing factor (1-ε) to simulate antiviral effects on the infection rate $\beta$.

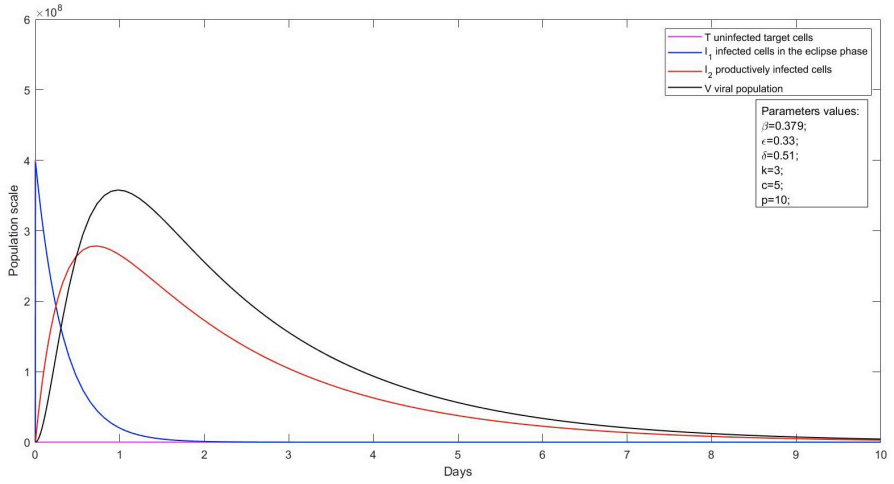


**Figure 7:** Target cell-limited model (5) with the reducing factor(1-$\varepsilon$) to simulate antiviral effects on the viral production $p$.

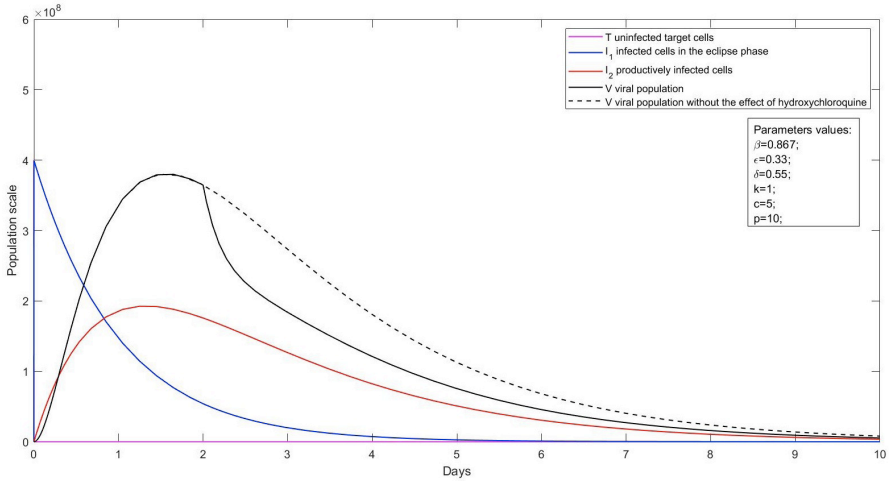


**Figure 8:** Simulation with hydroxychloroquine administrated 2 days after the infection outbreak.

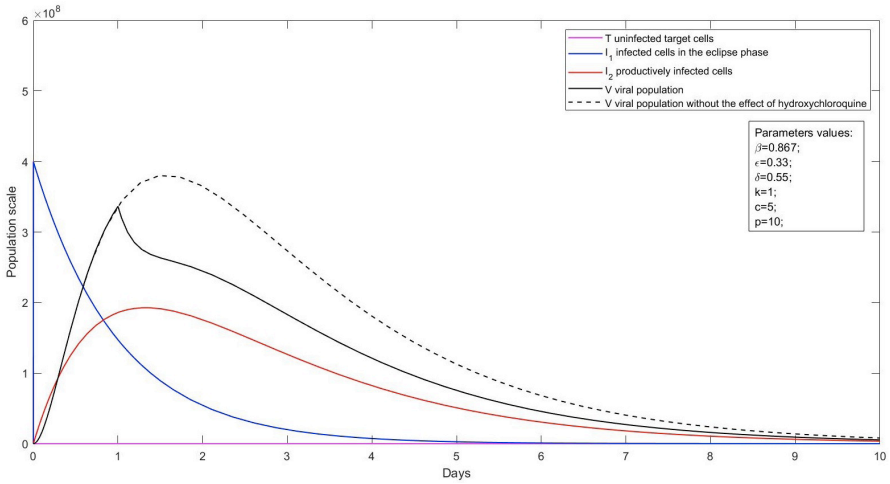


**Figure 9:** Simulation with hydroxychloroquine administrated 1 day after the infection outbreak.

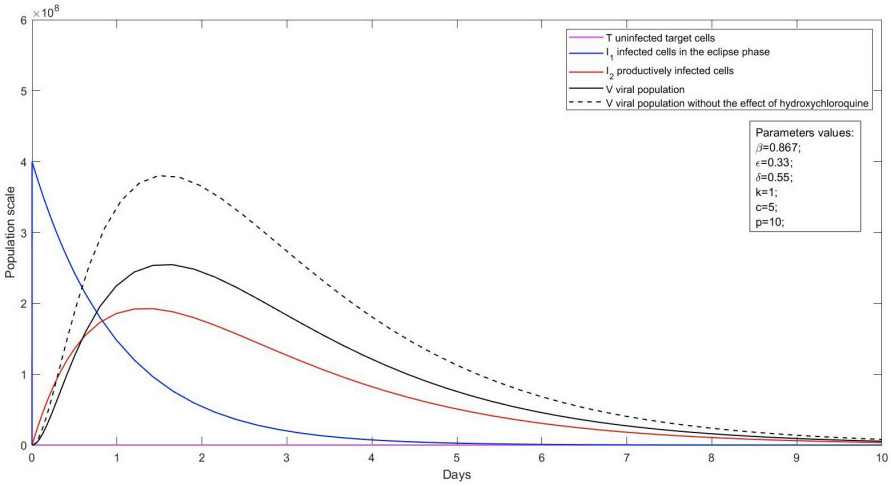


**Figure 10:** Simulation with hydroxychloroquine administrated at the infection outbreak.

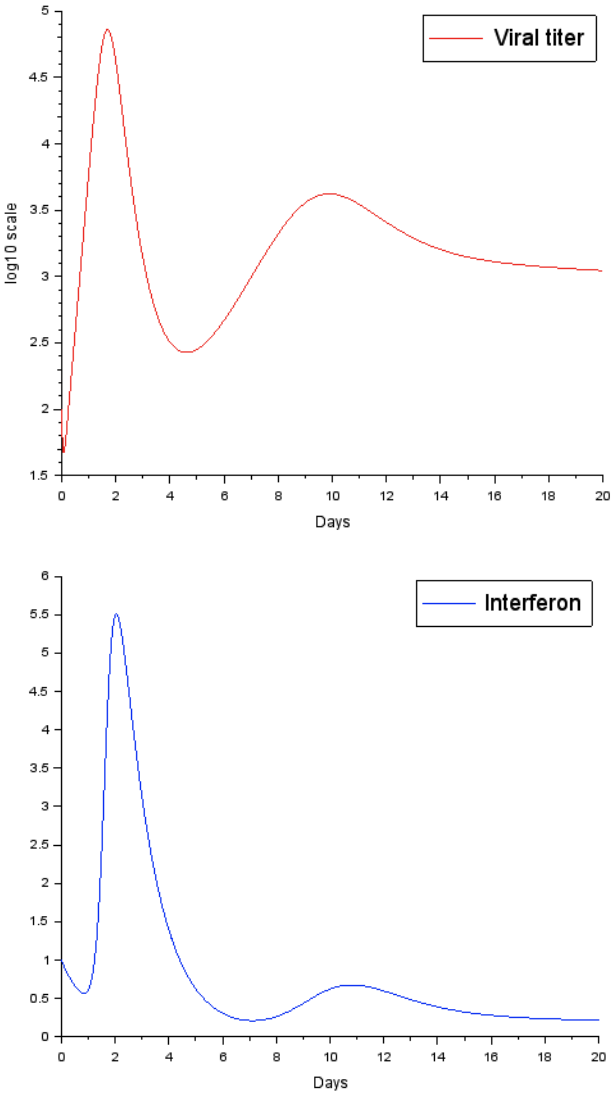


**Figure 4:** Simulation of viral titer double peaks obtained by using the target cell-limited model with IFNs action proposed in [15] (this model with differential delay does not consider the eclipse phase of infected cells). The graphs illustrate the viral titer curve (red) with two peaks and the INF dynamics (blue) during the course of the infection.

tency: among drugs which have effect on the same receptor system, the ones with lower values of $EC_{50}$ are more powerful.

More specifically for chloroquine and hydroxychloroquine, experimental results from *in vitro* studies have proven that both have good antiviral activity [17, 20, 5]. In particular, it has been found in [21] that they are able to decrease the viral replication depending on their concentration, and therefore we can assert that the expression in (6) correctly describes this phenomenon, because the decrease of viral replication is $\varepsilon$-dependent and $\varepsilon$ is itself concentration-dependent. Once a mathematical model of viral dynamics has been fitted to experimental data, we can combine the estimates of viral replication parameters with the specific properties of the drug candidates, in order to formulate possible conclusions regarding the effects of the treatments for various dosing regimens [4]. For that issue, we consider the $EC_{50}$ value of the drugs under investigations during various time intervals. According to [21], the $EC_{50}$ values for chloroquine are 23.90 $\mu M$ and 5:47 $\mu M$ at 24 hours and 48 hours after the administration, respectively; on the other hand, the $EC_{50}$ values for hydroxychloroquine are 6.14 $\mu M$ and 0.72 $\mu M$ at 24 hours and 48 hours after the administration, respectively. Hence, we conclude that hydroxychloroquine exhibits a superior *in vitro* antiviral effect in comparison to chloroquine and, from now on, we consider only results concerning the treatment based on hydroxychloroquine. For the experimental setting, following the approach by Gonçalves et al. [4], we take into account published data from four different Singapore hospitals, to run and examine several simulations concerning hydroxychloroquine efficiency. The patients from the trial group (13 patients) were hospitalized, on average, 3 days after the onset of symptoms (range of the real data: 1-10 days) and had not yet started any treatment. Adopting the real time Reverse Transcription Polymerase Chain Reaction (RT PCR) technique, viral loads in nasopharyngeal swabs, to which patients have been tested, were measured at multiple time points. The observed data showed a peak of viral load at 5 days after the onset of symptoms, on average (range of the real data: 2-27 days).

As the parameter values of our simulations, we assume the initial target cell concentration to be $1.33 \times 10^7$ cells/ml distributed over 30 ml of nasopharyngeal volume, which gives a total number $T_0 = 4 \times 10^8$ of nasopharyngeal target cells. Following previous models of viral infection [2], we consider the clearance rate of virus as $c = 5$ d$^{-1}$ and virions are supposed to be released from infected cells ($I_2$) at rate $p = 10$ d$^{-1}$. When data availability is limited to the viral loads, not all parameters can be estimated. For this reason, several values of $k$ were tested in [4], recalling that all the data from Computed Tomography (CT) were reported into a $\log_{10}$ scale, since this transformation does not change the quality of the parameters to be identied. The values $\{k_1, k_2, k_3\} = \{1, 3, 5\}$ d$^{-1}$ provide good fitting of the model, therefore we use the estimates deduced from them for the remaining parameters (refer to [4] for supplemental information). In particular, we choose the death rate of productively

infected cells as $\{\delta 1, \delta 2, \delta 3\} = \{0.55, 0.51, 0.52\}$ d$^{-1}$ (consequently, the average life span is about 1.88, 1.96 and 1.92 days, respectively) and the (normalized) infection rate as $\{\beta 1, \beta 2, \beta 3\} = \{0.867, 0.379, 0.302\}$. For each set of different parameters, we calculate the basic reproduction number $R_0$ which is found to take the values 27.1, 12.8 and 10.0, respectively. The mean antiviral effectiveness of a drug in seven days of treatment is given by the integral formula, namely

$$\bar{\varepsilon} = \frac{1}{7}\int_0^7 \frac{C(t)}{C(t) + EC_{50}}dt\,. \qquad (7)$$

Concerning the COVID-19, the characteristic value of $\bar{\varepsilon}$ is about 33% for the hydroxychloroquine [4], and we used this value as parameter $\varepsilon$ in our numerical simulations. We analyze how the hydroxychloroquine treatment affects the viral load peak when the drug is administrated at different times after the infection outbreak, with the set of parameters related to $k = 1$.

All the figures show an early viral load peak followed by a progressive decrease of the virions. According to the experimental data, the value of this peak without any treatment is about 3.8 x 10$^8$ and it occurs about 36 hours after the infection outbreak (dashed line).

The action of hydroxychloroquine reduces the viral load in different ways, depending on when the treatment is administrated: for instance, providing the hydroxychloroquine 2 days after the infection outbreak helps the abatement of the viral load, but it does not have any effect on the peak intensity (see Figure 8). Instead, initializing the treatment 1 day after the infection outbreak reduces the viral load peak to about 3.4 x 10$^8$ (see Figure 9). Finally, we observe the most signicant effect when the hydroxychloroquine is administrated immediately at the infection outbreak, and in this case the peak value decreases to about 2.5 x 10$^8$ (see Figure 10).

We deduce from these results that hydroxychloroquine does not have relevant antiviral effects if administrated more than 2 days after the infection outbreak. Indeed, it is advisable to initialize the treatment earlier, sometimes as a prophylactic agent to decrease the viral load peak, thus attenuating the viral replication and mitigating the disease progression, even if it could be not a complete protection.

However, it is worthwhile noticing that the correction by the factor (1-$\varepsilon$) of parameter $p$ modifies only the behavior of the variable $V$, while leaving completely unchanged the others. This is justied by observing that parameter $p$ is present only in the last equation of the model (1) and its variants, and then $V$ appears in the first two equations, but always multiplied by the variable $T$ which, as already explained, rapidly goes to zero (in almost an infinitesimal time). Following a purely qualitative approach, we simplify the model (5) by considering $T(0) = 0$ (hence $T(t) = 0$ is a stable solution to the first equation), and we solve the remaining (triangular) system, for $I_1(0) \neq 0$ in order to initialize the dynamics, together with $I_2(0) = 0$ and $V(0) \neq 0$, so that we obtain the following explicit solution:

$$\begin{cases} I_1(t) = I_1(0)\, e^{-kt} \\ I_2(t) = I_1(0)\dfrac{k}{k-\delta}\left(e^{-\delta t} - e^{-kt}\right) \\ V(t) = V(0)\, e^{-ct} \\ \qquad + I_1(0)\dfrac{k}{c-\delta}\cdot\dfrac{(1-\varepsilon)p}{k-\delta}\left(e^{-\delta t} - e^{-ct}\right) \\ \qquad - I_1(0)\dfrac{k}{c-k}\cdot\dfrac{(1-\varepsilon)p}{k-\delta}\left(e^{-ct} + e^{-kt}\right) \end{cases} \qquad (8)$$

Therefore, the parameter $p$ and its reducing factor (1 - $\varepsilon$) determine only the dynamics of the variable V from (8), and the difference between the numerical simulations with or without the hydroxychloroquine effects is observable only in the behavior of $V$.

In conclusion, we have to point out that our model is not really predictive: indeed, the simulations above show the viral peak already after 1 - 2 days, differently from the average period of 5 days actually observed for the 13 patients analyzed in [4]. In order to become more realistic, an agreement is necessary on both the parameters to be used and their appropriate estimate to be inserted into the numerical algorithm, because the models presented in this article are highly unstable with respect to the initial data. Rigorous studies on these aspects are still under development, as well as those related to hydroxychloroquine and its experimentation as a medicinal product for the SARS-CoV-2 virus. In fact, all these qualitative results are valid under the hypothesis that the treatments act according to the modeling assumptions, we seems not to unanimously confirmed by recent studies [3, 21].

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# COVID-19 Calls for Mathematics, Part 2: Interleukin IL-6 and Myo-Inositol, Suicide-substrate Enzyme Inhibitors

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**Abstract**

Interleukin IL-6 is a cytokine produced in response to various types of damage (infections, tissue injuries, autoimmune diseases, etc.) whose action contributes to host defense through stimulation of acute phase responses and immune reactions. However, experimental data show that high concentration levels of interleukin IL-6, and the subsequent inflammatory cascade induced into the organisms, could lead to several complications, such as organs progressive deterioration. The abnormal release of interleukin IL-6 is also a consequence of SARS-CoV-2 virus contagion, usually causing interstitial pneumonia and respiratory failure, which appear to be the source of decease in most of patients. Tocilizumab and myo-Inositol are two possible remedies proposed in the clinical literature to contrast the uncontrolled synthesis of interleukin IL-6, although the former exhibits non negligible collateral effects, differently from the latter. For this reason, the mathematical investigation of myo-Inositol interaction with other relevant substances involved in the SARS-CoV-2 could support the bio-medical research in determining, the optimal doses and administration timing necessary to minimize damages, in order to support the clinical results. The preliminaries of this approach are the subject of this article.

Moreover, because a vaccine against the SARS-CoV-2 virus will not be available shortly, we consider another possible pharmacological strategy to contrast its activity. There exist several candidate medicines which may inhibit infection and replication of SARS-CoV-2, and we focus our attention on the process of enzymatic inhibition through mechanism-based enzyme inactivators. The so-called suicide-substrate strategies constitute a fascinating area of interdisciplinary research. We explain the procedure to represent mathematically how a hypothetical drug would act to inhibit the essential enzymes used by the SARS-CoV-2 virus to replicate and spread.

Considering the biological aspects and the mathematical applications presented in this article, the more relevant questions which arise are: 1) does the design of these drugs deserve a more detailed investigation? Could they be a viable alternative to the strategy of massive vaccine campaign against the SARS-CoV-2 virus?

**Keywords:** SARS-CoV-2, interleukin IL-6, anti-inflammatory mediators, enzymatic inhibition, suicide substrates, mathematical modeling, ordinary differential equations, fixed points, stability analysis, quasi-steady-state assumption, numerical simulations, experimental data

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## 1. Interleukin IL-6 and myo-Inositol

As the outbreak of SARS-CoV-2 spreads around the world, it is noted that most of the deceases attributed to the virus are actually caused by its consequent Acute Distress Respiratory Syndrome (ARDS). Comparing the present situation to other coronavirus infections, it seems possible that a deregulated response from cytokines constitutes the principal cause of this respiratory failure. Indeed, patients with severe SARS-CoV-2 typically display a cytokine storm syndrome, unleashed by CD4 T lymphocytes which become activated as T helper-1 cells [4]. The lungs are infiltrated by inflammatory cells, such as lymphocytes, and examinations on patients in critical condition show diffuse alveolar damage with exudates [34]. This also produces disruption in the interstitium and in crosstalk between cells, endangering oxygen exchanges. In fact, from previous studies on pathogenic coronavirus epidemics [6], the exuberant immune response by cytokines is known to induce apoptosis in both endothelial and epithelial cells, thus causing vascular leakage and alveolar oedema, ultimately resulting in hypoxia.

Particularly, the cytokine which seems to play a central role is interleukin IL-6, mainly because during cytokine storms the amount of other inflammatory cytokines is not comparable to the major boost observed in IL-6 production. It is thought that this pathological increase in IL-6 levels may be determined by the altered balance between Regnase-1 and Arid5a proteins in post-transcriptional modification of the IL-6 mRNA, and some studies indicate that Arid5a predominance over ribonuclease Regnase-1 promotes inflammatory processes [14, 25]. Furthermore, most patients incurring in respiratory failure are already affected by unfavorable medical conditions, such as hypertension, cardiovascular disorders and cancer, also associated with IL-6 high levels [10, 19], whose release can be exacerbated by the virus: as a matter of fact, these are the patients who demonstrate an adverse clinical outcome after hospitalization.

Everything thus far suggests that, in order to block the inflammatory storm, it is necessary to down-regulate specific cytokines, both by decreasing the rate of their production and increasing the rate of their destruction, and some promising clinical trials with tocilizumab, an IL-6 receptor inhibitor, have shown a significant improvement in patients clinical outcome [25, 1, 28]. Tocilizumab is a humanized anti-IL-6 receptor antibody which blocks IL-6-mediated signal transduction by inhibiting IL-6 binding to transmembrane of the soluble IL-6 receptor (refer to Figure 4). However, as interleukin IL-6 plays an important role in both innate and adaptive immune response, clinical studies also revealed the insurgence of infections due to the use of IL-6 inhibitors [17].

As suggested by Bizzarri et al. [4], a valuable alternative to tocilizumab is provided by myo-Inositol, a naturally occurring polyol which has been proven to reduce IL-6 levels and to mitigate the inflammatory cascade, interestingly without peculiar side effects. Among the arguments for promoting the myo-Inositol as a possible remedy to IL-6-induced damages, its application for the treatment of Respiratory Distress Syndrome (RDS) in the newborn [11] and the beneficial effect against lung cancer [9] support its experimental administration in the combined treatment of other diseases of the upper and lower respiratory system, thus opening new perspectives for the case of SARS-CoV-2.

Mathematical modeling can considerably improve the understanding of endogenous mechanisms of inflammation and the delicate equilibrium between all participants in the human body immune response [13, 23, 15]. In particular, this methodological approach makes it possible to explore the circumstances which could lead to an excessive inflammation, hence resulting in tissue damage, organs dysfunction and possibly decease.

Contrary to previous models, such as the one advanced by Kumar et al. [13], where solely the pathogen and its pro-inflammatory mediators are taken into consideration, the model introduced by Reynolds et al. [23] aims to understand the robustness of the process with time-dependent anti-inflammatory mediators and to determine whether their utilization is advantageous. Anti-inflammatory mediators limit the harm caused to tissues by the inflammation and suppress the activity of phagocytes. Nevertheless, this action may have drawbacks, since too much efficiency would lead to a period of vulnerability of the immune system to pathogens: in fact, while exogenous anti-inflammation activity may impede the spreading of pro-inflammation agents and the tissue damage they would have induced, it also weakens the ensuing production of endogenous anti-inflammatory mediators [3, 2]. Thus, its effects on the outcome of pathogenic infections may be difficult to predict and, therefore, the problem requires a dynamical analysis such as the one presented in this article. The model proposed in [23] actually uses smaller subsy-

stems to build up more complex schemes, the smallest ones being coherent with the experimental results of previous studies and validated within the already known interaction between components of the immune response. For the cases discussed in Section 2, elements of the adaptive immune response are not included, such as specific antibodies, and also the anti-inflammatory mediators are not characterized.

The activation of anti-inflammatory mediators can be modeled by building a differential system in which $P$ denotes the density of pathogen, $N^*$ represents the activated phagocytes, $D$ describes the amount of damaged tissue and $C_A$ is the concentration of anti-inflammatory mediators. These variables evolve in time, and their growth depends non-linearly on the value of all the others. In particular, we consider the fact that phagocytes respond to the presence of a pathogen and try to eliminate it. Doing this causes collateral injuries, such as inflammation and damage to tissues, but the latter also activates more phagocytes, and therefore $N^*$ and $D$ actually fuel reciprocally. The anti-inflammatory mediators, such as interleukin IL-10 [22, 12], respond to the presence of activated phagocytes and other damages, and try to suppress them. A way to investigate more specifically the effects of $C_A$ administration is to assign different functions which replicate the variation of anti-inflammatory mediators in time [23], in order to improve the clinical situation or at least avoid patients decease. This approach makes it possible to select the more plausible strategy to achieve favorable outcomes with the system returning to a stable healthy state.

At the first attempt, $C_A$ can be seen as a parameter, constant in time. Then, the speed of evolution for $P$, $N^*$ and $D$ is simplified by considering various recurrent real life situations, to obtain the following system of nonlinear ordinary differential equations:

$$\begin{cases} \dfrac{dP}{dt} = k_{pg}P\left(1-\dfrac{P}{p_\infty}\right) \\ \qquad\qquad - \dfrac{k_{pm}s_mP}{\mu_m + k_{mp}P} - k_{pn}f(N^*)P \\ \dfrac{dN^*}{dt} = \dfrac{s_{nr}f(R)}{\mu_{nr}+f(R)} - \mu_n N^* \\ \dfrac{dD}{dt} = k_{dn}\, f_s(f(N^*)) - \mu_d D \end{cases} \tag{1}$$

where $C_A$ appears implicitly through the definition of the reaction function, which is chosen as

$$f(V) = \frac{V}{1+(C_A/c_\infty)^2}\,, \tag{2}$$

together with $f_s(V) = V^6/(x^6_{dn} + V^6)$.

The action of anti-infiammatory mediators, as well as the inhibition of $N^*$ and $D$, are represented by the linear (empirical) function $f$ given in (2), which leaves its argument unchanged if $C_A$ is null and diminishes constantly, tending to zero, if $C_A$ is increasing.

The variation of $P$ depends on its logistic growth, with rate $k_{pg}$ ranging from 0.021 $h^{-1}$ to 2.44 $h^{-1}$, which accounts for the reproduction of pathogen, but considering that an excessive amount (namely, above a given threshold $p_\infty$) leads to more spontaneous self-deterioration. The second term in the first equation describes a non-specific local response, such as the action of tissue macrophages and other biological defenses. This activation is typically fast, i.e. occurring only during a short period of time, hence that term can be deduced by simplifying a smaller model under the quasi-steady-state assumption (QSSA) that the speed of the response is almost null [23]. Finally, the effect of activated phagocytes is incorporated through the last term and it depends on the interaction between the phagocytes themselves and the pathogen.

The second equation summarizes the speed of $N^*$ in two terms. The first one considers the awakening of resting phagocytes, that is a rapid process, and in fact this term is obtained by simplifying another smaller model [23] again by means of the QSSA hypothesis. The auxiliary variable $R$ grows linearly with the presence of $P$, $N^*$ and $D$ according to an empirical law [23], namely $R = k_{nn}N^* + k_{np}P + k_{nd}D$. Then, the second term illustrates the deterioration of activated phagocytes in time.

The temporal variation of $D$ is represented in the last equation of system (1) and includes two terms. The $S$-shaped function $f_s$ is intended to suggest that low amounts of phagocytes cause few damages to the surrounding tissue but, while increasing, the damage spreads until its saturation, meaning that it will not exceed a certain value (even in the presence of a large amount of phagocytes). Indeed, $f_s$ is null if there are no activated phagocytes, and tends monotonically to a constant as shown in Figure 1. The last term imitates the spontaneous healing process of the system.

We point out that some of the parameters used to build the model (1) are the results of experimentation,

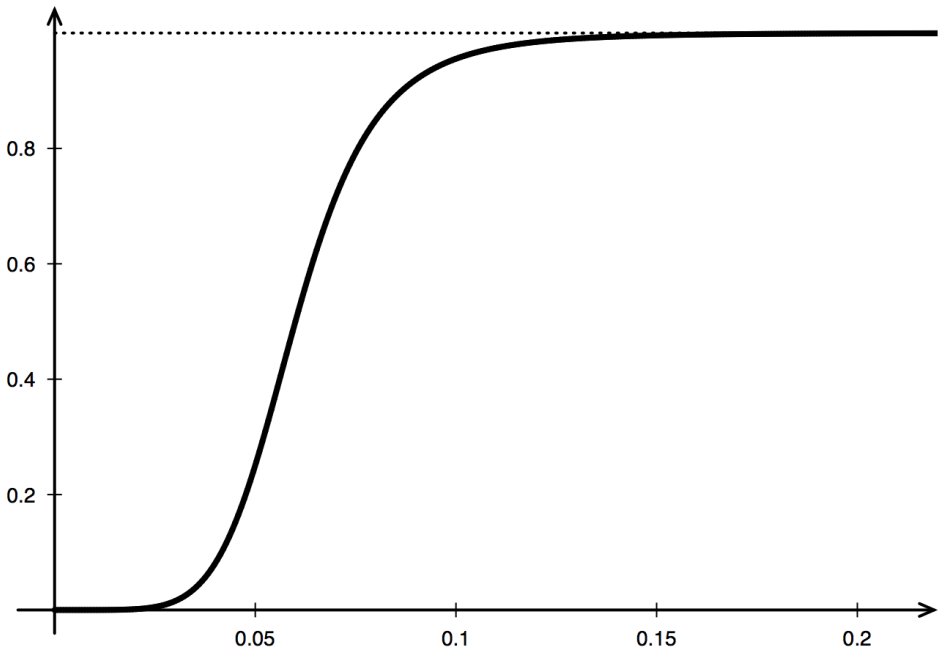



**Figure 1:** Graphic shape of the function $f_s$ used to describe the speed of $D$ in the model (1).

while others, and especially $k_{pg}$ inside the first equation, are varied in order to study different situations (all their values are positive or null). Unless specified otherwise, the numerical values for the simulation parameters are those reported in Table 1.

| parameters | values | units |
|---|---|---|
| $p_\infty$ | 20 | $10^6$ cc$^{-1}$ |
| $k_{pm}$ | 0.6 | $N^*$-units$^{-1}$ h |
| $s_m$ | 0.005 | $N^*$-units h$^{-1}$ |
| $\mu_m$ | 0.002 | h$^{-1}$ |
| $k_{mp}$ | 0.01 | $P$-units$^{-1}$ h |
| $k_{pn}$ | 1.8 | $N^*$-units$^{-1}$ h |
| $s_{nr}$ | 0.08 | $N^*$-units h$^{-1}$ |
| $\mu_{nr}$ | 0.12 | h$^{-1}$ |
| $\mu_n$ | 0.05 | h$^{-1}$ |
| $k_{dn}$ | 0.35 | $D$-units h$^{-1}$ |
| $\mu_d$ | 0.02 | h$^{-1}$ |
| $s_c$ | 0.0125 | $C_A$-units h$^{-1}$ |
| $k_{cn}$ | 0.04 | $C_A$-units h$^{-1}$ |
| $k_{cnd}$ | 48 | $N^*$-units D-units$^{-1}$ |
| $\mu_c$ | 0.1 | h$^{-1}$ |
| $k_{nn}$ | 0.01 | $N^*$-units$^{-1}$ h |
| $k_{np}$ | 0.1 | $P$-units$^{-1}$ h |
| $k_{nd}$ | 0.02 | $D$-units$^{-1}$ h |
| $c_\infty$ | 0.28 | $C_A$-units |
| $x_{dn}$ | 0.06 | $N^*$-units |

**Table 1:** Numerical values for the simulation parameters (refer to [23] for more details).

By studying analytically the system (1), starting from the smaller models which led to its construction [23], various (but at most three) *fixed points* are found according to different values of $C_A$ and $k_{pg}$, whose existence and stability properties sensibly depend on these parameters. In particular, the fixed points are endowed with a *basin of attraction*, namely a region of attractiveness for the overall dynamics.

The so-called *health state* is reached when pathogen, phagocytes and tissue damage are all null, and the amount of anti-inflammatory mediators is a specic constant; it is stable only if $k_{pg}$ is less than 1.5 h$^{-1}$, otherwise it is unstable. The *aseptic death state* is reached when the pathogen has been eradicated but the inflammation has not been stopped, so phagocytes and tissue damage have high values; it exists if $C_A$ is smaller than 0.626 pg/ml and the region of its existence is divided into two parts by some value, depending on $k_{pg}$ and ranging between 1.707 h$^{-1}$ and 3.8 h$^{-1}$, which, if bigger than $C_A$, makes the *aseptic death state* stable. The septic death state is reached when the phagocytes have been unable to stop the pathogen growth; it exhibits stability if $k_{pg}$ crosses a positive value, which decreases while $C_A$ increases but is never bigger than 0.276 pg/ml. For more details about the regions of stability and their dependence upon the changing parameters $C_A$ and $k_{pg}$, we refer to [23]. These results suggest that, even with the administration of anti-inflammatory mediators, the stability of a health state depends strictly on $k_{pg}$, namely the growth rate of pathogen, and thus any effective strategy must include a

combination of various treatments [22, 12, 20, 24].

Moreover, while increasing $C_A$ leads to the aseptic death state becoming an unstable fixed point, it also makes the septic death state a more plausible outcome, as portrayed for the example in Figure 2, because it opens a period of time in which the pathogen is free to multiply.

Dealing with the presence of anti-inflammatory mediators as a time-dependent variable leads to a new model, which is a variant of the system (1) already studied in [23], with a fourth equation describing the speed of evolution of $C_A$ as follows:

$$\frac{dC_A}{dt} = s_c + \frac{k_{cn} f(N^* + k_{cnd} D)}{1 + f(N^* + k_{cnd} D)} - \mu_c C_A \,, \qquad (3)$$

where the first term indicates a constant source of anti-inflammation mediators, possibly provided by myo-Inositol [4], while the two others replicate its activation in response to the presence of activated phagocytes and tissue damage, and its deterioration in time, respectively.

Comparing the model (1), by fixing the constant $C_A$, with its variation by adding equation (3), for which $C_A$ is given as initial value, and studying the relationship between $k_{pg}$ and the initial amount of pathogen $P_0$, a finer description of the stability for the case of an health state is possible. Indeed, when $k_{pg}$ is large enough to represent a threat to the system, but it is also small enough to ensure stability, the basin of attraction of the fixed points is wider if we assume the evolution model (3). An example of this phenomenon is shown in Figure 3, and these results indicate that an adaptive administration of anti-inflammatory mediators makes the patients recovery more plausible.

Finally, the model outcomes underline the importance of both anti-inflammatory and inflammatory response rates, also suggesting that they should be carefully tuned to overcome the more critical scenarios and restore health. Furthermore, the numerical simulations point out the narrow compromise between administering anti-inflammatory mediators and hindering the immune response: experimental studies and simulations have stressed out the importance of quantity and timing of the administration of anti-inflammatory me-

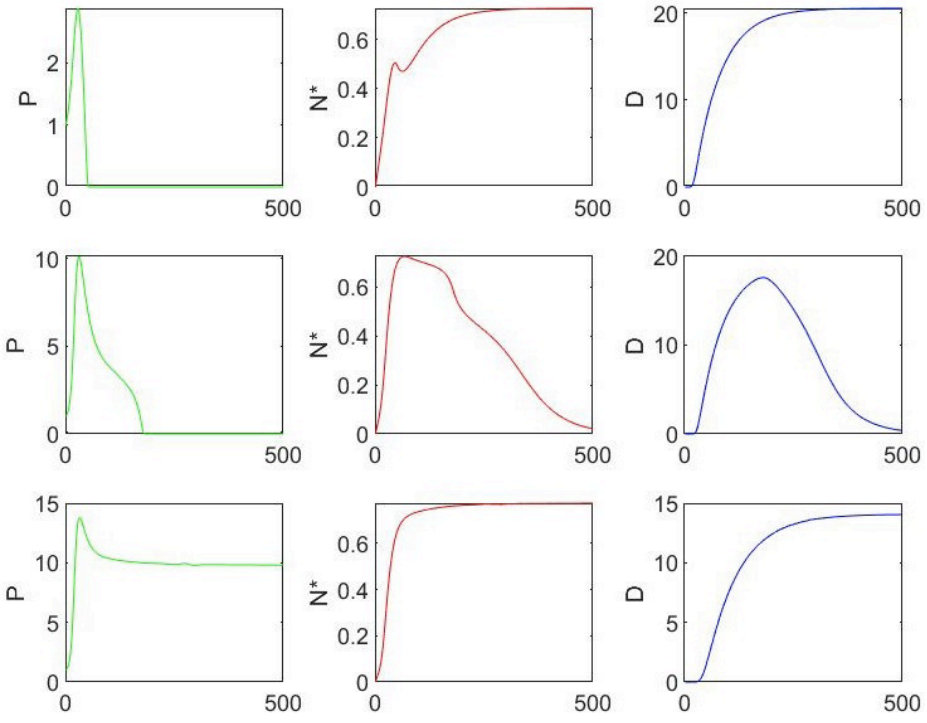



**Figure 2:** These graphs illustrate that, if $k_{pg}$ is below 1.5 $h^{-1}$, the value of $C_A$ plays an important role in the dynamics outcome. The results of three simulations of the model (1) are shown, with initial values $P$ = 1.0 x $10^6$ $cc^{-1}$, $N^*$= D = 0 and $k_{pg}$=0.3 $h^{-1}$, while $C_A$ has dierent values (displayed according to the rows). The time, represented along the horizontal axis, is measured in hours, therefore the expected results are reported over three weeks. The first row graphs corresponds to $C_A$ = 0.5 pg/ml and the outcome is the aseptic death, the second row corresponds to $C_A$ = 0.7 pg/ml and the dynamics tends to the health state, while the third row corresponds to $C_A$ = 0.9 pg/ml and represents the case of septic death.

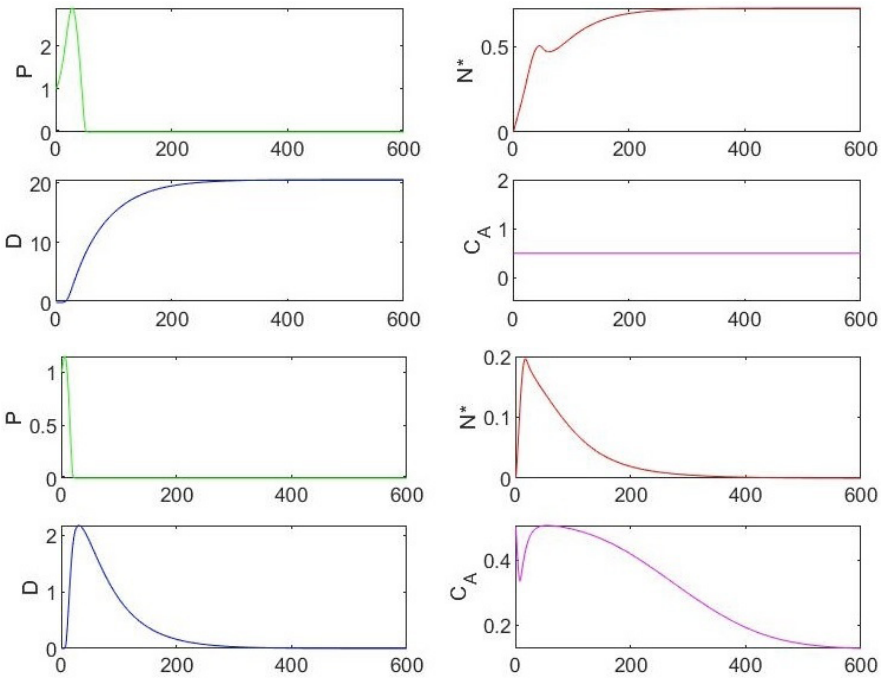



**Figure 3:** These graphs illustrate the expansion of the basin of attraction for the case of an health state. The first four charts correspond to the static model (1), while the others correspond to the dynamic case with additional equation (3). Both simulations start with $P_0$=1.0106 cc$^{-1}$, $N^*$= $D$= 0 and $C_A$= 0.5 pg/ml, as initial values, with the parameter $k_{pg}$=0.3 h$^{-1}$, but the outcome is very different. The first set of graphs shows the solution converging toward an aseptic death state, whilst the others indicate convergence toward a health state. These results estimate the recovery expectations in 25 days.

diators in patients whose clinical condition is on its way to a healthy resolution but has not reached it yet [3, 2].

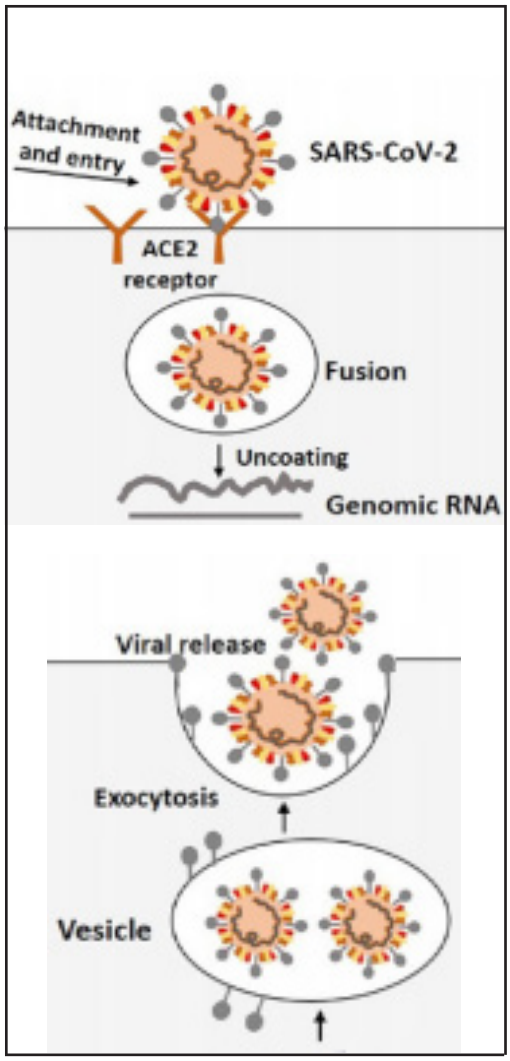
However, the oversimplication of a complex problem is maybe the greatest downside of the models presented in this article, although their affordable size permitted a detailed analysis of emergent behaviors and the description of the principal catalysts involved in the process. Guidelines for future research include a more in-depth biological characterization of anti-inflammatory mediators for application to the SARS-CoV-2 virus, taking into account their specic activity and operational time-scale, and it might also be useful to incorporate a rigorous mathematical analysis to design efficient strategy for administrating therapeutic agents.

## 2. Suicide-substrate enzyme inhibitors

Coronaviruses are enveloped RNA viruses and include those which cause the common cold as well as the highly pathogenic Severe Acute Respiratory Syndrome coronaviruses (SARS-CoV) and Middle Eeast Respiratory Syndrome coronaviruses (MERS-CoV). All coronaviruses contain specic genes which encode proteins for viral replication, nucleocapsid and spikes formation. The spike (S) glycoproteins on the outer surface of coronaviruses are responsible for the attachment and entry of the virus into host cells. The receptor-binding domain (RBD) is loosely attached to the virus particles and, therefore, the virus may actually infect multiple hosts. The entry mechanism of a coronavirus depends upon cellular proteases, including transmembrane protease serine 2 (TMPRSS2) which splits the S proteins and establish further penetration changes. SARS-coronaviruses require angiotensin-converting enzyme 2 (ACE2) as a key receptor. SARS-CoV- 2 possesses the typical coronavirus structure with S proteins and also other polyproteins, nucleoproteins and membrane proteins. The transmission rate of SARS-CoV-2 is presently higher than that previously observed for SARS-CoV, and the reason could be some genetic recombination event occurring at S proteins inside the RBD region, which may have enhanced its transmission ability [24].

The life cycle of SARS-CoV-2 in host cells begins when S proteins bind to the ACE2 cellular receptor and the conformation changes induced into the S proteins facilitate viral envelope fusion with the cell membrane through the endosomal pathway (see Figure 4, above). Then SARS-CoV-2 releases its genomic RNA inside the

host cell, in order to be translated into active DNA used by the host cell to replicate the virus. The mechanism allowing this process is performed by the reverse transcriptase (RT) enzyme, whereas the nucleic acid of the virus is also exploited for the synthesis of viral proteins. Both viral proteins and genomic RNA are subsequently assembled into virions in the endoplasmic reticulum (ER) and Golgi, and finally transported via vesicles to be released out of the cells (see Figure 4, below).




Among the several candidate medicines which may inhibit infection and replication of SARS-CoV-2 [30,1, 4, 16, 24, 26], we focus our attention on the phenomenon of enzymatic inhibition through *mechanism-based enzyme inactivators* [7, 18]. There are two types of inhibition mechanisms employed by ligands for a specic protein: the irreversible inhibition is accompanied by the formation of a covalent bond between the drug and its receptor, while the reversible inhibition is maintained by non-covalent intermolecular interactions. Binding of a reversible inhibitor would likely cause that one drug molecule hampers the activity of its target for a certain period; instead, binding of an irreversible inhibitor would generate a permanent bond between the drug and the related target macromolecule. In some cases of covalent inhibition, the enzyme also lends a hand to its own demise. Hence, the term "suicide" or "suicidal" inhibition —or rather mechanism-based inhibition— refers to that particular type where the enzyme prepares the ligand to commit suicide (these substrates are also called "Trojan horse inhibitors"). In recent years, suicidal inactivation has become a leading approach in studying enzyme-related mechanisms for rational designing of effective drugs in the pharmaceutical industry [7]. Physiologically, suicide-substrate systems target specific enzymes for inactivation, thereby reducing their activity, thus preventing substrate utilization and product formation [32, 31].

From a mathematical point of view, this process has been discussed in several studies [32, 31, 5, 27,8], which thoroughly analyze a modication of the classical *Michaelis-Menten* system shown in Figure 5, in order to account for the enzyme kinetics.

$$E + S \underset{}{\overset{k_1}{\rightleftarrows}} X \xrightarrow{k_2} Y \xrightarrow{k_3} E + P$$
$$\downarrow k_4$$
$$E_i$$

**Figure 5:** A simplified pathway of enzyme kynetics for suicide-substrate systems.

In that framework, the symbols $E$, $S$ and $P$ stand for enzyme, substrate and product, respectively, $X$ and $Y$ represent the enzyme-substrate intermediates, $E_i$ denotes the inactivated enzyme, and all reactions are mediated by (positive) rate constants. Within such system, $Y$ can follow two alternative pathways, namely toward $E+P$ with rate $k_3$ or toward $E_i$ with rate $k_4$. The quotient $k_3/k_4$ of these rates is called *partition ratio* and is usually denoted by $r$. Both pathways are considered to be irreversible over the time scale of the reaction. Then, irreversible inactivation of the enzyme implies that its form and functionality have been permanently modified; since it can no longer carry out its function, the enzyme thus commits suicide. The criteria for an inhibitor to be classified as *suicide substrate* include that inactivation

should be time-dependent, the reaction should be of first order type, and the enzyme should exhibit a saturation phenomenon [7].

The rate equations for the process illustrated in Figure 5 are deduced by means of the standard *law of mass action* as follows:

$$\begin{cases} \frac{de}{dt} = -k_1 es + k_{-1}x + k_3 y \\ \frac{ds}{dt} = -k_1 es + k_{-1}x \\ \frac{dx}{dt} = k_1 es - k_{-1}x - k_2 x \\ \frac{dy}{dt} = k_2 x - k_3 y - k_4 y \\ \frac{de_i}{dt} = k_4 y \\ \frac{dp}{dt} = k_3 y \end{cases} \tag{4}$$

where $e$, $s$, $x$, $y$, $e_i$ and $p$ denote the chemical concentrations, as functions of the time. Typical experimental initial conditions completing this mathematical formulation are given by $e(0) = e_0$, $s(0) = s0$, $x(0) = y(0) = e_i(0) = p(0) = 0$, for instance. Consequently, the system (4) is further simplied by adopting the following relations of mass conservation:

$$\begin{aligned} e_0 &= e + x + y + e_i \\ s_0 &= s + x + y + e_i + p \end{aligned} \tag{5}$$

Moreover, the equation for $p$ can be uncoupled from the others, because $p$ does not appear in any other equation, hence $p$ can be evaluated by direct integration after $y$ has been computed. Finally, the reduced model obtained from (4) thanks to (5) contains only four coupled ordinary differential equations, namely

$$\begin{cases} \frac{ds}{dt} = -k_1(e_0 - x - y - e_i)s + k_{-1}x \\ \frac{dx}{dt} = k_1(e_0 - x - y - e_i)s - (k_{-1} + k_2)x \\ \frac{dy}{dt} = k_2 x - (k_3 + k_4)y \\ \frac{de_i}{dt} = k_4 y \end{cases} \tag{6}$$

The quasi-steady-state assumption (QSSA) for the intermediate complexes, consisting in the hypothesis that $\frac{dx}{dt} = 0$ and $\frac{dy}{dt} = 0$ in system (6), yields an additional simplication, which implies the existence of explicit solutions given by

$$x = \frac{k_1 es}{k_{-1} + k_2}, \quad y = \frac{k_1 k_2 es}{(k_{-1} + k_2)(k_3 + k_4)}, \tag{7}$$

so that intermediate complexes computed from (7) bear the proportionality relation $x = Ky$, $K := \frac{k_2}{k_3 + k_4}$ .

However, these equations are still too difficult to be solved analytically, without other simplifying approximations. Therefore, numerical simulations are carried out to illustrate some particular points. The biologically more relevant questions which arise are: 1) will all substrate be converted during the process? 2) will all the enzyme be inactivated? As indicated in [31], the factor determining whether the substrate is exhausted before all the enzyme is inactivated is $r \times m$, where $m$ denotes the ratio of the initial concentrations of enzyme and substrate, namely $e_0/s_0$. On the other hand, it is claimed in [27] that characteristic factor is $(1+r) \times m$, which is deduced by applying the conventional QSSA reduction method to obtain a two-dimensional system. In particular, when $(1 + r) \times m \geq 1$, then the substrate is totally exhausted, whilst for $(1 + r)\, m \leq 1$ all the enzyme is inactivated, so that both occur when $(1 + r) \times m = 1$ (this result has been also achieved more recently in [8] without any QSSA simplifying hypothesis).

Although the results obtained in [27] seem to be more consistent with the numerical solutions to the full system of rate equations (4) than those presented in [31], both conclusions deviate from the solution when $m$ approaches 1 (see Figure 6). This fact highlights a shortcoming of most QSSA approximations for enzyme kinetics, since their validity decreases for increasing values of the ratio $e_0/s_0$. Consequently, a further analysis is needed because these indicators do not always lead to a correct conclusion.

For a quantitative estimation of the effectiveness of suicide substrates, a systematic mathematical study of these models may serve as an indicator to enzymologists, and especially to pharmacologists, during drugs administration and delivery, similarly to the methods applied for identifying indicators of suicidal inactivation in antibiotic kinetics (refer to [7] for more detailed information). In the scientific literature, there are few articles concerning the theoretical understanding of enzymatic reactions involving suicide substrates, possibly because of the severe lack of data from realistic ap-

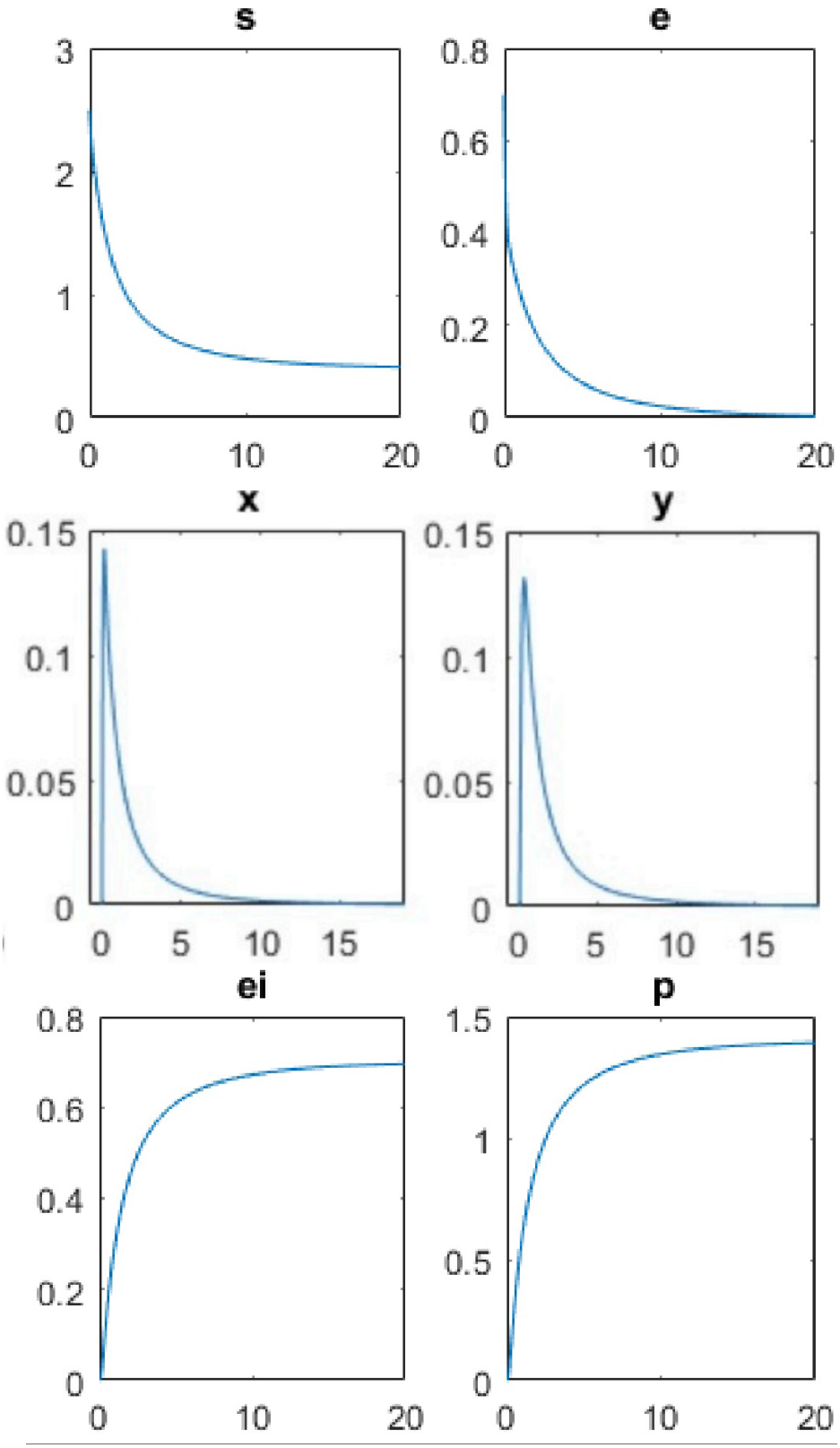



**Figure 6:** Temporal proles for enzyme ($e$), substrate ($s$), intermediate complexes ($x$) and ($y$), product ($p$) and inactivated enzyme ($e_i$). The experimental data for numerical simulations are $e_0 = 0.7$, $s_0 = 2{:}5$, $k_1 = 2$, $k_{-1} = 4$, $k_2 = 10$, $k_3 = 6$, $k_4 = 3$, and these values are chosen to reproduce the case $(1+r) \times m < 1$. The curves show that all the enzyme is inactivated, and indeed the concentration $e_i$ tends to $e_0$, while there exists an excess of substrate. The intermediate complexes exhibit similar trends, characterized by an initial phase of rapid growth, before they rapidly run out toward the final product.

plications. Simultaneously, experimentalists are often holed up because of the lack of theoretical background to design experiments and perform a posteriori analysis of experimental data.

Several drugs acting as suicide inhibitors are currently demanded for the prevention and treatment of different diseases: popular clinical examples of suicide substrate therapies include Aspirin, Exemestane for the treatment of breast cancer, the azidothymidine (AZT) and other nucleoside analogues used against AIDS/HIV, Penicillin, etc. and have been also investigated for the treatment of depression (monoamine oxidase inhibitors) and epilepsy (inhibitors of brain GABA aminotransferase, for example).

Our hypothesis of suicide inhibition to neutralize the SARS-CoV-2 is not unfounded, since this approach has already been pursued in 2003 for the inactivation of the SARS-CoV Main proteinase (Mpro) by means of the benzotriazole esters [29, 21]. Indeed, the SARS-CoV-related Mpro plays a central role in the formation of the viral replicase/transcriptase complex and it appears to be an ideal target for the development of suitable drugs. The structure of complexes resulting from reaction of the SARS-CoV-related Mpro with two aromatic benzotriazole esters provided a promising starting point for designing more specific inhibitors for this proteinase [29]. Although benzotriazole esters, which act as suicide inhibitors, have been reported as potent non-peptidic inhibitors of the enzyme [33], their exact mechanism of action remains unclear. Unfortunately, the currently available protease inhibitor drugs do not seem to work correctly, and sometimes at all, for the treatment of SARS-CoV-2 virus. This is mainly due to the fact that proteases are a family of enzymes with many different characteristics and the drugs already developed for other pathologies are not perfectly compatible with specific proteases of the SARS-CoV-2 virus.

Of course, the creation of this type of drugs could be extremely beneficial, without neglecting different controversial aspects. Suicide-substrate inhibitors are harder to be identified and synthesized because they usually require a fully description of the enzymatic behavior for their design, to provide a base-structure target for the transition state of these enzymes. Due to their high potency and selectivity, as well as a lower propensity for the development of drug resistance, mechanism-based inhibitors can constitute a valuable outlook on the problem of drug resistance. Moreover, there are only a restricted number of enzymes which are capable of being targeted by a mechanism-based inhibitor compared with thousands of synthetically feasible or available compounds in databases used by medicinal chemists. Suicide-substrate inhibitors are particularly invaluable for targeting proteins of bacterial cells when there is a low homology and similarity with human proteins, and the risk of toxicity is appreciably lower [18].

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## History

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# When Politics Freed Science from Task Force Impositions

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The survival of many London entrepreneurs is endangered by a new British government law. It's the Nuisances Removal and Diseases Prevention Act, a bill proposed by the technical task force established by the Queen and the Government with the aim of curbing the spread of the epidemic.

The task force believes that it is necessary to improve the quality of London's air and therefore proposes to impose the adaptation of their production and smoke emission systems on many factories. Which obviously involves huge investments.

The alternative would be the total closure of the factories. We are in London, in 1854. In the midst of the cholera epidemic that would have killed 23,000 people across Great Britain.

In those years the dominant theory in explaining the causes of cholera was that of miasms, which we could also call "fetid airs". The basic idea was that polluted air was the vehicle of the epidemic. Stables, slaughterhouses, and many factories that processed organic materials of both animal and vegetable origin, were the main sources of these pollutions. In fact, gas and boiling production plants dispersed fumes from the nauseating odors into the air, fumes that were believed to be the carriers of cholera.

Dr.John Snow, however, was not convinced by this explanation.

## John Snow's research on cholera

Snow, in those years was an anesthesiologist already well established, he was the one who administered cloroform inhalations as an anesthetic to Queen Victoria on two of her parts.

Precisely because he was an expert on gas inhalation, he was immediately unconvinced by the theory of miasms. According to him, the transmission of the disease occurred through the waters.

In 1849 he began to make his first epidemiological investigations by making appropriate maps on the spread of cholera among Londoners and published a short treatise entitled *On The Mode of Communication of Cholera*. In 1854, when the epidemic recurred, John Snow, in general skepticism, proposed the removing the Broad Street water pump in the Soho neighborhood. The origin of cholera was there for him. A few days after the pump was removed, the epidemic outbreak in that neighborhood actually subsided.

It was because of these ideas against the tide that the London entrepreneurs, whose business was threatened by the new bill, asked Snow to testify before the British Government's Health Commission. The aim was to convince politics to block that law that could have been the end of any activity for them.

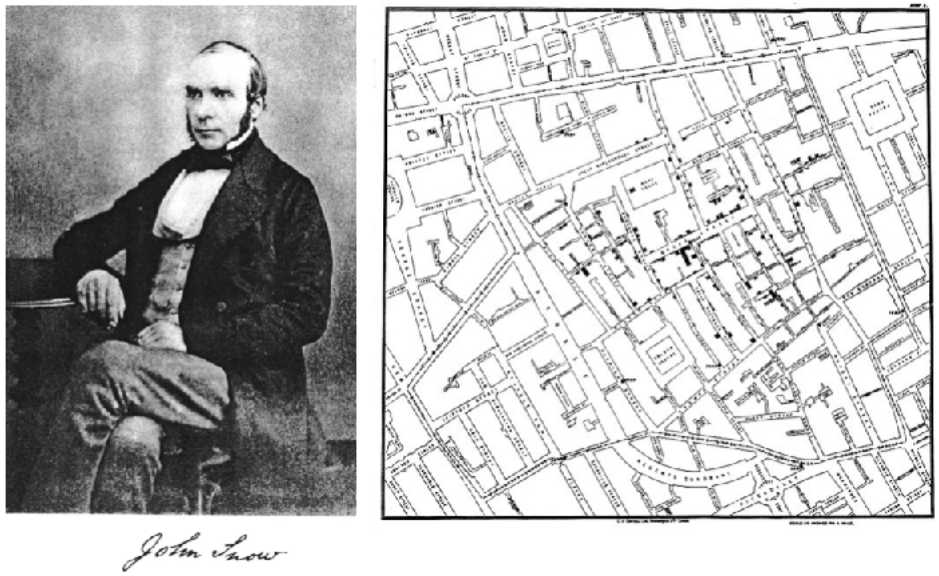


**Figure 1:** John Snow and one of his epidemiological maps

## The interrogation of the Health Commission of the British Government

The hearing was set for March 5, 1855, the President of the Commission was Mr Sir Benjamin Hall, the very Ben from whom the Big Ben of the London clock was named.

Sitting in front of the commission, dr. Snow is immediately clear and honest in explaining the reason why he offered to testify:

*"I received a request from Mr. Knight. I was asked if I would give evidence on behalf of the manufacturers whose interests are threatened by the Nuisances Removal Act."*

*"To what points would you desire to draw the attention of the Committee as regards the sanitary question?" Snow is then asked.*

*"I have paid a great deal of attention to epidemic diseases, more particularly to cholera – says Snow – (...) and I have arrived at the conclusion with regard to what are called offensive trades, that many of them really do not assist in the propagation of epidemic diseases, and that in fact they are not injurious to the public health. I consider that if they were injurious to the public health they would be extremely so to the workmen engaged in those trades, and as far as I have been able to learn, that is not the case; and from the law of the diffusion of gases, it follows, that if they are not injurious to those actually upon the spot, where the trades are carried on, it is impossible they should be to persons further removed from the spot."*

The hearing continued with many more questions and the commission proved cold towards those explanations. The president, Sir. Benjamin Hall, was a strong supporter of the theory of miasms and he held in great consideration the opinion of dr. William Farr, director of the British Statistics Society and the British Government Statistics Department. We could say that Farr was one of those experts with the right profile to lead a task force. Obviously Farr was also a strong supporter of the theory of miasms and pushed for the commission to distance itself from the theories of Dr. Snow.

## The scientific community against Snow's theories

Even *The Lancet* published a tough editorial against John Snow on June 23, 1855. An editorial that spared no criticism even from the direct political commission

Sir Benjamin Hall, who had wasted time listening to these absurd theories.

With these words *The Lancet* reconstructs the hearing of dr. John Snow of March 5, 1855:

> *"They (businessmen) bring before the Committee a doctor and a barrister. They have formed an Association. They have a Secretary, a bone merchant, who has read the writings of* ***Dr. Snow****. Now, the theory of* ***Dr. Snow*** *tallies wonderfully with the views of the "Offensive Trades' Association" — we beg pardon if that is not the right appellation — and so the Secretary puts himself in communication with* ***Dr. Snow****. And they could not possibly get a witness more to their purpose.* ***Dr. Snow*** *tells the Committee that the effluvia from bone-boiling are not in any way prejudicial to the health of the inhabitants of the district; that "ordinary decomposing matter will not produce disease in the 'human subject.'"*

The editorial was not signed but was probably written by James Wakley, son of the founder of the newspaper, Thomas Wakley, and succeeded him as editor:

> *"Why is it then, that* ***Dr. Snow*** *is singular in his opinion? Has he any fact to show in proof? No! But he has a theory, to the effect that animal matters are only injurious when swallowed! The lungs are proof against animal poisons; but the alimentary canal affords a ready inlet. (...)* ***Dr. Snow*** *claims to have discovered that the law of propagation of cholera is the drinking of sewage water. His theory, of course, displaces all other theories. (...) In riding his hobby very hard, he has fallen down through a gully-hole and has never since been able to get out again. And to* ***Dr. Snow*** *an impossible one: so there we leave him."*

## The final decision of the Health Commission of the British Government

Despite all these pressures, on 14 August 1855 the British government officially took the decision not to impose any restrictions on factories that produced fumes, thus giving credit to John Snow's theories.

Maybe because he was not an expert on the subject, Benjamin Hall remained more open than the established scientists of the time to consider theories other than conventional ones. In doing so he has guarded, perhaps unconsciously, that margin of doubt and that openness to the inconceivable indispensable for science to progress.

In the following years, in fact, science produced new evidence to confirm Snow's hypotheses, William Farr When himself changed his mind in 1866 by converting to the theory of the transmission of cholera by water. In 1884 when Robert Koch isolated and studied the cholera bacterium, *Vibrio cholerae* in detail, it was finally understood that transmission could not take place by air.

Today John Snow is universally recognized as one of the fathers of modern epidemiology.

## The late apology of The Lancet

More than 150 years after these events, on April 13, 2013, The Lancet published an article to apologize to John Snow using these words:

*The Lancet* wishes to correct, after an unduly prolonged period of reflection, an impression that it may have given in its obituary of Dr.John Snow on June 26, 1858. The obituary briefly stated:

> *"Dr. John Snow: This well-known physician died at noon, on the 16th instant, at his house in Sackville Street, from an attack of apoplexy. His researches on chloroform and other anaesthetics were appreciated by the profession."*

The journal accepts that some readers may wrongly have inferred that *The Lancet* failed to recognise Dr.Snow's remarkable achievements in the field of epidemiology and, in particular, his visionary work in dedu cing the mode of transmission of epidemic cholera. The Editor would also like to add that comments such as "In riding his hobby very hard, he has fallen down through a gully-hole and has never since been able to get out again" and "Has he any facts to show in proof? No!", pu-

blished in an Editorial on Dr.Snow's theories in 1855, were perhaps somewhat overly negative in tone.

## History, science and the human soul

History always proves to be an excellent teacher to better understand the present and avoid repeating certain mistakes. Thinking today of being immune to these dynamics would however be too naive, it is something inherent in the human soul. The same story however shows us how Science has always managed to evolve freeing itself from all those human conditionings that wanted to control it.

But what is a year for humans can be decades for science.

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## History

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# Art, Plague and Fear

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*They were given power over the fourth part of the earth to exterminate with the sword, with hunger, with the plague and with the fairs of the earth.*
*Revelation VI, 1-8*

Human frailty is one of the omnipresent themes in the art of any time and of any people, and therefore with it the vicissitudes of human life which also include disease and death. Therefore, whether it is a way to exorcise it, to document it or more simply because through the representation of physical evil, of the disease, the artist can somehow approach it unharmed, privileged spectator of a tragic moment that brings with it on the same level pain anyone in the same way. Illness, especially the dreaded plague makes no distinction between princes, prelates and simple commoners, treating everyone with the same regard. Chronically recurrent disease of the plague, will generate a state of anxiety that will spread in Europe for at least three hundred years, eventually becoming a commonplace of human fear.

We then try to give an image to this invisible enemy, and here is where painting intervenes, with its ability to concretize, to coagulate something that is impregnable, rarefied as a disease, in a painting. It is the power of pictorial art, making visible what otherwise would not be. And seeing the "evil", in this case the plague, means knowing it and perhaps even being able to dominate it. So it is above all the painters from the Middle Ages onwards to render the "plagues" in images, with the representation of emphasized bodies that soften the

**Figure 1:** The Triumph of Death, fresco, Buonamico Buffalmacco, 1336-41 5.6 x 15.0 m, south-east wall of the Camposanto of Pisa.

**Figure 2:** The Plague, Gaetano Zummo, 1690, polychrome wax, 76 × 93.5 × 47.8 cm, La Specola Museum, Florence.

cities or clouds that, moving from one country to another, sow death as they pass. It is also the fault of the stars which astrologically cause the deadly contagion. There is therefore a conspicuous source of iconographic material that shows the suddenness of the attack of the disease, its striking in an undifferentiated manner with an obscene death, often deprived of the usual funeral rituals.

We also observe, at least in some artists, more or less veiled, a curious look at the limit of morbidity, on body swellings, on sores, pustules and ulcerative wounds which they sometimes almost enjoy depicting, insisting on them as a stigma of the bad and therefore, ultimately of the demonic. Disease, Plague or other always comes from Hell, even if someone may consider it a divine punishment for the sins of humanity, the disease, the younger sister of Death is, without any doubt, of infernal origin, and not by chance it joins often at the work of the Witches[1].

Hence the idea of a "poisoning" emerges: an extreme attempt to give a face to the unknown but ubiquitous and deadly enemy by finding him in the greaser, the stranger or the witch, who has come to poison the city. The days of the plague are presented as an open-air theater that has the city as its stage and where the actors are sick humans, contemplated in their death by those who are immune.

But this imaginary, which is not often the result of mere imagination, but of a careful look at the reality of the time, is not only relegated to past centuries, to the Middle Ages and especially to the Renaissance and the Baroque age, but we also find it, changed in form but no less disturbing in some contemporary works. Man

**Figure 3:** Fresco of Porta S. Gennaro, The plague of Naples, Mattia Preti, 1657-1659, Porta S. Gennaro, Naples.

changes over the centuries, it is true, but very slowly, and at the bottom of his soul he fears, trembling, with the same fears of his ancestors gathered around a bonfire to protect himself from the night beasts.

Today, this ancient theme has always coexisted with human life: the epidemic, or for those who prefer the most apocalyptic Pandemic, has reappeared in our lives. Expelled from the collective imagination in recent decades, especially if seen in such an all-pervading way in our country, the disease has reappeared unexpectedly, presenting itself at the door of our homes as an uninvited and unwelcome guest. Only in catastrophic movies, in various forms and formulas, the danger of a new

plague was considered, in a flood of anthropophagous zombies, vampires and viruses from deep space[2].

However, not being able to say yet how this Coronavirus, also known as Covid 19, will affect the artistic creativity of the 21st century, we will limit ourselves to taking into consideration how similar events, indeed certainly much more dramatic than ours, have affected the art of the past, starting from one of the best known paintings that deal - also but not only - with this theme: *The Triumph of Death* by Pieter Brueghel the Elder[3], an oil on panel painted in around 1562 and which is currently preserved in the Prado Museum in Madrid.

The work shows the spectator precisely the triumph of Death over the things of our world, herself queen, followed on a reddish horse by an army of living skeletons, in a real hymn that refers to the macabre dances, to the four knights of the Apocalypse and resurrection of the dead.

Against the background of Brueghel's painting, there is a barren, burnt and devastated landscape in which scenes of destruction are still taking place that do not spare any social class and there is manifested the violent and unstoppable action of Death that brutally annihilates humanity, proving itself impartial and ruthless and that arrives and dominates everything by killing men in various ways, in an allegory of war, pestilence and human misery. The warm tones of the oil colors used by the artist evoke an arid and infernal atmosphere in which men face the transition with the most varied moods: with surprise, dismay, resignation and even in the throes of a vain rebellion.

An immense terror and a chilling silence - interrupted only by the echoing of a distant bell - emanate from the painting. In front of the viewer, a parched and sunless world extends, immersed in a twilight whose pale light is enlivened only by the funeral reverberation of the fires. It is not the end of the world that is represented, but the conquest of life by death, so that, in a certain sense, a way of being is replaced by another form of existence.

The artist depicts Death as a skeleton riding an emaciated horse that, with its scythe, kills anyone who crosses its path, just like the plague that decimated entire European populations. And Brueghel represents his fear, while in the distance we see men hanged, perhaps

**Figure 4:** The Triumph of Death, Pieter Brueghel the Elder, 1562, oil on panel, 117 × 162 cm, Prado Museum, Madrid.

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**Figure 5:** The Plague, Arnold Böcklin, 1898, tempera, 149.5 cm x 104.5 cm, Kunstmuseum Basel.

**Figure 6:** St Mark on the throne or St Mark and the Saints, oil on board, Tiziano Vecellio, around 1510-1511, Santa Maria della Salute, Venice.

unjustly: they are some Jews accused of causing the serious epidemic.

The painting is therefore like a story that unfolds before the eyes of the viewer in numerous small episodes, in which Death triumphs over man.

The landscape of Brueghel takes on deforming and unreal characters in this picture: in a desolate land, interspersed with dying trees and macabre hangings, the final struggle of men against the Army of Death takes place, composed of disturbing skeletons wrapped in white shrouds. The outcome of the chaotic battle is already decided; the last alive are surrounded by the funeral form, with no possibility of escape. The horizon is obscured by an intense and black smoke, coming from the bonfires of the Hell.

Brueghel probably had the opportunity to learn originally the macabre theme from the fifteenth-century frescoes that stood on the south-facing wall of Palazzo Sclafani in Palermo and which bear the same title as his painting. Today the Palermo fresco has been divided into four portions and repositioned at the National Gallery of Palazzo Abatellis, after having miraculously survived the bombings of the last World War. Again, therefore, preceding Brueghel's masterful brush, Death brings with it various forms of destruction, and among them, not least the pestilence, fatal and unstoppable.

However, even before Brueghel the Elder and the Sicilian frescoes, the theme of the Triumph of Death is found already between 1336 and 1341 in the Camposanto of Pisa, executed by Buonamico Buffalmacco[4]. The fourteenth-century painter is remembered as the protagonist of some short stories by Giovanni Boccaccio in his Decameron and in this work the topic of pestilence returns with the usual paraphernalia of deaths between priests, merchants and nobles whose souls are disputed between angels and demons and on which the signs of putrefaction are already manifesting.

Still in the sixteenth century, the Medici Florence was devastated by the plague in the year of the Lord 1523.

The city is in its full splendor of beauty, culture and art, which make it one of the nerve centers of Europe, and therefore of the world of that time, when sudden, pestilence comes within its walls. Who can leave the town as quickly as possible, seeking refuge elsewhere and one of them is Jacopo Pontormo[5], who finds shelter from the disease in the Certosa del Galluzzo, where he will spend the most peaceful period of his life constantly

marked by a perennial melancholy. Influenced by the visions of Albrecht Dürer[6], in those days Pontormo frescoed his cycle called *Stories of the Passion* in the large cloister of the Certosa, but he is not alone, escaping from the plague with him there is also the student and friend Agnolo Bronzino[7]. In that sad predicament instead Jacopo finds himself and discovers the true essence of his life. The plague was therefore benevolent with him, transforming his forced exile into a rediscovery of freedom and inner peace.

Andrea del Sarto[8] also sought refuge from the black disease, but he did so by going further away from Florence. Thus, one of the greatest mannerists, one of the most eccentric, unprejudiced and unconventional artists who followed the school of the "modern way", formed in the climate of intellectual freedom of the republic governed by Pier Soderini[9], sheltered in the monastery of Luco in Mugello, for who paints an altarpiece depicting the body of the dead Christ, naked abandoned on a white sheet that covers the sepulchral stone. Immediately under the lifeless body of Jesus, a mass chalice is painted covered with a paten, on which stands the consecrated host. The death of Christ is therefore the source of eternal life, this is the clear meaning of the work created precisely during the epidemic. Having escaped from the disease of 1523, Andrea Del Sarto, for a mocking joke of fate, however, will die during the subsequent wave of plague that will hit the City of the Lily in 1530.

In those same years, another city among the richest and most important of the peninsula, Venice, the dogale and most peaceful, placed under the aegis of the Evangelist Mark and his winged lion, would be violently affected by the epidemic, perhaps facilitated in this from being that golden gateway to the sea that faces East and West. Perhaps, however, precisely for this reason, the lagoon city was also the first to have a real prevention against diseases, even if this did not preserve it from the epidemic of 1510, during which, once again and as always, the works of art related to miraculous healing grew considerably in number.

In this particular event there is the great ex voto, created by Tiziano Vecellio[10] for the church of Santo Spirito in Isola, entitled *San Marco and the Saints* with good probability commissioned by the Doge himself and by the Senate of the Republic to commemorate the end of the plague.

The composition of the painting follows an invisible design in the form of a pyramid, whose summit is San Marco, or Venice itself, while at the base there are the saints Cosma and Damiano - both doctors who treated the sick without compensation - caught in the act of indicating the wounds of San Rocco and San Sebastiano, the latter always invoked to obtain healing from the plague. The face of San Marco was deliberately painted in the shade by Titian, to remember the sad event that hit the lagoon.

**Figure 7:** San Rocco heals the plague victims, Jacopo Tintoretto, 1549, 307 × 673 cm, Church of San Rocco, Venice

**Figure 8:** Yield of Grace after the plague of Naples, Micco Spadaro, 1657, National Museum of San Martino, Naples.

**Figure 9:** The Triumph of Death, unknown author, around 1446, detached fresco, 600 × 642 cm, regional gallery of Palazzo Abatellis in Palermo.

**Figure 10:** San Rocco and the victims of the plague, Simone de Wobreck, 1576, oil on panel, 200 x 300 cm, S. Cosma e Damiano, Palermo.

Today the painting is in the Basilica of Santa Maria della Salute, also built after 1630 to dissolve the vow of having defended the city from the plague of that time.

Also in the city of Leone di San Marco, Jacopo Robusti[11], better known as Il Tintoretto, the last of the great Venetian painters of the Renaissance, in just over a decade will paint over fifty sacred canvases for the Scuola Grande di San Rocco, a ancient building that became the seat of the school of the brotherhood dedicated to the saint revered for his talents as a thaumaturge. One of these paintings, painted in 1549 for the presbytery of the church annexed to the school, is entitled *San Rocco heals the plague*, painted in oil, it presents in a nocturnal atmosphere, some plague victims who turn their suffering gaze towards the saint by impetrating his Help.

Much further south, in beautiful Sicily, a plague epidemic spread and claimed countless victims in Palermo in 1575. It was the Flemish painter Simone de Wobreck[12] who painted it for what was then the homonymous church today rededicated to Saints Cosmas and Damian, showing also San Rocco and the plague victims. In this work, the epidemic is represented as a divine punishment for the sins committed by humanity: At the top, God himself was painted, together with the Christ and the Virgin; the first bearing the signs of the Passion and finally the saints Rocco, Sebastiano, Cristina and Ninfa, who ask them for the grace of the salvation of the people. On the lower part, you can see the procession of the crucifix of Cristo Chiaromonte of the Cathedral, with all the population, among whose people you can recognize the confreres of the Compagnia dei Bianchi.

Instead in alemannic lands, more precisely in Alsace, in Colmar, the work is kept, considered the masterpiece of Matthias Grünewald[13], the *Polyptych of Isenheim*, painted between the year 1512 and 1516, today at the Musée d'Unterlinden.

This singular "altar machine", or altarpiece, if you like, is able to offer the viewer three different configurations, but it is only the third that we will analyze, or that relating to the *Temptations of Saint Anthony*.

In a nightmare scenario, the anchorit of the Egyptian desert is subjected to the violence of some demons with horrible and obscene forms that attack him, beating him and mocking him in every way.

However, the most attentive observer will notice a character painted in the lower left corner. At the feet of the hermit saint, on a cartouche we read the Latin phrase *"Ubi eras, Bone Jhesu, ubi eras, quare non affuisti ut sanares vulnera mea?"* or "Where were you or good Jesus, where were you and why didn't you come to heal my wounds?". The character of the painting remains shrouded in mystery, and therefore even more disturbing is his presence, but the vulnerable refers to the fact that in the vicinity of Isenheim there existed at that time, a monastery of monks faithful to the Antonian rule[14], whose main task was assistance to Ergotism[15] patients, also known as Fire of Saint Anthony[16], but which at the time did not correspond to the disease known by us with the same name and which is actually *Herpes Zoster*[17]. The Fire of Sant'Antonio, or even of San Marziale, of the medieval and later ages, is probably a set of various ulcerative pathologies that led those who had been affected, to gangrene and from there to a slow and painful death.

Matthias Grünewald was able to reproduce the effects of this disease in such a detailed way, as he could freely access the mortuary of the convent and therefore see with his own eyes the devastating effects of evil on the patients that the monks, with the blue Tau sewn on the habit, they tried to heal by soothing their wounds with lard and feeding them with uncontaminated bread.

The golden age of the Renaissance vanishes, fading into the twisted darkness of the Baroque, in a time much darker than the previous ones and yet still affected by the scourge of the diseases, as happens in Naples in 1656, and Mattia Preti[18] will paint its terrible and bloody atmosphere in his work *The plague of Naples*. Another neapolitan painter, Micco Spadaro[19], will also create a votive picture entitled *Yield of Grace after the plague of Naples* to thank the saints for ending the plague. The rendering of these paintings is documentary, real, no longer immersed in a fantastic context as previously happened. The classic example of this different approach, which characterizes the art of the seventeenth century, is that of the works of Gaetano Zummo[20], now exhibited at the Museo della Specola in Florence,

**Figure 11:** The Plague of Azoth, Nicolas Poussin, 1631, oil on canvas, 148x198 cm, Louvre Museum, Paris.

**Figure 12:** The Family, Egon Schiele, 1918, oil on canvas, 152.5 × 162.5 cm, Österreichische Galerie Belvedere.

**Figure 13:** The Plague, Gaetano Zummo, 1690, polychrome wax, 76 × 93.5 × 47.8 cm, La Specola Museum, Florence.

considered not by chance the founder of the renowned Florentine anatomical school.

Of Syracusan origin, Zummo was formed by studying the bodies in the morgues to become the best wax doctor in all of Europe. His most famous work is *La Peste*, probably created in Naples in the year 1690 on the still close memory of the plague of 1656.

The three-dimensional scene created by the artist is simply infernal, with bodies of living and dead tangled together in a chaotic and shapeless mass. Corpses in an advanced state of decomposition are clinging to those who are about to die, in a sort of obscene, hideous multiple embrace, in which bodies corroded by putrefaction and others painfully in agony are intertwined. In the midst of this macabre bedlam, a monk with a hidden face, with superhuman strength, carries the ulcerated bodies towards the funeral pyre that stands out in the background.

Also in the seventeenth century, Nicolas Poussin[21], also buried in Rome in the church of San Lorenzo in Lucina, will perform in 1631 a work entitled *The plague of Azoth*, today in the Louvre, for a nobleman from Palermo, such as Fabrizio Valguarnera, but then purchased by Cardinal Richelieu for his own art collection from which he finally came to that of Louis XIV.

The painting depicts a biblical passage taken from the first book of Samuel, in which God strikes the Philistines, guilty of having stolen the Ark of the Covenant from the chosen people, with a terrible plague. Also in this case, an atmosphere of tragedy envelops everything. The ruins of the temple of the god Dagon[22], the Ark and other architectural vestiges, are the backdrop for people fleeing the bodies of the plague scattered everywhere on the ground, between mice and the smell of putrefaction. Probably the artist had the opportunity to see the epidemic that killed Milan the year before and was shocked to such an extent that he could reproduce that gloomy and oppressive atmosphere on the canvas.

However the disease will certainly not end with the *Siglo de Oro*, it will simply take other forms, mutant, but always faithful to its man living in this world and here in the field of pictorial art we find it with *La Peste*, a 1898 painting executed in tempera by the Swiss artist Arnold Böcklin[23], one of the greatest exponents of german symbolism, today on display at the Kunstmuseum in Basel.

The work depicts Böcklin's obsessive fear of the apocalyptic nightmares of War, Death and Pestilence which in this case is depicted by a parched skeleton who rides a fantastic and monstrous creature, with membranous wings like those of bats, while fly over a medieval-looking city. The entire pictorial composition is rendered in a tone of acid green and bruise, as if to recall the swollen aspect of the decomposition of the meat.

**Figure 14:** Isenheim Altar or Isenheim Polyptych, Matthias Grünewald, 1512-1516, oil and tempera on panel, Musée d'Unterlinden, Colmar, Alsace.

Twenty years later, it will be the turn of the Austrian Egon Schiele[24] to deal with the theme of pandemic and fatal disease, with his painting *The Family*, dated 1918 and today exhibited at the Österreichische Galerie Belvedere in Vienna. One of his last works in which he depicts himself, with his wife and their child in a future projection, since his wife Edith, while he was painting, was in the sixth month of pregnancy.

The terrible flu disease called "The Spanish"[25] raged throughout Europe, which killed the world population causing the death of fifty million people in a few months, just before the end of the Great War.

**Figure 15:** The Plague, Gaetano Zummo, 1690, polychrome wax, 76 × 93.5 × 47.8 cm, La Specola Museum, Florence.

The happy scene hoped for by Schiele in his auspicious painting will not take place, as Edith, still with their baby in her lap, will die of that terrible disease on October 28, 1918. Her husband Egon will follow her on October 31, also killed by the pneumonia caused by the pandemic flu virus.

Since then, the step of Time and that taken by humanity, even if rapid, has been too short for substantial changes to take place in the relationship between man, art and social disease, so it is difficult for us to imagine in what way and with what aspects the representation of the epidemic will mark our future in the image, the only immediate example, almost a snapshot is what appears in these days on social media where someone with a fine irony, is having fun to modify the works of art considered iconic, such as Leonardo da Vinci's *Mona Lisa* for example or *The girl with a pearl earring* by Rembrandt Harmenszoon van Rijn and other famous ones like these, making them wear masks and gloves... So here, this strange technological and postmodern man of the contemporary world of the first twenty years of the new century, reacts to the viral attack no longer by creating art, albeit with an apotropaic, devotional or documentary purpose, but with a u this is a digital virality that

perhaps tries, with a bitter laugh, to exorcise the evil, of which it is still afraid, as always.

## Endnotes

1 Witches are in fact attributed with the powers of evil that also entail the ability to spread diseases thanks to their servitude with the devil, therefore they are accused of poisoning the wells, causing famines and deaths of cattle as well as spreading the infection. All activities punished with being put to death at the stake.

2 There are many films that have as a theme a global epidemic infection, among the many we want to remember some of them, for example Paul W.S.'s *Resident Evil*. Anderson, *28 days later* by Danny Boyle, Terry Gilliam's *The 12 Monkeys Army*, John Hillcoat's The Road, Steven Soderbergh's *Contagion*, *I am legend* of Francis Lawrence, remake of *The Last Man on Earth* with Vincent Price directed by Italian Ubaldo Ragona and *The city will be destroyed at the dawn* of George A. Romero.

3 Pieter Brueghel (Breda, c. 1525/1530 - Brussels, 5 September 1569), was one of the greatest Dutch Renaissance painters, generally referred to as the Elder, to distinguish him from his eldest son, Pieter Bruegel the Younger. The information in our possession on the life of Bruegel is poor, incomplete, and sometimes contradictory because, for example, the exact date and place of birth, they are still unknown and therefore it is known only that in 1551 Brueghel is mentioned for the first time in writing, when he joined the Guild of San Luca in Antwerp qualifying as a master.

4 Buonamico di Martino, called Buffalmacco (Florence, about 1262 - 1340), was a Florentine painter, a prominent representative of Gothic painting in Tuscany in the first half of the fourteenth century. Long believed to be only a protagonist of mocking novels of the Decameron, the most recent research has attributed to him the frescoes of the Camposanto of Pisa.

5 Jacopo Carucci, better known as Jacopo da Pontormo or more simply Pontormo (Pontorme, 24 May 1494 - Florence, 1 January 1557), was a Florentine painter of early Mannerism. Student of Andrea del Sarto, together with Rosso Fiorentino, he was in turn a master of other artists including Bronzino.

6 Albrecht Dürer (Nuremberg, May 21, 1471 - Nuremberg, April 6, 1528), was a German painter, engraver, mathematician and treatise writer, considered the greatest exponent of Renaissance painting in his country. In Venice the artist came into contact with neo-Platonic and therefore esoteric environments that initiated him to hermetic symbols.

7 Agnolo di Cosimo, better known as Agnolo Bronzino or simply the Bronzino (Monticelli of Florence, 17 November 1503 - Florence, 23 November 1572), a pupil of Pontormo, lived all his life in Florence at the court of Cosimo I de' Medici. He was among the most refined portrait painters of the early Mannerism, of religious and allegorical subjects.

8 Andrea del Sarto, pseudonym of Andrea d'Agnolo di Francesco di Luca di Paolo del Migliore Vannucchi (Florence, 16 July 1486 - Florence, 29 September 1530), was a Florentine painter with a great executive formality and master of Pontormo and Rosso Fiorentino, but he was less bold and controversial than they were.

9 Pier Soderini (Florence, 18 May 1450 - Rome, 13 June 1522), was a gonfalonier for life in Florence from 1502, a position he maintained only until 1512, coming from an ancient Florentine family who had given many political figures to the city, he was responsible for the decoration of the Salone dei Cinquecento in Palazzo Vecchio, commissioned to the two greatest Florentine artists of the time: Leonardo da Vinci and Michelangelo Buonarroti.

10 Tiziano Vecellio (Pieve di Cadore, 1488/1490 - Venice, 27 August 1576), was the most important painter of the Venetian Republic and an exponent of the Venetian school.

11 Jacopo Robusti or perhaps Jacopo Comin, known as Tintoretto (Venice, September or October 1518 - Venice, May 31, 1594), was a painter of the Republic of Venice and one of the greatest exponents of Venetian painting and Mannerist art in general. The nickname derives from his paternal profession, a textile dyer, he was also nicknamed "The furious" or "The terrible" for his character and for the dramatic nature of the perspective and the light that made him believe by critics an anticipator of Baroque art.

12 Simone De Wobreck (Haarlem, around 1557 - Palermo, around 1587), was a flemish painter active in Sicily for more than thirty years, so much so that he was more connected to that island than to Holland.

13 Mathis Gothart Nithart, better known as Matthias Grünewald (Würzburg, about 1480 - Halle, 31 August 1528), is one of the greatest and most original german painters, for the visionary drama of his paintings. Doubts about his date of birth and the certain lack of documents also make Grünewald's artistic training problematic. It is not known who his teacher was either.

14 In the Middle Ages a religious order arose dedicated exclusively to the care of Ergotism sufferers, known as the regu-

lar canons of Saint Anthony of Vienne which in 1774 was incorporated into the Order of Malta.

15 Ergotism would be an intoxication from ergot or the spur of the rooster, in French, already known in medieval times with the name of "Saint Anthony's Fire" or also "sacred fire" or with the more courtly term of "evil of ardent" up to the most fearsome "Flames of Satan". The ergot, or *sclerotium*, is horned rye.

16 The Fire of Saint Anthony owes its singular appellation to the fact that Saint Anthony the Abbot was invoked for his recovery. Around the remains and relics of the saint, a real thaumaturgical cult arose in 12th century France, which changed it into the unique eponym of the disease, while other saints and the Virgin Mary herself, continued to be invoked as healers.

17 *Herpes zoster*, commonly called St. Anthony's Fire, is a viral disease of the skin and nerve endings, caused by the childhood chicken pox virus. Its name derives from the greek words, "snake" and "belt", which synthetically describe the painful disease, like a fire snake placed inside the body, which sometimes produces a painful rash with blisters, usually in a belt-like strip.

18 Mattia Preti (Taverna, 25 February 1613 - Valletta, 3 January 1699), was a Neapolitan painter also known as "The Calabrian Knight" because he was born in Calabria and later as a Knight of Grace of the Order of St. John of Jerusalem, by Pope Urban VIII in Rome. Active on the Italian peninsula and on the island of Malta, he was one of the most important representatives of seventeenth-century Neapolitan painting.

19 Domenico Gargiulo, known as Micco Spadaro as the son of a sword maker (Naples, 1609/1612 - 1675), was a Baroque painter, active above all in Naples as a landscape painter and known above all for having documented many tragic events in his city such as the insurrection of Masaniello.

20 Gaetano Giulio Zumbo, or Zummo (Syracuse, 1656 - Paris, 22 December 1701), was a Sicilian abbot who became famous in the seventeenth century for his works in waxwork and for his nativity scene. The information about this artist trained at the Jesuits and in Bologna, a famous anatomical study center, is scarce and fragmentary.

21 Nicolas Poussin (Les Andelys, 15 June 1594 - Rome, 19 November 1665), was a french painter of classical approach who came to Italy in 1624, under the protection of Cardinal Barberini, a wealthy collector and patron and thanks to his friend the poet Giambattista Marino became the fashionable painter of many wealthy roman families.

22 Dagon is an important Mesopotamian deity of the Canaanite fertility with the appearance of a bearded man with the lower part of the fish body, adopted as the main deity by the Philistines, in the *Bible* an episode is mentioned in which the statue of Dagon collapses before the Ark of the Covenant in the Philistine city of Ashdod. In demonology texts, Dagon is the name of a second level demon and finally a supernatural entity with that name appears, in a novel by Howard Phillips Lovecraft, published in the magazine *Weird Tales* in 1923.

23 Arnold Böcklin (Basel, 16 October 1827 - San Domenico di Fiesole, 16 January 1901) was a Swiss painter, one of the main exponents of German Symbolism, but who spent most of his life in Rome and Italy, drinking from art Renaissance in the country.

24 Egon Leon Adolf Schiele, better known as Egon Schiele (Tulln an der Donau, 12 June 1890 - Vienna, 31 October 1918), was an Austrian painter and engraver who was a pupil of Gustav Klimt. Schiele was the absolute exponent of early expressionism and in particular in the Viennese Secession movement; early talent, he dies at the age of twenty-eight.

25 The flu commonly referred to as "Spanish" or also as "The Great Flu" was an unusually fatal flu pandemic that killed tens of millions of people around the world between 1918 and 1920 after infecting nearly five hundred million people, including inhabitants of some remote islands of the Pacific Ocean and the Arctic Ocean, ending up causing the death of fifty million people out of a world population of about two billion. This fact made it considered the most serious form of pandemic in the history of mankind, as it made many more victims of the XIV century black plague, even among robust young adults and not, as usually happens, with weaker elderly people.

One of the causes of death of the Spaniard was a rapid progressive respiratory failure whose viral aggression perhaps was enhanced by some particular circumstances such as war, malnutrition, or perhaps the same overcrowded hospitals with widespread poor hygiene.

The flu was given the name "Spanish" because the first to give official news were the Spanish newspapers which, not being involved in the censorship of the Great War, spoke freely of it, contrary to what happened in the belligerent countries where the rapid spread of the disease was hidden from the press, which described it as an epidemic limited to Spain.

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## News in focus

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# EGOI is Taking Off

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*Organisms* reports the recent birth of EGOI platform, namely the website of the Experts Group on Inositol in Basic and Clinical Research.

This group involves 31 international personalities from 12 different countries, skilled in the field of inositol physiology and therapy, with the aim to discuss scientific contents relating to their own expertise in the field.

Inositol is a natural molecule that is found in the phospholipids of cell membranes, in the lipoproteins of the plasma and, in the form of inositol-phosphates, in the cell nucleus [1,2].

When we speak about inositol, we mean a group of nine different stereoisomers, so that it would be more correct to use the plural "inositols".

Among these, however, the term inositol is generally used to refer to the most bioavailable type, myo-inositol. In addition to Myo-inositol there is also the isomer D-chiro-inositol.

Both, in the form of inositolophosphoglycans, are "second messengers" of the insulin hormone [3].

Even if their biological functions are often confused, we need to remember that Myo-inositol and D-chiro-inositol play different roles in the body.

Myo-inositol is involved in the cellular absorption of glucose, meanwhile D-chiro in metabolism and storage of glucose in the form of glycogen [3].

The benefits of both Myo- and D-chiro-inositol are now well established.

These isomers demonstrated to be effective in the prevention and in the treatment of many different diseases, such as polycystic ovary syndrome (PCOS), insulin resistance (IR), metabolic syndrome, gestational diabe-

tes mellitus (GDM) and neural tube defects (NTDs) [4-7].

Recently, the interest in inositol has also involved other areas, such as cardiology [8] and oncology [9,10].

Moreover, inositol phosphates derivatives, especially those downstream the activation of specific inositol kinases, play critical roles in chromatin remodelling and DNA methylation. Overall, the participation of those metabolites seems to exert unexpected key functions during morphogenesis and cell fate commitment, in both natural and pathological processes.

However, even though data and results on the use of inositols are progressively increasing, some key concepts are still unclarified, especially concerning the proper use and combination of inositol isomers in different clinical settings.

EGOI focus its activity in fostering advanced studies and scientific debate on these arguments, by enhancing the cooperation and scientific networking among scientists from different countries.

To visit the EGOI website: www.inositolgroup.com

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## Books

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# Pharmacology Studies: Hints for a Change in the Paradigm

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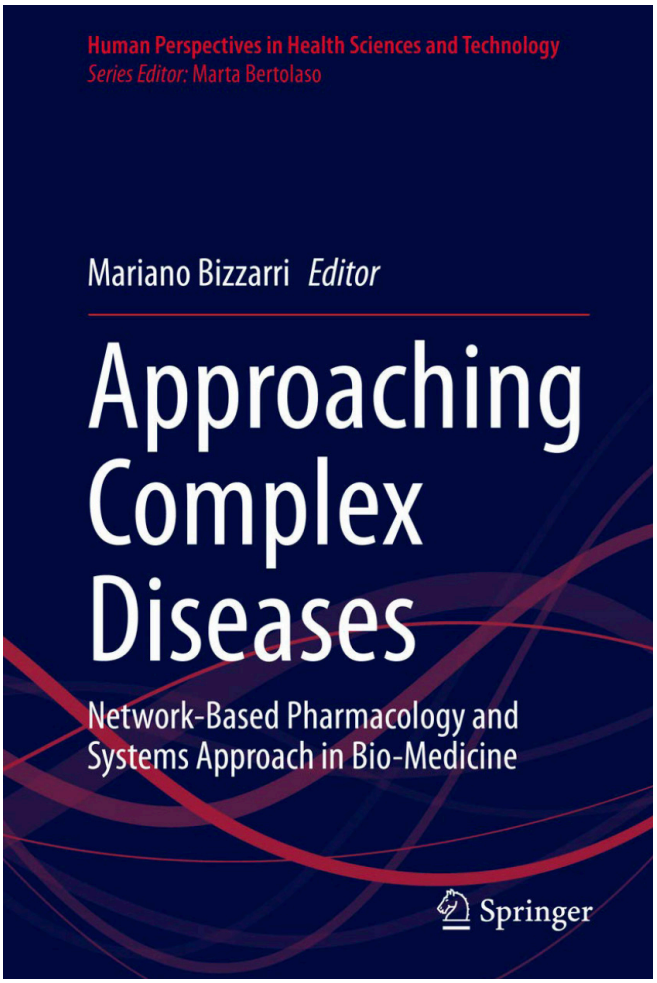


**Approaching Complex Diseases: Network-Based Pharmacology and Systems Approach in Bio-Medicine**
**Mariano Bizzarri** (Editor)
**Springer Nature**
**Series:** Human Perspectives in Health Sciences and Technology Book
**Volume No.:** 2
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The pharmaceutical industry is currently facing unparalleled challenges to develop innovative new drugs. Although the annual number of new drugs has not changed much, research and development (R&D) investment per drug is escalating at a marked rate. However, this relevant involvement in drug discovery is unfit to cope successfully with new challenges, as those provided by recent advances in basic and applied medical sciences.

While pharmacological research performed impressive results in treating cardiovascular, cerebrovascular and infective diseases, no proportional benefits have been recorded in the cure rates of neoplastic, metabolic and degenerative diseases.

Indeed, despite the increased investment in R&D by the industry, the number of new molecular entities achieving marketing authorization is not increasing. Contrary to expectations, high investment, development of technology and omics approaches - such as those based on proteomics and genomics - neither have reduced the R&D risk, nor have enhanced efficiency.

Three drug-discovery fads have driven the industry's R&D programs in the past thirty years: computer aided drug design, combinational chemistry linked to high throughput screening and genomics.

Until the 1990s, drug discovery and development was largely based on a phenotypic approach or observation-based ('empirical') approach. However, the accumulation of knowledge in biochemistry and molecular biology, led to a shift toward the target-based model, which entirely rely on a reductionist-based theoretical framework. Consequently, target-based drug discovery has been the main research paradigm used by the pharmaceutical industry during the last 30 years and billions of dollars have been invested into this approach. However, recent industry data strongly indicate that the

target-based approach is not an effective drug discovery paradigm and is likely to be the cause of the productivity crisis the industry is experiencing.

While drug-developing chemists and biologists in the 1990s mostly welcomed the transformation into a target-based approach (which was surmised more predictable and science-driven), two decades of experience shows that this model is failing to boost both drug discovery and efficiency. Selected targets were often not druggable and with poor disease linkage, leading to either high toxicity or poor efficacy. The off-target effect of a drug was much more difficult to predict in comparison to the phenotypic approach. Because the whole industry was using similar compound libraries for druggable targets, the diversity of pharmaceutical companies' portfolio has been spoiled. This has led to intense competition, where speed of clinical trials and marketing were the main attributes in determining the first-in-class or best-in-class.

Moreover, this approach will likely focus on non-essential targets, thus producing more failures through lack of efficacy. However, there are no evidence that any of these is or will be capable of replacing the old techniques. Namely, the basic premises on which gene-based pharmacological approach is increasingly questioned, as no one of the bewildering results hitherto anticipated have been so far achieved. For instance, the possibility of finding so-called synthetic-lethal drug targets, which are only essential in cancer cells that carry mutations in so-called tumor suppressor genes, is attractive only in theory as many objections stand out against that hypothesis. Indeed, a classical genetic approach is unlikely to be a solution as this model underestimates the importance of environmental milieu in shaping health boundaries. The second reason is the great complexity of gene/gene, gene/environment interactions, and the third reason is the high individual variability.

The purpose of drug design is to find the optimal structure that possesses high specificity around the target and interferes less with other sites to decrease the likelihood of side effects. However, in many, if not in most diseases, such unique target simply does not exist. For instance, in cancer, several pathway are deregulated, none of which is as specific enough to be a 'hallmark of cancer'. Moreover, by utterly inhibiting/activating this/these target(s) would seriously impair also the functioning of normal tissues, which usually rely on the same pathways.

Some attempts have been made to deal with these challenging hurdles, even if a rational strategy is still lacking.

We need a conceptual revolution. This 'paradigm change' will have profound scientific and philosophical consequences, given that it implies the search for general principles on which a cogent theory of biology might rely. Because much of the logic of living systems is located at higher levels, it is imperative to focus on them. Indeed, both evolution and physiology work on these levels. A Systems Biology approach is needed to catch such a complexity. Accordingly, this new perspective will entail epistemological and methodological issues as well.

*Industry synergy*. Based on the R&D level and progress made, new small, molecular entities will still be dominated in drug innovation for the next decade. This strategy is primarily thought to reduce the burden of financial investments. However, still confusing is the class of compounds on which we have to focus. Currently, this approach mostly relies on perspective of 'industrial synergy', aimed at multichannel integration of small/medium size enterprises.

*Nanotechnology*. In recent years, nanotechnology has been increasingly applied in drug development throughout the drug development chain. Nanoparticle-based therapeutics can confer the ability to overcome biological barriers, effectively delivering drugs and biologics, and preferentially target sites of disease. However, despite the potential advantages of nanoparticles, only a relatively small number of nanoparticle-based medicines have been approved and marketed for clinical use. The safety and efficacy of nanomedicines can be influenced by minor variations in multiple parameters and need to be carefully examined and controlled in preclinical and clinical studies, particularly in reference to their biodistribution, pharmacokinetics and potential toxicity.

*Natural products*. Natural products and their derivatives have historically been invaluable as a source of therapeutic agents. Despite the disbelief that such class of potential drugs encompassed in the last decades, recent updates and technological advances, coupled with unrealized expectations from current lead-generation

strategies, have led to renewed interest in natural products in drug discovery. Indeed, many natural molecules, prone to be eventually engineered to amplify their efficacy, have already proven to be effective in the treatment of several diseases.

*Network polypharmacology*. The dominant paradigm in drug discovery is the concept of designing maximally selective ligands to act on individual drug targets. However, many effective drugs act via modulation of multiple proteins rather than single targets. Advances in systems biology are revealing a phenotypic robustness and a network structure that strongly suggests that exquisitely selective compounds, compared with multitarget drugs, may exhibit lower than desired clinical efficacy. This new appreciation of the role of polypharmacology has significant implications for tackling the two major sources of attrition in drug development—efficacy and toxicity. Integrating network biology and polypharmacology holds the promise of expanding the current opportunity space for druggable targets.

*Tumor reversion*. Tumor reversion, a new testable paradigm in drug discovery, constitutes a remarkable case in point of the aforementioned strategy. An increasing number of reports has ascertained the occurrence of cancer reversion, both in vitro and in vivo. This process encompasses mandatorily a change in the cell-stroma interactions, leading to profound modification in tissue architecture. As cancer can be successfully 'reprogrammed' through the modification of the dynamical cross talk with its microenvironment, the overall cell-stroma interactive network must be recognized as the 'target' for pharmacological intervention. This new approach bears huge implications, from both a theoretical and clinical perspective, as it may facilitate the design of a novel anticancer strategy focused on mimicking or activating the tumor reversion pathway.

What we have to do now? Clearly, the looming difficulties will be primarily on the premises on which therapies are planned. For these, the companies may well have to go back to academia or, at least, to academics studying new and unexplored paths. For instance, systems biology, which today is still largely an enterprise of "academic" interest may find itself increasingly incorporated into the research programs of industrial enterprises.

We believe that the needed approaches are not simply to flog individuals to try harder but to build systems and infrastructures that enhance creative effort. Lateral thinking can and should be taught. Indeed, time is gone to address such challenging issues and to restore both confidence and efficiency to the pharmaceutical industry.

The volume we are proposing herewith in the Springer series, points to address such questions, by providing a full assessment of the premises underlying a radical shift in the pharmacology paradigm.

Communication

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# A Manifesto from the "Gravity Center": Beyond the COVID-19 Pandemic in Italy

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## Plan for scientific clarity

We are a group of doctors and scientists who want to examine how the fight against Covid-19 is developing at global level with the intention of making the situation clearer, with especial regard to new therapies that are showing themselves to be useful in saving many human lives.

In the last few months, we have seen an improper use of science presented in the media as irrefutable dogma, which is a betrayal of every true scientific spirit. We have also observed that - faced with widespread subservience in the name of science at the service of politics and money – the voice of an increasing number of free scientists and doctors on the front lines have taken a stand apart from the "truths" bandied about in talk shows that are a thousand miles removed from the operational realities in hospitals and research institutes.

These colleagues have often done so, unfortunately, individually and timidly, for fear of reprisals.
This has happened in particular in the last few weeks, thanks to the courage of a few therapists who – having realised that the clinical approach to Covid-19 had various large gaps – risked everything to save their patients by adopting innovative care treatment and refusing the use of potential harmful treatments.

We therefore believe it is tremendously urgent to exercise every effort to overcome the fragmentation of individual voices by setting up a platform on which the greatest number of scientists and doctors who have been able to set aside their own ideological differences can all contribute – based on the present document that has been shared and countersigned. The aim is to present a united front against the "official truth" - defending against outside interests – which are being rammed down our throats daily in the name of a science that in fact is being despised and violated.

## The Italian experience

### 1. Delay

A great deal remains to be made clear about the emergency and the spread of the Covid-19 epidemic. It is not in the scope of this document to broach these issues.

This document is an analytical proposal.

Italy, and especially its regions in the North, has been one of the hardest-hit countries. We believe there are weighty and well-founded responsibilities, both in the regions and centrally, which have aggravated the situation created by the Covid-19 virus. There are grave doubts about the public health measures that were adopted in Italy. We have been taken by surprise, but we had been warned in good time, in various forms and at various times.

To sum up: the Government activated – but only in part – a programme of emergency action around 50 days late, which then revealed itself to be deadly.

The first public report from the Istituto Superiore di Sanità [Health Academy] was only made on the 16th January.

It is certainly true that the behaviour of the World Health Organization was strangely wavering, uncertain and often confused. Nevertheless, the succession of news coming out from China was already enough to be

able to give rise to serious alarm. Instead, the Giornale della Protezione Civile [Emergency Planning Journal] dated 24th January informed readers that *"Italy has a plan against the coronavirus, but for the moment there is no cause for concern"*. It seems that nobody at the Istituto Superiore di Sanità [Health Academy] had read the Report on Health Security from the *John Hopkins Bloomberg School of Public Health*, dated September 2019. That report examined the *"state of preparedness"* when confronted with a pandemic caused by *"widespread impact respiratory pathogens"*, with a *"high potential transmissibility and high recorded death rate"*. The precision of the forecasts in such a study could only be measured a *posteriori*, that is, today. In addition, that document is worth examining with extreme care at a time when new international safety issues have to be faced. In fact, that document highlighted "if a pathogen with *high respiratory impact were to emerge, either as a result of accidental or deliberate release, it would probably have a significant impact upon public health, on the economy, on society and on politics"*. Moreover, not only. It also added a further hypothesis: "*the combined possibilities of short incubation periods and asymptomatic spread could have the effect of only having a very short window of opportunity available to act to stop transmission, making the outcome difficult to contain"*, and *"making it able to strike several countries simultaneously and to demand approaches that differ from the usual"*.

Whoever reads these forecasts cannot fail to be struck by the precision of the description, in advance of what then happened in reality.

On 20th January, China declared an increase in contagion of 60% in two days. Americans evacuated from China. On 30th January the WHO declared Coronavirus to be a *"Global Health Emergency"*. The next day, on the 31st January, the Italian Council of Ministers declared a state of emergency for six months, and set aside 5 million Euros for initial needs.

However, another fifteen days would pass before a WHO delegation went to China (on February 16th) to perform an in-depth investigation. Italy, strangely, did not take part. However, the mission report shows that in sporadic cases of abnormal pneumonia (suspected of being traceable to influenza of a Sars type) had already been reported since the previous October. Therefore, in the first fortnight of February there were already signs showing a high probability that a new virus had been in circulation for months. Similar results, of anomalous pneumonia, were later recorded in Northern Italy.

Having gone un-noted at the time, they could mean that the virus had reached Italy and was circulating long before the epidemic exploded.

## 2. Data Uncertainty: Diagnostic Unreliability—Lack of Preparation and Technical Resources

Four orders of uncertainty have compromised analyses, Government choices and media narratives. The latter turned out to be catastrophic in nature and largely unreliable.

a) Data arriving from China, the number of deaths and infections, did not permit any realistic evaluation of the situation, which was then to arise in Italy. Here the responsibility of the Italian Government is limited. Much greater is that of the WHO.

b) But where instead there was an accumulation of errors, all of them serious, was the absence of a sampled screening programme; the precise initial identification of the areas struck; and the assessment of the number of people infected. In that way the extent of the infection was underestimated and gross calculation errors were made about the lethal nature of Covid-19.

c) Technical limitations influenced all aspects of data collection. The greatest confusion has characterised the criteria of assessment of causes of death. Italy adopted the *"all inclusive"* approach. The almost total absence of autopsies has hampered the understanding of the pathogen mechanisms that caused death, and in particular, impeded identifying the most important cause of death. This is in part where the enormous difference in mortality rate between Italy and Germany at the end of March 2020 derives (11,40% versus 0.9%).

d) The use of testing has been unexplainably irregular and not uniform across the territory. That makes the validity of results uncertain. Culpably, no priority was given to serologic analysis (evaluation of M & G immune-globulin) which was the only test that could give us real information on the numbers infected and those who were still hosting an active pathology.

## 3. Ambiguous and Confused Communications

The model for communications adopted by the Government was the least thought out possible. A mix of official notices, unofficial and casual news, spread across communiqués and individual interviews, mostly distributed by entertainment channels, television panels and various chat shows, which in turn were populated by experts, mixed in with random views from laymen, characters from show-business and people in general, so as to be indistinguishable from infotainment. The overall result of that kind of communications, instead of producing an effect of responsible alertness, has led to widespread scaremongering and concern. In this way, the habit of conveying a regular *"war bulletin"* daily – together with an endless procession of experts – each of whom was mostly concerned with providing their own personal view, often in contradiction with those of others – gave rise to worry, anxiety and confusion well beyond what was due and desirable.

## 4. World Health Organization: Missing Fulfilment, Political Inappropriateness, and Enormous Conflicts of Interest

1) It did not perform monitoring of the Wuhan laboratory, despite the activities of the latter having been targeted for some time by the scientific community. It should be recalled that the WHO frequently attended and inspected *Lab-4* in that Chinese region.

2) It did not raise the alarm in a timely manner, which action should have been taken as soon as the new viral strain was isolated (9th January 2020).

3) It issued pandemic warnings about 40 days late: on the 15th January, cases of Covid-19 had already been declared in Thailand, Japan and the United States, in addition to the Wuhan epicentre. By the 20th January, it was already clear that the infection was transmitted human-to-human through respiratory pathways. And yet the WHO, in its communications and final report following the inspection visit made to China (24th February), persisted in declaring the situation under control and in praising China for the measures applied. At the same time the WHO – through a statement by Walter Ricciardi, member of the WHO *Executive Board* and currently advisor to the Italian Health Ministry – calmly stated that blocking flights from China *"was an error"*. The idea was taken up again by the WHO's Director General - Tedros Adhanom Ghebreyesus – according to whom *"these indications risk increasing fear and discrimination and have little results in public heath terms"*.

4) In the meantime, the WHO ignored the warnings issued by Japan, Korea and Taiwan. It is further incredible that the WHO, who was ready to praise China, did not instead stress the efficient protocols put in action in Korea and in Taiwan to halt the epidemic, as models of good practise. It is even more incredible that the WHO deliberately ignored the alarm raised by Taiwan at the start of December with regard to the development of a possible epidemic originating in China.

To sum up, it is beyond doubt that murky relations exist between China and the WHO. It is also beyond doubt that opaque relations exist between the WHO and a few private foundations, and all the major multinational companies that can be summed up in the term *Big Pharma*. The entire episode deserves further investigation and assessment within the United Nations, with a request that on this topic a specific investigation be made. That initiative can be triggered either by the UN Security Council or by Italy itself.

At the same time, the Italian Government must reexamine all its relations with the WHO and make its own proposals, to be discussed in international forums, to replace the WHO with an international body, which is entirely public, and funded exclusively by nation States.

## 5. Urgent Tasks for the Italian Government

The government has shown (and continues to show) errors and uncertainties that need to be expressly exposed.

First, as has been stated, we have seen a glaring lack of awareness of the incumbent danger of dangerous epidemics. One could use the same expression as in the *Lancet* review: "trained incapacity" to describe the behaviour of politics and of the crucial apparatus of State machinery. Political power has demonstrated that it has been unable to adapt even to the directions of the scientific community that had forecast the rise of a potentially lethal new pandemic of influenza virus over at least fifteen years.

Secondly, it is embarrassing to observe how, faced with the information available to it – both public and confidential -the Government delayed taking advantage of the Piano *Nazionale per la Prevenzione* (2014-2018) [National Prevention Plan] and above all triggering the Piano *nazionale di preparazione e risposta a una pandemia influenzale* [National Plan for Preparedness and Responding to an Influenza Pandemic], published in 2007 and later updated 2016.

We shall see later how that plan was disregarded, point by point:

- The purpose of the Plan– structured into six stages of activation – was strengthening preparedness for a pandemic at national and local level, so as to:

Identify, confirm and **rapidly describe cases of influenza caused by new viral subcategories**, in such a way as to recognise the start of a pandemic in good time (Stage 2).

*This did not take place notwithstanding that in the course of the months of December and January, trouble spots of atypical viral pneumonia were recorded in several areas in Lombardy, in the Veneto and in Emilia Romagna. In particular, from the 28th December no less than 40 anomalous cases of viral pneumonia were reported in the hospital in Piacenza. A later study by Milan University enabled the debut of the epidemic in Italy to be placed between October and November, thus well before the first verified case in Codogno. The warnings sent to the Minister of Health would then form the scope of a specific circular dated 5th January 2020, in which the risk of a possible epidemic was explained, and a request was made to pay attention to the connection between viral pneumonia and China. That circular was reiterated on the 12th January, stressing once again the connection between viruses and epidemics, whilst pointing out– in a completely paradoxical way – that the WHO, based on news received from China, was by then "reassured of the quality of the investigations in progress [in China] and by the response measures implemented in Wuhan". In the circular dated 16th January we witnessed an about-face, an utter outright manipulation of the truth, given that it went back to talking of a possible epidemic, but cancelled the link to China and even picked out Japan as a source, adding the words "Japan (ex-China)"!*

- During the course of *"Inter-pandemic Stages 1-2"*, as set out in the plan, the following was due to be performed: "Health information to the population to promote the adoption of common hygienic standards, which include: frequent hand-washing, cleansing domestic surfaces with the usual products, and covering the mouth and nose when sneezing or coughing. Adopting measures to limit the transmission of infection in communities (schools, rest homes and meeting places), where excessive crowding should be avoided, and providing premises with adequate ventilation. Preparing appropriate measures for controlling the spread of the influenza pandemic in hospitals. Providing Personal Protection Equipment for health workers. Checking sanitizing and dis-infecting systems are functioning. Identifying appropriate pathways for the infected or those suspected of so being. Surveying hospital bed availability and that of rooms with negative pressure. Surveying the availability of mechanical devices to assist patients. Minimizing the risk of transmission and limiting morbidity and deaths due to the pandemic. Reducing the impact of the pandemic on the health and social services and ensuring that essential services are maintained".

*None of this took place, so much so, that the Italian Government actually sent out/gave away huge quantities of health equipment (including facemasks) to China and other countries.*

- In the course of Stages 3-5 the Plan further recommends: "creating and implementing surveillance protocols for: travellers coming from infected areas; health workers who assist patients with suspected or confirmed influenza of a potentially pandemic strain; the laboratories that handle clinical samples at risk; defining and implementing surveillance protocols for clusters of influenza syndromes that are potentially attributable to a pandemic virus, either through general practise doctors and family paediatricians and through hospitalisation Institutions".

*As is obvious, all of this was widely disregarded, especially as regards the protection of health workers and the involvement of territorial medicine, which only a few months later turned out to be a winning card in limiting the progression of the disease, and in reducing the number of patients requiring hospitalisation in intensive care units*

To sum up, the Plan for Pandemic Management was ignored, disregarded, and implemented late, and then only in part.

## 6. Rethinking the Entire Health Structure of the Country

Over the last few years, successive governments have degraded the idea of medicine that we have inherited from the great Greco-Roman and Christian traditions. The hospital – once known as *Hotel de Dieu* – has become a Health Enterprise, where choices are made by measuring them against financial and management efficiency criteria. This has meant the abandonment of territorial medicine and the creation of multi-specialty centres of attraction, placed in large cities, deemed to be fully comprehensive terminals for health demands of entire macro-regions. The development of territorial medicine would have enabled better care and handling of patients in a home environment, responding to *"primary"* specialist demand" (neonatal care, maternity, accident and emergency), for which citizens should not of necessity be forced to turn to macro-hubs in major cities. This would also have made resources available – both economic and human – that could have been used to diversify health service offerings. In particular, the need to provide a larger number of beds in intensive care had been known for some time (especially after the Monti Government had irresponsibly cut 2/3 of the bed units then available), but nothing was done to fill this gap. This ended up causing thousands of deaths. It should also be considered that, since the 1990s, the term ***"prevention"*** - which was often referred to in the 1970s and 1980s to face up to complex issues such as environmental (and professional) pollution, degenerative (cancer) and metabolic conditions (obesity, diabetes) - has gradually disappeared from the vocabulary of health service managers. Surrendering the paradigm of prevention has resulted in underestimating the risks arising from new, foreseeable, pandemic waves.

This emergency could offer an opportunity to rethink the health model that has developed in the last decades. It would be appropriate to launch a debate and proposals on this topic, which is able to translate into a project for re-founding public health.

## 7. Investing in Scientific Research

This issue affects all vital sectors of the Country. For years Italian scientific research has received laughable funding, bot compared with GDP, and when compared with its levels in other European Union countries. Italy has, furthermore an absolutely prime international reputation, being three times more productive than Germany, even though it one quarter of the research funding. One would have expected, in conjunction with Covid-19 emergency, that an emergency scientific research plan would have been launched immediately, if only to start essential research to identify possible cures. None of all this has been as much as tabled. It turns out that the Health Ministry has put up a prize of 5 Million Euros, reserved exclusively for IRCSS and formally excluding any contributions from Universities. Only in July was launched an FISR program for universities, for around 20 Million Euros [1].

## The status of the diesease: what should be done?

At the current stage of the epidemic, it has been established without any possible denial that Covid-19 is a disease – certainly highly contagious – that can be treated in most cases, but which at times can be serious, as unfortunately happens with many other pathologies.

Guidelines for tackling it should therefore be made up of the following points:

a) **Prevention**: great stress should be laid upon enhancing innate immunity and checking silent chronic inflammation. Such goals can be reached first by means of a suitable diet and lifestyle. In individual cases, specific supplements can be resorted to. In particular, it is necessary to take account of the psychic-emotional state of persons, in the knowledge of its fallout on the immune system. It is clear that at the moment that there are no adequate scientific studies on the impact of such approaches on the illness. On the other hand, there are harmful effects on all viral diseases of a compromised immune system, and since silent chronic inflammation occurs in the second stage of the illness, this enhances the cytokine storm.

b) Avoiding viral transmission, by **controlling areas of major contagion** (in particular assessing people without symptoms in the areas at greatest risk, intervening in a timely way for isolation, testing, and supplying all health workers and those at major risk of infection with appropriate Personal Protective Equipment.)

c) The possibility of acting early on a territory with **therapies that have already shown to be effective** even in the absence of randomised studies that are expected shortly (hydroxychloroquine, heparin, and

corticoids). The possibility of intervening in the most difficult cases with hospitalisation and second-degree care (antiviral agents, ozone therapy, hyper-immune plasma, myo-inositol, and oxygen with various approaches to its administration) should also greatly decrease the need for hospitalisation in Intensive Care Units.

d) **Increasing intensive care numbers**, even if, with data in hand, much less should be needed if there was a widespread use of therapies that have shown to be effective to date.

e) Learning to **live** with it in the same way as we live with many other diseases, paying more attention to hygiene standards. It is certain that major gatherings form a danger, also through increased viral load. Thus, they should be avoided in periods of a major virus presence. For this reason, as for other diseases, **constant monitoring** is needed.

## What have we learned in the last two months?

From clinical results, it has emerged that Covid-19 is a mild disease in the majority of cases (roughly 85%). It is major but not dangerous in a further 10% of cases, in which various approaches to treatment are available. In the 5% that could have a deadly outcome, the following various therapies have been tried with success:

**1) Ozone Therapy with Antiviral Agents**
**2) Therapy using Anti-coagulants**
**3) Hydroxychloroquine and Heparin**
**4) Hyper-Immune Plasma**
**5) Myo-inositol**

Even if modern medicine is based on proven effectiveness (*EBM – Evidence-Based Medicine*) aimed at guaranteeing that before a newly created remedy is place on the market one is certain that the advantages outweigh the side effects, we know that this research method obviously requires very long periods.

At the same time, given that is well known that publications receive funding from Pharmaceutical Companies that pursue profit at all costs from the remedies they patent, the absence of publication is often a good approach to burying innovations that are not of interest.

Today we find ourselves before a dilemma of whether to continue researching a new molecule, which could potentially be toxic and which will require long periods in order to be approved (for example, a vaccine); or opting for a therapeutic approach which has been known for years and considered to be well tolerated (such as hydroxychloroquine for example). Another opportunity is offered by well-experimented approaches, known to be harmless and efficacious (for example, hyper-immune plasma or ozone therapy).

It is obvious that faced with grave situations, even a few cases are enough to declare a therapy as *"likely"* to be effective. That is precisely what is happening with the therapies set out above.

Furthermore, such therapies should be available in very short order, and not only in emergencies.

For this reason, it is supposed that insisting on waiting for a published scientific study is a system for burying this kind of approach, with the sole purpose of pursuing economic gain.

In addition, the desire to bet on a vaccine - *whatever it takes* – presents various criticalities as stressed by Ernesto Burgio, *"when we proceed in too much of a hurry, under the pressure of an epidemic emergency – as happened in the Philippines with Dengue – there is a risk that a new vaccine, or one that has not been tested enough can even trigger grave forms of the infection being combated: or may cause a kind of immune activation mediated by Th2 lymph cells (a kind of allergic reaction) or by a paradoxical reaction, triggered precisely by the antibodies prompted by the vaccine (ADE, Antibody Dependent Enhancement). In the foreseeable rush to a vaccine against SARS CoV2 similar incidents are possible and it is necessary to be prudent".*

Considering the therapeutic successes – see the links shown above – which are not being made known to the public – save fleetingly and, we would say, almost unwillingly – by means of the media or official statements, by the present document we intend to request that the Ministry **convene the doctors who are already experts in these kinds of therapies.** Accordingly, in the shortest possible time, the advantages and disadvantages of each of them can be set out for colleagues, even in the absence of double blind studies. In the case of a second wave, during the course of **Stage 2** it will be necessary to be ready to act, using:

**A) Public prevention and health worker training courses.**
**B) Effective protection systems (masks, gloves, etc.) in adequate quantities.**

**C) Diagnostic asseveration procedures to be implemented in case of well-founded clinical suspicion. This can avoid most infections.**
**D) Creation of specific centres - out and out military style *task forces* – that can act in a few hours in the case of emergency.**
**E) Protocols that are actionable by the *USCA* (Unità Speciali di Continuità Assistenziale) [Special Welfare Continuity Units] across the whole Country to support physicians on the territory with continuous monitoring of infected patients even if they are not hospitalised.**
**F) Availability of medicines that have been shown to be effective and tested by various front-line colleagues.**

**Whilst waiting for this, the signatories of this document commit to gathering the relevant data and making it available to everyone.**

Rome, 18th May 2020

[1] Update of 10th July

## Attachment 1

**Plan for Scientific Clarity** was drawn up by:
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**Associations that have joined the Plan:**
**Medicina di Segnale (circa 850 associati)**
www.medicinadisegnale.it
FIAMO (circa 200 associati)
http://www.fiamo.it/

**The Plan has also been joined by:**
Piero Priorini (Psychotherapist), Franco Lugnani (M.D.), Carmelo Samonà (M.D.), Paolo Baron (M.D.), Stefano Clauti (M.D.), Fabrizio Fiorini (M.D.), Danilo Toneguzzi (M.D.), Mauro Alivia (M.D.), Carlo Mocci (M.D.), Stefano Gasperi (M.D.), Guido Cantamessa (M.D.), Eva Rigonat (M.D.), Andrea Basili (M.D.), Anna Maria Cebrelli (Psychologist)

## Attachment 2

**Update as of 10th July 2020**

Little more than a month after drafting the **Piano di Chiarezza Scientifica** [*Plan for Scientific Clarity*], we have been forced to assess what has happened in that period of time, given the hectic unfolding of events and of research results in the field related to the Covid-19 epidemic. During that month in fact, some major events have taken place and the epidemiological situation has changed noticeably, as can be deduced from the following points:
1) The numbers of infections are continuing to drop in Italy and in a much more significant extent; severe cases of Covid-19 have diminished, **almost to the point of reaching zero**;
2) The usefulness of certain therapeutic approaches has emerged and a few **controlled trials** have confirmed their effectiveness. Amongst these are anti-coagulant therapy, cortisone-based therapy (Dexamethasone), hyper-immune plasma and ozone therapy;
3) **Endothelium involvement** by the virus has been confirmed, with major implications for all stages of therapy, from treatment at home to intensive care;
4) In Italy, we still notice the **absence of territorial medical planning** for diagnosis and treatment guidelines for handling Covid-19, and that of a precise **prevention protocol** to avoid infections especially in healthcare surroundings, in which it is still not yet possible to have **adequate quantities of PPE available**. We further observe inconsistencies in the handling of asymptomatic cases, who turn out to be positive for Co-

vid-19 after serological test.

5) Furthermore, we find ourselves confronted with a **proposal for wide anti-influenza vaccination, without any appropriate scientific basis** for its effectiveness against Covid-19.

To sum up the situation, today we find ourselves faced with the **lack of a common strategy** not only in Italy, but also in the rest of the world, both at the level of scientific research and that of social containment. On the other hand what is not missing – and continues still today when infections have reduced drastically (suggesting a probable 'weakening' of the viral load) – is the complete and utter **media terrorism** that tends to amplify every single case – even in the so-called "Phase 2" – without however, producing any evidence that might justify this kind of alarmism.

Totally distorted scientific information – the **Lancet / WHO case** was typical – demonstrates beyond any reasonable doubt the **enormous economic interests** that are concentrating on producing and distributing a vaccine to the entire population of the world. Incorrect information on the harmfulness of hydroxy-chloroquine, was in fact, published in the Lancet journal and confirmed by the WHO and by our ISS (National Health Institute) – but was later unmasked forcing both the journal and the WHO into a sudden U-turn. This was a decidedly striking case in the history of reference scientific journals and has demonstrated that **scientific publications cannot be trusted and the institutions in charge of public health even less so.**

Faced with the above, in our opinion there are **three main issues** that must be treated today with maximum urgency by the relevant authorities:

1) **The issue of freedom to choose treatment**

The principle of freedom to choose treatment, laid down in section 32 of the Constitution is not negotiable, and cannot be removed on the pretext of real or fictitious states of emergency. The Centre of Gravity is committed to defending this indefeasible right with all its powers.

2) **The senseless policy of social distancing in schools**

With school lessons starting in September an out-and-out assault on the humanity of our children is being planned. It is criminal to see the project for social distancing between students or the use of facemasks in classes. The harm from these measures goes beyond any possible imagination – even over the long term in the later course of life. Given that, to date, no studies exist that demonstrate with certainty that children were infected by the disease in Phase 1 at school and that they brought the infection home, the approach should be one of performing check-ups based on well-grounded suspicions and asking families not to send children to school if they are unwell. To this end, preventive medical equipment across the territory, in schools and workplaces is indispensable.

3) **A wider vision of the approach to treatment**

Lastly, a very little importance has been given by the media and the so-called "experts" to the enhancement of immune response by **prevention** thanks to **diet** and **lifestyle**, and to **supplements** about which scientific evidence exists, with vitamin D being first and foremost. On this topic, it should also be recalled that an inner attitude of **calm and courage** should also be promoted to defend against the media bombardment that has been raging during the last months. The **spiritual dimension** of humankind should not be underestimated, despite a certain world of financial interests that has today taken possession of science and politics.

In conclusion, Covid-19 is a disease that we must get to know better, a disease that is much less serious than many others but which must be handled in an appropriate way. It would therefore be suitable for physicians and for the population to receive regular technical updates on the situation. For this reason, it is considered useful to form a **group of independent experts** to work alongside those selected by the Government. That group must commit to conveying the results of its own **independent research** to the Government, including by the means of creating a website, which can be freely accessed by both doctors and citizens.

# Organisms

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