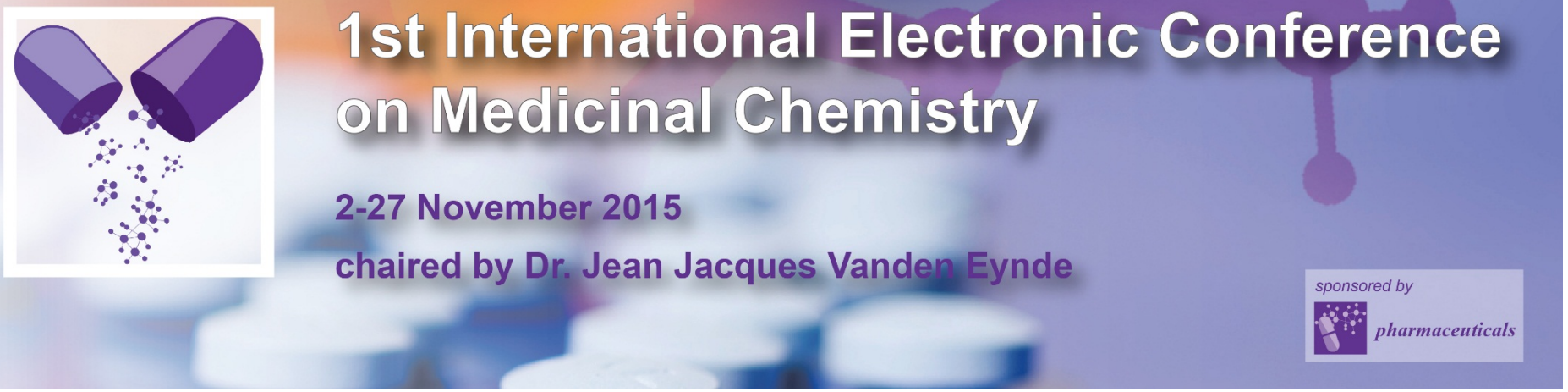

Ebola Virus Disease: Questions, Ideas, Hypotheses and Models

Francisco Torrens [1,*] and Gloria Castellano [2]

[1] Institut Universitari de Ciència Molecular, Universitat de València, Edifici d'Instituts de Paterna, P. O. Box 22085, 46071 València, Spain;

[2] Departamento de Ciencias Experimentales y Matemáticas, Facultad de Veterinaria y Ciencias Experimentales, Universidad Católica de Valencia *San Vicente Mártir*, Guillem de Castro-94, 46001 València, Spain.

***** Corresponding author: torrens@uv.es

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Employed Entry Mechanisms
- Lipid-raft-dependent mechanisms
- Receptor-mediated endocytosis
- Macropinocytosis
- Receptor binding and atttachment mediated by GP1

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Binding Receptors for Entry into Target Cells
- Folate receptor
- Glycan-binding proteins of the C-type lectin family
- B1-integrins
- T-cell immunoglobulin and mucin domain 1 (TIM-1)
- Tyro3/Axi/Mer (TAM) receptor family

↓

Initial Infected Cells
- Monocytes
- Macrophages
- Dendritic cells

Secondary Infected Cells
- Fibroblasts
- Endothelial cells

↓

APOPTOSIS, AUTOPHAGY, NECROSIS

Abstract: How is Ebola virus disease (EVD) contagious? What does it happen at the host/pathogen interface? Why are certain viruses capable of jumping to new species? The genetic plasticity is key if the virus is to overcome a host immune attack. Double-stranded ribonucleic acid (dsRNA) triggers release of cytokines. What is the possibility of an outbreak in Spain? What is the alert level that can have a country as Spain? Does the Law of Labour Risks observe the protocol? The model of Ebola virus transmission dynamics is reviewed, with the aim to provide a broad sketch of the fundamental human-Ebola-virus biophysical forces that enable and constrain EVD. Kumar group reported a model of Ebola evading the immune system. What are the factors of the emergence, rapid spread and uncontrolled nature of 2014 virus outbreak? How to treat EVD? How does the Ebola virus replicate? Why have bats evolved to resist fatality in the face of the Ebola virus? How have bats evolved to resist fatality in the face of the Ebola virus? Did Ebola Zaire either exist in the African landscape before 1976, or evade detection and documentation? How does Ebola evade the immune system?

Keywords: Seasonal distribution; Deforestation; Socio-political infrastructure; Micronutrient deficiency; Vaccine

The aim of this presentation is to initiate a debate by suggesting a number of questions (Q) that can arise when Ebola virus disease (EVD) is evocated and providing, when possible, answers (A) and/or hypotheses (H).

GENERALITIES

A 60% of all human pathogens are of zoonotic origin, *e.g.*, those that have recently jumped to humans (Ebola, identified in 1976). A pathogen spread relies on perpetual contact with susceptible-individuals groups. Conditions for zoonotic-viruses emergence and spread are socio-economic and environmental changes, long-distance mobility and changing climate.

Q1. How is EVD contagious?

A1. It passes on by contact with secretions of a patient in active phase.

Q2. What does it happen at the host/pathogen interface?

Q3. Why are certain viruses capable of jumping to new species?

H1. Genetic plasticity is key if virus is to overcome a host immune attack.

Q4. Is EVD a growing threat?

Q5. What will happen if phase-3 trials have insufficient power to determine efficacy as or if incidence continues to wane as hoped?

Double-stranded (ds)ribonucleic acid (RNA) (dsRNA), an intermediate in viral replication, triggers release of cytokines, primarily interferons, which in turn causes upregulation of antiviral genes and antibodies. Viruses developed ways to inhibit interferon response: interferon antagonists [in Ebola, viral membrane-associated (VP24) and polymerase complex (VP35) proteins].

Mas-Coma and González-Candelas (personal communication) raised the following questions on EVD arrival at Spain on 2014.

Q1. What are causes of the infection?

Q2. What is the risk for the health staff?

Q3. What is the possibility of an outbreak in Spain?

Q4. Is there a possibility that the virus mutate and become its transmission by the air?

Q5. Is it necessary to put to sleep the dogs of infected people?

Martín-Moreno (Rodríguez, E. Entrevista: José M.ª Marín Moreno. In: *Anuario SINC 2014*; FECYT: Madrid, Spain, 2015; pp. 122-123) raised the following questions on 2014 EVD outbreak.

Q1. What steps must one take before potentially infected people?
Q2. What is the alert level that can have a country as Spain?
Q3. Does the Law of Labour Risks observe the protocol?
Q4. Why has this infection happened?
Q5. What has it happened?
Q6. Was there a human error?
Q7. What health action must be activated after an infection?
Q8. The suits that were used, are the adequate ones for this type of virus?
Q9. Had a suit of higher-level protection better covered the exposure to the virus?
Q10. What information must be provided to worried neighbours?

The model of Ebola virus transmission dynamics

In earlier publications it was reported the phylogeny of anthropoid apes, fractal and hybrid-orbital analyses of protein tertiary structure, modelling of complex multicellular systems and tumour-immune cells competition, structural classification of complex molecules by information entropy and equipartition conjecture, molecular classification, diversity, complexity and emergence, periodic classification of human immunodeficiency virus (HIV) inhibitors, molecular classifications of thiocarbamates with cytoprotection activity *vs*. HIV, styrylquinolines as HIV integrase inhibitors and *N*-aryloxazolidinone-5-carboxamides as HIV protease inhibitors, a hypothesis explaining how acquired immunodeficiency syndrome (AIDS) destroys immune defences, a tool for interrogation of molecular structure, mucoadhesive polymer hyaluronan as a drug delivery vehicle, reflections on the nature of the periodic table of the elements and cultural history of nano/miniaturization and (quantum) simulators (computers). In the present report, the model of Ebola virus transmission dynamics is reviewed, with the aim to provide a broad sketch of the fundamental human-Ebola-virus biophysical forces that enable and constrain EVD.

Evaluation of ViroCyt® Virus Counter for Filovirus Quantitation

Development and evaluation of medical countermeasures for diagnostics, vaccines and therapeutics requires production of standardized, reproducible and well-characterized virus preparations; *e.g*., for filoviruses, plaque assay for quantitation of infectious virus, transmission electron microscopy (TEM) for morphology and quantitation of virus particles, and real-time reverse transcription polymerase chain reaction for quantitation of viral RNA (qRT-PCR). The ViroCyt® Virus Counter (VC) 2100 (ViroCyt, Boulder, CO) is a flow-based instrument capable of quantifying virus particles in solution. *Via* a proprietary combination of fluorescent dyes that stain nucleic acid and protein in a single 30 min step, Rossi *et al*. (C. A. Rossi, B. J. Kearney, S. P. Olschner, P. L. Williams, C. G. Robinson, M. L. Heinrich, A. M. Zovanyi, M. F. Ingram, D. A. Norwood and R. J. Schoepp, Evaluation of ViroCyt® virus counter for rapid filovirus quantitation, *Viruses*, **7** (2015) 857-872) showed a rapid, reproducible and cost-effective quantification of filovirus particles. *Via* a seed stock of Ebola virus variant Kikwit, they determined the linear range of the instrument: 2.8×10^{6}-1.0×10^{9} virus particles per millilitre (mL) with coefficient of variation 9.4-31.5% for samples tested in triplicate. Particle counts of VC for various filovirus stocks resulted within one log of TEM particle counts. They established a linear relationship between the plaque assay, qRT-PCR and VC. Results of VC correlated with plaque assay and qRT-PCR. The VC is an easy, fast and consistent method to quantify filoviruses in stock preparations.

Elena (personal communication) raised the following questions on 2014 EVD outbreak.

Q1. Are there equipment and experience needed to treat EVD patients?
Q2. What is a virus?
Q3. What factors do favour the appearance of new viral diseases?
Q4. Why is it so difficult to control determined infections or find vaccines?
Q5. How does one interfere a virus without doing anything to the cell?
Q6. Why have people no vaccine?
Q7. Why is nowadays when the most virulent outbreak is occurring?
Q8. Could the epidemic be spread to Europe?
Q9. Could an epidemic begin?
Q10. Must one be critical with protocols followed by Spanish Government?
Q11. To put to sleep the dogs of infected people?
Q12. Does it represent a danger or an opportunity?
Q13. Is there the equipment needed to have a dog infected by Ebola?
Q14. Are the media sensationalist?
Q15. Laying on of hands, seawater, *etc*. How must one act *vs*. these calls?
Q16. In who have they tested them?
Q17. With what probability do they function?
Q18. Are people close to eradication of viral diseases or is it a long way?

Zoonoses

Ecology of zoonoses: Natural and unnatural histories

Karesh *et al*. (Karesh, W.B.; Dobson, A.; Lloyd-Smith, J.O.; Lubroth, J.; Dixon, M.A.; Bennett, M.; Aldrich, S.; Harrington, T.; Formenty, P.; Loh, E.H.; Machalaba, C.C.; Thomas, M.J.; Heymann, D.L. Zoonoses 1 Ecology of zoonoses: Natural and unnatural histories. *Lancet* **2012**, *380*, 1936-1945) proposed Q/Hs on zoonoses ecology (*e.g*., EVD, original HIV).

Q1. How do zoonotic diseases result from natural pathogen ecology?

Q2. How do other circumstances change disease-exposure dynamics?

Q3. Where does one stand in marginalized infectious diseases of poverty?

Q4. How do these pathogens survive and change?

Q5. Why do pathogens what they do?

H1. The ecological principle of competitive exclusion is the basis for approaches to control of zoonotic pathogens in livestock and poultry.

Q6. What techniques are animals slaughtered and processed with?

Q7. How are products stored, packed, transported and prepared?

H2. Genes conferring antimicrobial resistance originated as an evolutionary response to antimicrobial drugs produced by free-living bacteria, fungi and plants to protect themselves from infection or competition.

H3. (Bennett). Resistance patterns, and the genes encoding resistance, are much the same in wildlife and livestock.

H4. Whatever the original sources of resistant bacteria and genes, differences in the ecology of wildlife species produce selection pressure on the microbes, rather than differential exposure to anthropogenic antimicrobials or presence of different resistant strains in the environment.

Q8. Has agriculture antibiotics use exacerbated drug resistance in people?

Q9. What is the extent of transfer of antimicrobial-resistant organisms from animals to people?

Q10. What is the potential for reversal of resistance?

Q11. Would it occur in clinical settings after a change in antimicrobial use?

H5. Reversion to drug susceptibility depends on occurrence of natural dilution of microbial populations with susceptible strains and fitness costs of resistance.

H6. None of the approaches commonly used to search for potential new human pathogens would have identified SIV as a potential risk to man.

Q12. How is the environment changing?

Q13. How do these changes affect microbial dynamics across the system?

H7. Enhancing the role ecologists play in control programmes includes production of more accurate mathematical model outputs by collaboration with clinicians with real-time data, participation in prospective and retrospective study design, and field studies to identify key risk factors to target surveillance and interventions.
H8. Disease ecology approaches are particularly useful in driving advances in prediction of the emergence and spread of novel zoonoses.
Q14. How can dynamics of a wildlife-host pathogen change seasonally?
Q15. How does it function the microbiome from people?
Q16. How does it function the microbiome from animals they contact?
Q17. What does it cause zoonotic microbes to proliferate?
H9. The One Health approach provides a wider, holistic view with which to achieve this aim.
Q18. Where do zoonoses occur?
Q19. How do zoonoses occur?

Drivers, dynamics and control of emerging vector-borne zoonotic diseases

Kilpatrick and Randolph (Kilpatrick, A.M.; Randolph, S.E. Zoonoses 2 Drivers, dynamics, and control of emerging vector-borne zoonotic diseases. *Lancet* **2012**, *380*, 1946-1955) revised and proposed Hs/Q on drivers, dynamics and control of emerging vector-borne zoonotic diseases.

H1. Climate change will lead to more widespread and abundant vector-borne pathogens (VBPs) as more of the planet starts to resemble closely the tropics where VBPs are presently most abundant.

H2. The arrival of exotic and upsurges of endemic VBPs are because of climate changes.

H3. Effects of climate change on VBPs will be variable, as is expected from all such complex systems.

Q1. What will global warming do?

H4. Feeding on additional alternative hosts results in risen vector densities, which result in higher transmission even if a smaller proportion feed on people.

H5. Dilution effect. Naturally occurring biodiversity diverts vectors from infectious hosts.

Viral Virtuosos of Persistent Infection vs. *Acute Infection*

Sullivan (Sullivan, C.S. Viral virtuosos. *Scientist* **2015**, *29*(2), 33-39) raised questions on viral virtuosos of persistent *vs*. acute infection.

Q1. How do viruses orchestrate lifelong infections?

Q2. How do the viruses of the human body use these micro (mi)RNAs, and their own, to establish and maintain long-term persistent infections?

Q3. How could miRNAs and other noncoding regulatory (nc)RNAs function spawn therapies, while yielding insights about the evolutionary forces that dictate parasitism, mutualism and other multiorganismal relationships?

Q4. Does RNAinterference (i) serve as a meaningful antiviral response in mammals?

Q5. How does the human virome contribute to health and disease?

Viruses and Cell Death Programmes

Lamkanfi group (Lamkanfi, M.; Dixit, V.M. Manipulation of host cell death pathways during microbial infections. *Cell Host Microbe* **2010**, *8*, 44-54) raised a Q on the regulation of apoptosis during infection.

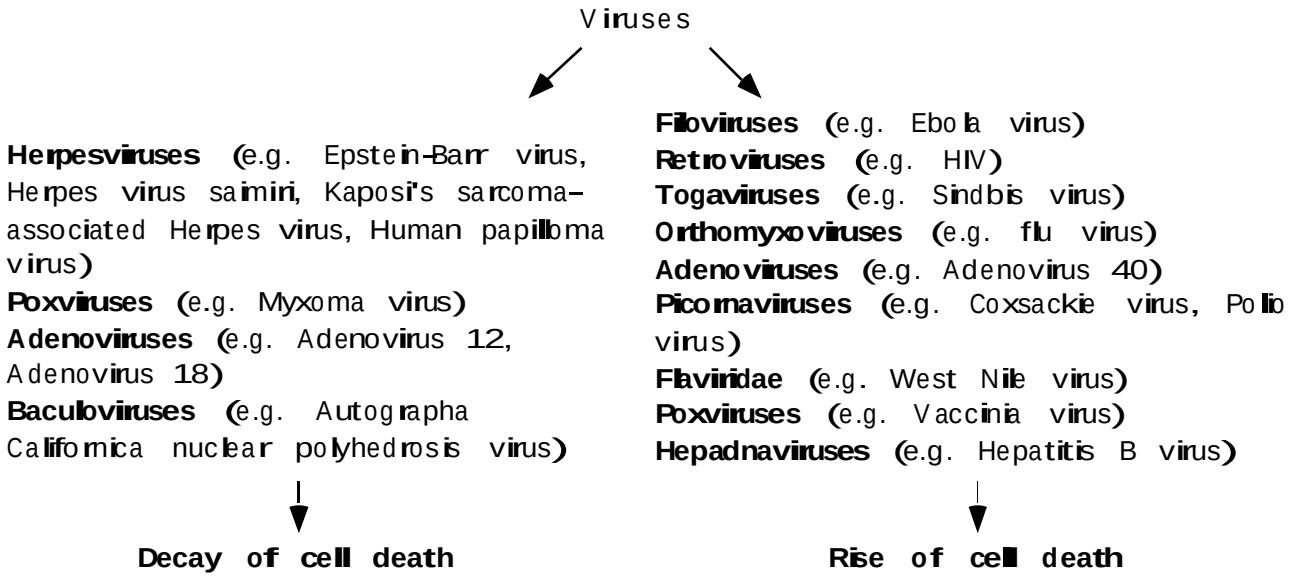
Q1. How do viruses evade host cell apoptosis?

Kaminskyy and Zhivotovsky (Kaminskyy, V.; Zhivotovsky, B. To kill or be killed: How viruses interact with the cell death machinery. *J. Intern. Med.* **2010**, *267*, 473-482) proposed a Q/H on cell-death consequences.

Q2. How do viruses interact with the cell death machinery?

H1. Necrotic cell death is regulated by a specific set of signal transduction pathways and degradative mechanisms.

Q3. How does the Ebola virus induce massive apoptosis of lymphocytes?
H2. Inflammatory mediators or NO secreted by infected macrophages are capable of inducing bystander cell death.
H3. Viral proteins induce lymphocytic cell death.
Figure shows cell death-related consequences of viral infection.



Intracellular Events and Cell Fate in Filovirus Infection

Mühlberger group (Olejnik, J.; Ryabchikova, E.; Corley, R.B.; Mühlberger, E. Intracellular events and cell fate in filovirus infection. *Viruses* **2011**, *3*, 1501-1531) proposed Qs/Hs on intracellular events and cell fate.

Q1. What is known about the intracellular events leading to virus amplification and cell damage in filovirus infection?

Q2. How may cellular dysfunction and cell death correlate with disease?

H1. Adaptive immune responses may occur.

H2. Lymphopenia, resulting from apoptosis as the infection progresses, contributes to the failure to clear the infection.

H3. A well-regulated cytokine response early in the course of the infection is critical to the outcome of the disease.

Q3. How do filoviruses enter their target cells, replicate their genomes and assemble progeny viruses exploiting cellular machineries?

Q4. How do filoviruses interact with cellular signalling pathways?

Q5. What is the current understanding of the fate of infected and non-infected cells in filovirus detection?

Q6. What are the morphological changes in ultrastuctural data of infected and non-infected cells, in filovirus infection?

Q7. Why does little or no inflammatory cellular response occur at the sites of viral replication?
Q8. What does it happen in the infected cell?
H4. The folate receptor is a significant filovirus receptor.
H5. Thymus-dependent lymphocyte (T-cell) immunoglobulin and mucin domain-1 (TIM-1) is a receptor for Ebola and Marburg viruses type-I transmembrane glycoprotein (GP).
Q9. What is the fate of infected and non-infected cells in filovirus infection?
Q10. How do viruses interact with the cell death machinery?
H6. In extensive filovirus infection, the cells are unable to maintain a normal water-ion balance.
Q11. Do filoviruses manipulate signalling pathways involved in apoptosis or cell survival?
H7. The inhibition of retinoic acid-inducible gene-1 (RIG-I) signalling by VP35 prevents the induction of apoptosis in infected cells.

H8. Cells using Toll-like receptors (TLR)-mediated antiviral pathways, *e.g.*, plasmacytoid dendritic cells (DCs), are less prone to the inhibitory effects of VP35 Ebola virus than cells relying on RIG-like signalling pathways.
H9. The GP-induced cytotoxic effects are caused by GP-guanosine-5'-triphosphate (GTP)ase dynamin interaction, leading to interference with the intracellular trafficking of cell surface proteins.
Q12. What is dynamin involvement in GP-induced cytopathic effect (CPE)?
H10. Extracellular signal-regulated kinase-2 (ERK2) signalling cascades are involved in the induction of GP-mediated CPEs.
H11. A mechanism of GP-induced cytotoxicity is induction of endoplasmic-reticulum (ER) stress associated with the unfolded protein response.
Q13. Are proposed mechanisms to explain GP-mediated CPE connected?
Q14. How are the different proposed mechanisms to explain GP-mediated CPE connected?
Q15. What is the ability of GP to induce cell death?
Q16. What does it happen in the infected cells?

H12. Host-derived proteins contribute to cluster of differentiation of T_4 receptor (CD_4) T-cell subtype death.
H13. An accumulation of incomplete viral transcripts during abortive infection of resting CD_4 T-cells was shown to activate intrinsic pathways, which lead to apoptosis during HIV infection.
H14. Some generalized mechanisms contribute to lymphocyte apoptosis in filovirus disease.
H15. Both intrinsic and extrinsic apoptotic pathways contribute to lymphocyte depletion.
H16. The loss of lymphocytes contributes to the failure to generate fully protective adaptive immune responses in human and nonhuman primates.
H17. Dysregulated DCs and macrophages contribute in other ways to lymphocyte apoptosis.
H18. Programmed death-1 (PD-1) signalling results in decayed T-cell proliferation because of induction of apoptosis *via* PD ligand-1 (PD-L1) binding.

Evading the Host's Immune Response

Kumar group (Akhtar, A.; Befkadu, E.; Basu, P.; Kumar, P. Exposing the origins of the Ebola outbreak: Urging for a shift in response from reactive to proactive. *Am. J. Infect. Dis. Microbiol*. **2014**, *2*(6A) 1-18) reviewed the emergence of EVD outbreak proposed Qs and Hs.

Q1. What are the underlying factors contributing to the emergence, rapid spread and uncontrolled nature of 2014 virus outbreak?

Q2. How to treat EVD?

Q3. How does the Ebola virus replicate?

H1. Different genes playing a role in the early immune response to the virus impart bats resistance to viruses.

H2. Bat genes co-evolve with virus genes.

Q4. Could redirection of the human and nonhuman primates immune system lead to a reduction in Ebola's death rate?

Q5. Why have bats evolve to resist fatality in the face of the Ebola virus?

Q6. How have bats evolve to resist fatality in the face of the Ebola virus?

H3. The virus strain in 2014 epidemic shows signs of genetic mutations.

Q7. Did Ebola Zaire either exist in the African landscape before 1976, or evade detection and documentation?

H4. All emergences between 1976 and 2005 are descendants of Yambuku-like virus, rejecting the idea that the strains evolve independently.

H5. The descendents spread *via* outbreak regions.

H6. Ebola Zaire employs a mechanism of spread.

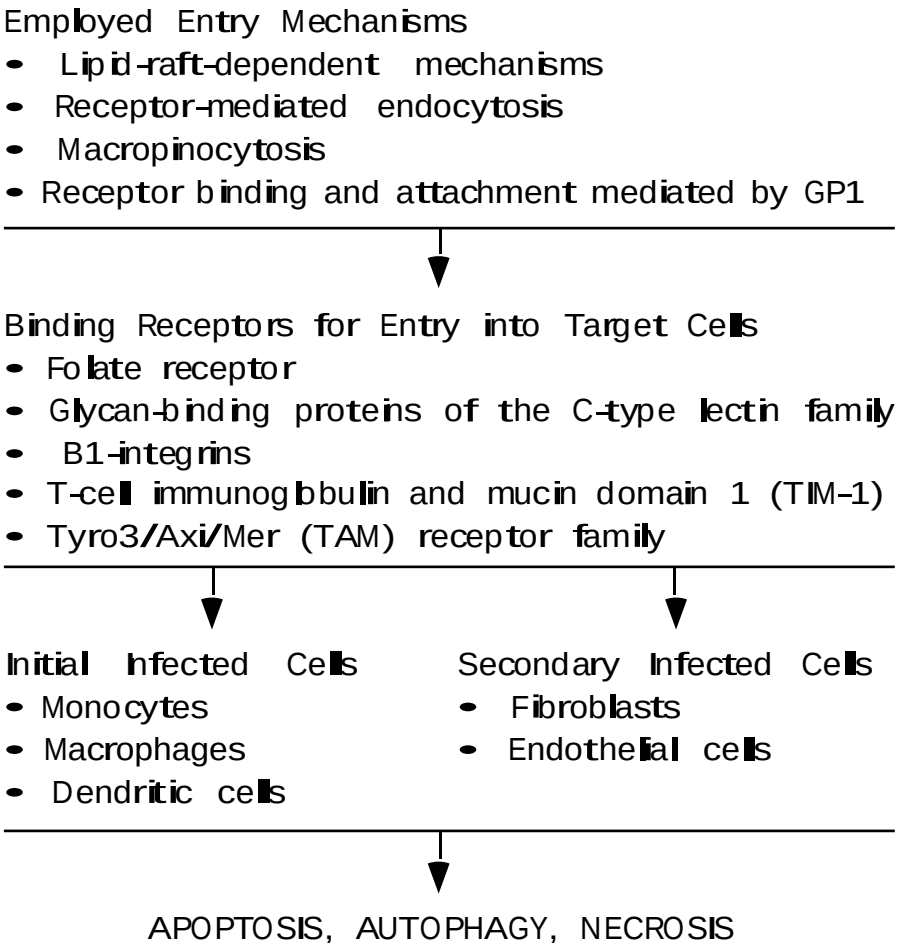
H7. West African Ebola virus variant of Ebola Zaire presents a substitution rate of 8×10^{-4} per site, per year.

H8. Outbreaks are indeed representative of independent zoonotic transmissions, yet are desired from a genetically diverse natural reservoir.

H9. The VP24 is critical in contributing to the virulence and plays a role in host adaptation.

Q8. How does Ebola evade the immune system?

Cells of the mononuclear phagocyte system are the first to be manipulated by the virus and responsible for providing the host with innate/adaptive immunity.



H10. Higher virulence levels exist in the initial cases of the infection.
Q9. What is the cause for initial virulence and subsequent chance of survival?
H11. 22 million Central and West Africans are at risk of an Ebola infection.
H12. Previous H. Strain virulence is solely a product of viral genetics.
H13. The socio-political and environmental climates are key variables in creating the flux in mortality rates and spread of infection.
H14. The second outbreak was initiated by an ill imam traveling from Guinea to Mali in an attempt to receive better care at the Pasteur Clinic in Bamako.
H15. 2013-14 epidemic varies in trends and final outbreak size between Sierra Leone, Guinea and Liberia, sharing common cultural and geographical traits.
H16. Bats are Ebola reservoir having behavioural associations with seasons.
H17. A trigger in 2014 outbreak is shift in the seasonal migratory route of bats.
H18. Ebola Zaire virus creates a Se demand on the infected host.
H19. Ebola virus survival *via* replication is increased by low [Se].
H20. T-cell disabling in Ebola Zaire is similar to that in Se-deficient hosts in flu.

H21. Fibrinogen, procalcitonin and cross matching of blood assays are unsafe.
H22. Colloid solutions, human albumins and synthetic starches are associated with adverse renal outcomes and provide no benefit to EVD infected patient.
Q10. A dead-end host: Is there a way out?
Q11. Why was not Zmapp made more widely available in Africa?
H23. Ethical H. Administering an experimental drug without safety is unethical.
H24. An advantage to the blood-transfusion recipient exists if the donor is from the same geographical region as EVD infected.
H25. Health care workers treating outside EVD treatment units are at risk.
Q12. What role should pharmacy play in epidemics like this?
Q13. Ethical Q1. Why transport aid workers to native countries for treatment?
Q14. EQ2. What are the benefit *vs*. risk profiles of experimental therapies?
Q15. EQ3. Is rushing an experimental agent to control an epidemic adequate?
Q16. EQ4. Is drug impact on epidemic containment positive or negative?

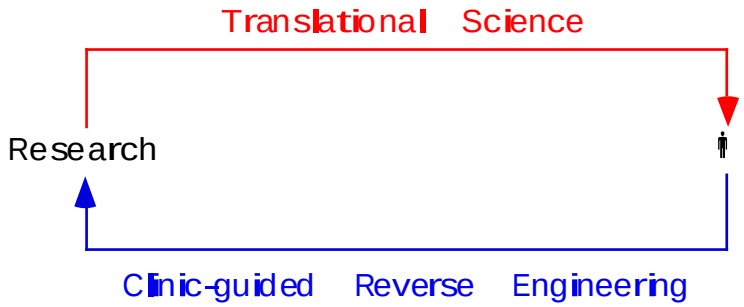
Conclusions

1. Understanding noncoding RNAs solves viruses orchestrating lifelong infections. MicroRNAs are tools that viruses use to manipulate our biology and immune response to hitchhike with us throughout our evolution. Virus-host co-existence involves control of viral life cycle: Ebola virus infection is flashy but persistent infection is elegant. Viral microRNAs optimize the location and timing of virus replication to fly under the radar of the host immune response.
2. How little one knows about why and how certain viruses spill over from their natural hosts, and how they interact with the human immune system.
3. Transmission among humans occurs *via* blood/body secretions exchange. Possible triggers associated with outbreaks are low temperatures, high humidity, seasonal change and its relationship with animal behaviours, socio-economic decline leading to deforestation, *etc*. A factor is outbreak delayed identification. While vaccines and antivirals are in development, preventative public health interventions (*e.g*., risk communication, protective-principles implementation) are a means to mitigate the spread of Ebola virus disease.
4. Get the means to control the outbreak in its origin to avoid it for expanding.
5. Trials need to reassume rapidly when and where next Ebola outbreak occur.

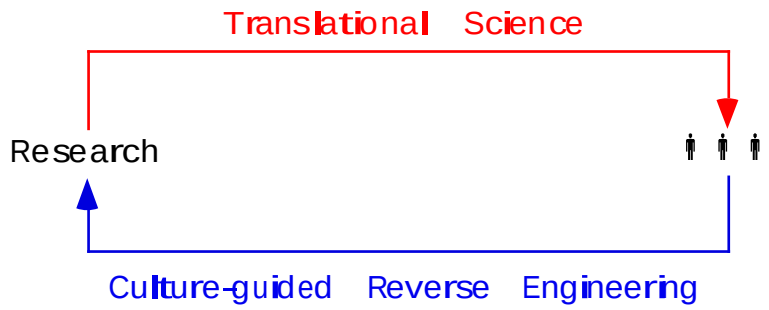
In the future, a holistic examination of the full spectrum of viral diversity, not just of the viruses that make us ill, may be factored into medical decisions; *e.g*., a rise in the levels of a benign persistent virus was suggested as an indicator for monitoring therapeutic immune suppression. Additionally, beneficial altering host viral communities (*provirotics*), could contribute to improved human health. Advancing the notion will require effort to catalog and exploit the human virome, and decipher the contributions of the humble but elegant persistent viruses (and ncRNAs regulating them). We are not prepared for viral outbreaks: (1) the state of knowledge about host-pathogen interactions is selective; (2) a deficit of trained medical and scientific personnel delays deployment to the established Ebola treatment centres in West Africa; (3) the public is unaware of the threat of emerging viral diseases. Funding for basic research in virology is insufficient. Ebola outbreak remembers people that an understanding of zoonotic viral infections is necessary, especially in the face of the changing environment. Interferon research is an emerging avenue that helps achieving the understanding and improving the quality of viral infection management.

Reorientation of Research in Translational (Marketable) Science

A reorientation of research in translational (marketable) science is necessary. In chemistry and physics the reorientation can be carried out by bench-guided reverse engineering. Ideas in biology should be valued by the number of questions that they generate. On one hand, in biology (science) the reorientation can be performed by clinic-guided reverse engineering. The same applies to medicine (the technology of biology). A scheme of the reorientation of research in translational science is shown.



Reorientation of Research in Translational (Marketable) Science in a Society
On the other hand, in a society the reorientation of research in translational science can be done by culture-guided reverse engineering. A scheme of this reorientation is shown.



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