PROJECT FINAL REPORT

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Project title: Preparedness, Prediction and Prevention of Emerging Zoonotic Viruses with Pandemic Potential using Multidisciplinary Approaches

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TABLE OF CONTENTS

1. Final publishable summary report

1.1 –Executive summary

PREDEMICS extended from November 1st, 2011 to April 30th, 2017.
To understand environmental, ecological and anthropological factors involved in crossing the species barrier, virus detection and/or serological data were collected by screening of environmental, wild and domestic host as well as human samples for HEV and shed light on HEV circulation over time, geographic spread and transmission. Factors and molecular determinants that influence IAV survival outside the host were identified. Analysis of the circulation of LYS in bats showed the importance of the number of species and population dynamics among bat colonies. The genetic structure of the red fox populations highlighted the role of ecological and physical corridors in virus spread and disease dynamics. Molecular phylodynamics studies on HEV, LYS and JEVr informed about the conditions of dispersal and contribution of multiple introductions to epidemiologic waves and establishment of endemic circulation and confirmed food-borne transmission of HEV. Detailed evolutionary and spatial analyses of the avian IAV H7 epidemics in Northern Italy, suggested which locations were the source and played a role in the spread of the highly pathogenic virus and revealed unexpected inter-farm transmission dynamics and role of partial cross protection. Next Generation Sequencing technologies, for which an analysis pipeline VIVAN (**Vi**ral **V**ariance **An**alysis) was developed, pointed to differences in genetic heterogeneity of H1N1pdm09 in patients in link with severity and revealed specific IAV subtype landmarks in the quasispecies distribution. Potential viral factors that govern virus polymerase fidelity were also identified. Computational methods for viral evolutionary inference were further extended for phylogeographic applications. These powerful tools for the analysis of viral evolutionary dynamics in relation to population dynamics of hosts and vectors were largely applied to study rabies cross-species transmission, examine the drivers of global IAV spread in human, swine or dispersal of H5N1 through migratory birds or, to study the origin of the 1918 IAV. They were also used for the HIN1pdm09 and avian H7N9 IAV or for the recently emerged MERS-CoV and EBOV, to trace cross-species transmissions and emergence into the human population and simulate real-time estimates of the evolutionary rate and intrinsic growth rate, or to understand CHIKV and ZIKV spread and evolution.
Replication efficiency, pathogenesis and transmissibility in natural hosts were studied in the different virus models. Results point to determinants involved in IAV transmission and adaptation from avian to mammalian hosts. Their impact was analysed in light of newly described interactors of the IAV polymerase. Determinants of pathogenicity of JEV were identified and vector competence was found to vary according to JEV genotype and mosquito species. Vector competence was also studied to evaluate the potential for transmission of ZIKV in Europe. Importantly, direct transmission of JEV by the oral route was demonstrated in pigs. Studies of the mechanisms of evasion of the host innate immunity by the different viruses (LYS, WNV, EBOV) focused on the modulation of different pathways. Mechanisms of broad adaptive responses were investigated, in particular the impact of host immune response on evolution and the contribution of opsonizing antibodies to (cross)-protection. New intervention strategies against JEV, based on innovative recombinant vaccines or attenuated strains that result from random codon re-encoding and infectious-subgenomic-amplicons approaches, were developed.
For the purpose of data integration and data-sharing, requirements for the various virus families, and the database scheme based on the the EpiFlu 2.0 platform were defined. Mapping methods for animal distributions and behaviours and models were developed that were used in various studies relevant to the dynamics of LYS, flavivirus, IAV and the newly emerging MERS-CoV, EBOV and CHIKV, ZIKV or YFV to explore the emergence and potential risk and conditions favoured for endemic maintenance of viruses and describe and assess strategies to control and mitigate the burden of those diseases. Activities within PREDEMICS were also dedicated to training and dissemination with exchanges of scientists, e-learning activities, and the organization of workshops.

1.2 - Summary description of project context and objectives

The PREDEMICS project was funded under the **FP7-HEALTH.2011.2.3.3-1 program** *"Identification of factors promoting the emergence of pathogens with human pandemic potential from pathogens with a zoonotic background and related prevention strategies"* under grant agreement N°278433. The project started on 1st November 2011 with an initial duration of 60 months, that was extended to 66 months. It benefited of a an EC funding of 11.75 M €.

The overall objectives of PREDEMICS were to:

- Identify key factors (considering environment, ecology, anthropology, virus evolution and virus-host interplay) associated with the highest risk of virus emergence at the 4 stages of emergence, i.e. exposure and introduction into a new host species, infection causing local chains of transmission, spread in human populations and post-transfer adaptation leading to widespread transmission and pandemics
- Determine the impact of the transmission route on viral evolutionary trajectories and cross-species transmission
- Unravel the mechanisms that govern interactions between a virus, its hosts and the environment to favour/limit cross-species transmission and adaptation to a new host
- Evaluate most effective intervention strategies to limit cross-species transmission and spread in the new host
- Identify risk patterns of emergence of practical relevance for disease surveillance control and intervention, and pandemic preparedness

Rather than addressing a wide variety of pathogens (viruses, bacteria, parasites), within PREDEMICS we chose to focus on selected RNA viruses which represent the majority of recently recognized emerging pathogens. Viruses to be studied were selected according to their relevance for the fundamental and practical questions posed by the four stages of emergence leading to successful cross-species transmission, namely:
1) **Exposure and introduction**: the initial single infection of a new host species (i.e. humans)
2) **Infection**: limited human-to-human disease transmission, including spillovers that progress to cause small local chains of transmission before epidemic fade out
3) **Spread**: the epidemic or sustained endemic human-to-human disease transmission with geographical extension.
4) Post-transfer **adaptation** leading to widespread transmission and pandemic.

Viruses that were the primary focus of PREDEMICS are:

- influenza A viruses (IAV)
- hepatitis E virus (HEV)
- arboviruses of the Japanese encephalitis virus (JEV) serocomplex and related viruse (JEVr)
- lyssaviruses (LYS)

These viruses are all pathogens with major impact in Public Health and relevance for Europe. They also represent the main routes of transmission: respiratory (IAV), faecal-oral (HEV and avian IAV) and vector-borne (JEV and related viruses: JEVr, transmitted by insect vectors) and contact (LYS transmitted by various species of carnivores). This is important, as factors promoting the emergence with human pandemic potential may be dependent on transmission routes. Likewise, the selected viruses involve the key sources and disseminators of emerging zoonotic agents in recent years: birds (IAV), pigs (IAV, HEV, JEV), bats (LYS), carnivores (LYS) and arthropods (JEV). In the course of the project work was extended to include studies on viruses responsible of major outbreaks and emergence events. In particular, work included emerging influenza viruses such as

the avian H5N8 viruses that reached Europe in 2016 and represent a major threat for poultry in Europe or the avian H7N9 viruses that have been responsible since their emergence in 2013 for some 1580 laboratory-confirmed human cases of infection in China and represent a potential pandemic threat.
Beyond initial plans, additional flaviviruses were included. These were either part of the JEV related viruses such as Usutu virus (USUV) for which recently increasing cases of infection have been reported in Europe, or Zika virus (ZIKV) in light of its unprecedented geographical extension in 2013/2016 reaching the Americas. It also included Yellow Fever virus, following the upsurge of cases in Angola and the Democratic Republic of Congo in 2016. In addition, according to a semi-flexibility clause "*In case of emergence of a zoonotic virus with pandemic potential, PREDEMICS partners will have the possibility to engage in research within the scope of PREDEMICS based on expertise, know-how and methodologies developed within PREDEMICS*", work was also extended to include studies on viruses from other virus fal-milies that hade not been foreseen initially. These included, Chikungunya virus (CHIKV) another arbovirus, an alphavirus that like Zika virus achieved extensive geographical spread in 2013/2014 with human cases also detected in Europe, but also Ebola virus (EBOV), an hemorragic fever virus member of the *Filoviridae* family responsible for an unprecedented deadly epidemic in Western Africa in 2013/2015 or the Middle-East Respiratory Syndrome coronavirus (MERS-CoV), a betacoronavirus, identified for the first time in the Middle-East in 2012, that has been responsible for more than 2000 laboratory-confirmed cases in humans, and or which dromedary camels have now been identified as the source of infection.

The following workplan divided in 7 work packages (WP) was conceived to address the specific questions and priorities of the project, with defined objectives for each work-package.

WP1: Environment, ecological and anthropological factors
Through specific selected examples of viruses (IAV, HEV, LYS, JEV and related flaviviruses (JEVr) with the addition of MERS-CoV) and situations, the objectives of WP1 are:
• To analyse the human/animal interface process in the initial zoonotic infections.
• To study the interface between environmental reservoirs and domestic animals or humans and between wild and domestic animals where it is the most appropriate for a given virus and a given situation.
• To study key environmental and biological factors for the persistence of viruses in the environment and in reservoir species.
• To analyse what anthropological factors and environmental disturbances can affect virus dynamics.

WP2: Virus and host evolutionary dynamics
The specific objectives of WP2 are:
• To characterize the molecular epidemiology of IAV, HEV, LYS, JEV and related flaviviruses (JEVr) and to reveal the tempo, mode and spatial patterns of the viral evolutionary dynamics using statistical phylogenetic approaches.
• To elucidate the patterns and variation of within-host viral diversification according to host species and upon cross-species transmission or during the process of adaptation to a new target host
• To develop new molecular models and inference tools connecting sequence evolution to spatial spread and the evolution of virus and host traits.
Additional activities were performed on MERS-CoV, and EBOV.

WP3: Host-virus interaction and anti-viral response
The primary aim of WP3 is to understand how viral and host determinants interact to favour/limit the potential for cross-species transmission and adaptation to a new host. Specific objectives are:
• To understand the fundamental molecular basis of virus-host interactions to identify potential targets for disease intervention and control.

• To evaluate the capacity of IAV, LYS, HEV and JEV/JEVr to infect relevant natural hosts and cells in culture.
• To identify (viral and cellular) molecular factors involved in the mechanisms of entry, replication activity and modulation of innate immunity during HEV, IAV, LYS, JEV and WNV infection.
• To understand JEV replication and transmission in domestic pigs and primary porcine cells.
• To determine opsonizing functions and targets of anti-IAV and -JEV antibodies and characterize their role in protection of disease to develop more efficient vaccines.
Additional activities related were performed on MERS-CoV and EBOV as well as on CHIKV.

WP4: Sharing platforms
The overall goal of this WP was to conceptualize and implement a platform for data sharing and data interpretation for the project partners and the scientific community at large.
In particular the set objectives are:

- To conceptualize database schemes and curation processes for the exchange of sequence data for HEV, JEVr and LYS along the principles of the influenza sequence database EpiFlu of the GISAID initiative
- To develop concepts and tools for sharing of NGS data
- Provide sequence annotation tools enabling linkage to literature references for HEV, JEVr and LYS

WP5: Modelling of ecological, genetic and anthropological data
The objectives of WP5 are for the diseases of interest to PREDEMICS, to which MERS-CoV, EBOV, CHIKV and YFV diseases were added:

- To develop quantitative modelling tools and techniques to assimilate, analyse and integrate data from the diverse ecological, anthropological and genetic sources in the Project. Specifically models will be:
  i) transmission models;
  ii) models for risk assessment and contingency planning;
  iii) evolutionary/epidemic models.
- To develop statistical analytical techniques to generate rigorous model parameter estimates from the Project data sources.
- To use the combined quantitative models in a predictive sense to investigate the interplay of spatio-temporal spread, pathogen evolution and human behavioural responses and anthropogenic influences to address specific public health motivated questions about the underlying pathogen transmission dynamics (within and between the host and human populations).

WP6: Training
The objectives of the training program of WP6 dedicated to Isabel Minguez-Tudela, are aimed

- To foster exchange of ideas, information and staff with a trans-disciplinary approach involving scientists within and beyond the consortium from both animal and human health sectors
- To offer training opportunities within and beyond the consortium
- To promote links with other relevant EU-funded projects, international organizations (FAO, OIE, WHO,...) and leading opinion leaders and universities in Europe.

Beyond the viruses that were the initial focus of the project, MERS-CoV, EBOV and CHIKV were also included.

WP7: Management and Communication
The overall goal of WP7 is to ensure the successful implementation of the PREDEMICS project and achieve the objectives set by:

- Coordinating the project, on a day-to-day basis, at scientific, administrative, logistics, financial and legal levels, and initiating/implementing strategic activities.

- Developing relevant and efficient communication and dissemination tools/activities so as to ensure effective communication and information flow within the Consortium and with the European Commission (EC) and facilitating the transfer of knowledge among the beneficiaries and to the scientific community, the various stakeholders and policy makers in Public Health and the general public
- Organising all project meetings: Kick-Off meeting, Steering and Scientific Committee meetings and General Assembly meetings.
- Ensuring the fulfilment of contractual obligations among beneficiaries and vis-à-vis the EC and providing modalities for relevant IPR protection.
- Making sure ethical aspects are properly considered and assuring gender equality.

To address these objectives, PREDEMICS brings together 23 internationally recognized teams from 18 institutions in 7 EU countries and one associated country with cross-disciplinary expertise in veterinary and human medicine.

As a whole, the unprecedented trans-disciplinary collaboration within PREDEMICS, bringing together the animal and human health sectors and integrating epidemiological, ecological and biological parameters on viruses with high potential relevance for Europe that represent the key routes of transmission and disseminators held its promises to advance knowledge about the factors involved in cross-species transmission of zoonotic pathogens with pandemic potential and provided evidence-based data for policy makers that could translate into the implementation of adequate control strategies contributing to prevention of emergence events and increased preparedness.

1.3 - Description of the main S&T results/foregrounds

Work Package 1: "Environment, ecological and anthropological factors"
WP Leader: Jean-Claude MANUGUERRA [Beneficiary 1 – Institut Pasteur (IP)]

The overall objective of WP1 was, through specific selected examples of viruses (IAV, HEV, LYS and JEV and related flaviviruses (JEVr)) and situations, to analyse the human/animal interface process in the initial zoonotic infections; to study the interface between environmental reservoirs and domestic animals or humans and between wild and domestic animals where it is the most appropriate for a given virus and a given situation; to study key environmental and biological factors for the persistence of viruses in the environment and in reservoir species and to analyse what anthropological factors and environmental disturbances can affect virus dynamics.

- Task 1.1: Persistence of pathogens in the environment
Beneficiaries: P1-IP, P2-IZSVe, P6-IMPERIAL, P7-ANSES, P16-ISS, P18-UB - Leader: I. Leclercq, P1-IP

Persistence of influenza A viruses outside their hosts
Transmission of influenza A viruses (IAV), either airborne in mammals or oro-faecal in aquatic birds, submits virus particles to a wide range of environmental conditions. In water, temperature and salinity are important factors modulating viral stability. The main objectives were to study the survival of IAV outside the host and to identify molecular determinants involved in the loss or maintenance of IAV viability.
Having previously shown that the susceptibility of IAVs to a given temperature or salinity was not due to genomic degradation (*Dublineau et al., 2011*), we first validated the use of IAV lentiviral pseudotypes as an experimental tool for this study, and we showed that the nature of the HA plays an important role in the stability of IAV in the environment (*Sawoo et al., 2014*). Focusing on pandemic 2009 (H1N1pdm) and pre-2009 seasonal (H1N1Prepdm) IAVs, no difference was observed between both pseudotypes at low salinities at a given temperature, as previously shown with their viral counterparts (*Dublineau et al, 2011*), while increasing temperature and salinity had a strong negative effect on the survival of the H1N1 pseudotypes, with a higher impact on H1N1Prepdm pseudotypes. Our studies also identified several amino-acids potentially involved in H1N1 virus survival in water. In survival kinetics of reassortant viruses bearing only the HA and the NA of the two H1N1 viruses, mutations at some residues were found to decrease stability of the whole particles in saline water at 35°C. Most of the destabilizing mutations were correlated with an important sensitivity to pH inactivation. Inactivation slopes of various reassortant viruses also revealed that both the external structures of the particle (the HA and the NA) and internal proteins such as the matrix proteins modulate the stability of viruses in water at 35°C. Finally, using immunofluorescence microscopy, we showed that viral particles exposed for a long period in saline water at 35°C were still able to bind their cellular receptor whereas the HA-mediated fusion within the endosome was not possible anymore (manuscript in preparation).
Another aspect of our study was focused on the role of the host species of origin in IAV survival. The structure of the virus is directly dependent on the genetic makeup of the viral genome except the glycosylation moieties and the composition of the lipid bilayer. Viral kinetics in water at 35°C with H5N1 and H1N1 viruses showed that viral strains grown on mammalian cells persisted longer in water than viral strains grown on avian cells, suggesting that the composition of the lipid bilayer could influence virus survival (*Shigematsu et al., 2013*).

Circulation of Hepatitis E viruses (HEV) in wastewaters in Italy
From 2011 to 2016, we collected 894 urban wastewater samples (influent) throughout Italy (20 regions). Fifty-one out of 894 samples were positive for HEV (5%) by broadly reactive nested-RT-

PCR. By sequencing 18 strains were classified as HEV-1 and 33 as HEV-3. Mapping of wastewater samples positive for HEV (at least one positive sample detected), showed a geographic distribution: North (4.9%), Center (6.4%), and South (6.2%). Northern Italy is an area of high-density pigs farming; despite this, HEV-3 strains were detected more frequently in South and Central Italy. Treated effluents (13/31) to the surface waters (1/27) also resulted positive for HEV. Surveillance on human wastewaters may help to generate data on circulation of HEV in the general population otherwise not available or only referable to acute cases requiring hospitalization. We can assume that HEV can be discharged at low titre but data are needed to evaluate its persistence and infectivity.

Rabies virus infectivity, antigenicity and nucleic acid conservation following disinfection of utensils used for brain collection

Cleaning and disinfection are essential steps to prevent not only cross-contamination of samples in the laboratory environment, but also the risk for laboratory personnel to be accidentally exposed. To assess the efficacy of biocide products to effectively inactivate virus and to avoid nucleic acid contamination, five disinfectants, all belonging to biocidal categories considered effective against RABV, were selected. Their efficacy was assessed in the presence of a heavy organic challenge at increasing concentrations and/or contact times against Challenge Virus Standard-11 (CVS-11). Among the tested disinfectants, Virkon®, phenol and sodium hypochlorite proved to be effective at the tested conditions. When comparing reference tests with molecular methods, the latter tested positive following all but three disinfection protocols, which means that besides the protein denaturation, RABV nucleic acid is barely disrupted. Results of this study demonstrate that the selection of a proper decontamination protocol is paramount in guaranteeing reliable rabies post-mortem diagnosis and the safety for the laboratory personnel.

- Task 1.2: Local environmental changes

Beneficiaries: P1-IP, P2-IZSVe, P4-AMU, P16-ISS, P18-UB - Leader: J. Serra-Cobo, P18-UB

HEV in animals in Italy

In Italy, the presence of HEV in wild red deer populations living in the Apennines was investigated. Two hundred fifty one deer sera were collected between October and January during three hunting seasons. Deer herds are thinned every year by hunting, as planned by the local authority. A total of 35 out of 251 sera tested (13.9%; 95% CI: 9.9-18.9) were positive for anti-HEV IgG antibodies by ELISA. Seropositive animals were identified only in family groups (14.8%). HEV RNA was detected by qRT-PCR in 10 out of 91 sera selected randomly, showing a prevalence of 11%. Sequencing of two positive samples, confirmed matching of deer HEV strains with HEV genotype 3, related to both humans and swine strains reported previously. Information on wild animal strains is still poor and the possible role of other mammals as either HEV reservoir or vessels for emergence of novel strains is unknown. Moreover, during the hunting season hunters handled about 70 tons of raw deer meat confirming the possible risk of transmission.

Study of the evolution of bat lyssavirus

Partners 1 and 18 continued the analysis of the data obtained in an eco-epidemiological network that includes several stations located in a transect ranging from the Pyrenees through Barcelona to Mallorca and Menorca. From 2012 to 2016, we obtained 925 sera samples from 8 Spanish localities and 12 bat species. The first 7 localities constitute a network for study eco-epidemiological changes in a large scale. Complementary, the San Pedro pothole was prospected to finish a longitudinal study about two poorly studied bat species (*López-Roig et al. 2014, Viruses*). We found differences in the percentage of *Miniopterus schreibersii* between hibernation colonies where the percentage is low and spring and summer colonies where it is high. These differences are probably due to the cold temperatures that suppress viral activity during the hibernation season. The results also showed a possible tendency to synchronism.

- Task 1.3: Natural movement of animals
Beneficiaries: P1-IP, P2-IZSVe, P4-AMU, P7-ANSES, P18-UB - Leader: R. Charrel, P4-AMU

Genetic characterization of the red fox (Vulpes vulpes) population affected by rabies (RABV) and canince distemoer virus (CDV) in Northern Italy and neighbouring countries
The main objective was to identify and genetically characterise the wild fox population in a wide geographical area encompassing North-Eastern Italy, Austria, Slovenia and Croatia, where RABV and CDV were co-circulating. The population structure was investigated through a microsatellite analysis with 21 markers on 627 samples collected between 2006 and 2012. The analyses showed modest genetic differences between groups of animals, which appeared to be distinguished on a geographical basis. Both environmental and artificial barriers have a statistical influence in fox distribution, highlighting their potential in shaping the geographical spread of epidemic infectious diseases such as rabies or distemper. The study highlights the importance of monitoring bordering areas. Consequently the identification of corridors and barriers can be useful to reduce the vaccinations costs, helping implementing surveillance strategies for infectious diseases in wildlife.

SHERPAxMAP: an innovative platform technology for improving serodiagnosis and serosurveys of JEVr infection in humans and animals
An easy, fast, and low-cost method, designated SHERPADES, was developed for the massive production of recombinant antigens from medically important arboviruses including dengue (DEN), Japanese Encephalitis (JE), West Nile (WN), Usutu (USU*)* and Rift Valley Fever (RVF) viruses. The viral antigens were fused in frame to SHERPADES protein and produced using the Drosophila Expression System. Color-coded MagPlex microspheres were irreversibly conjugated to the recombinant antigens produced through SHERPADES using an optimized coupling protocol, and combined to form a multiplex fluorescent microsphere immunoassay to detect serum antibodies. Specific serum immunoglobulins captured during the assay procedure were revealed by a secondary reporter antibody using a flow analysis tool. Together, these technologies allow the rapid and simultaneous detection of antibodies to a wide range of infectious pathogens in biological fluids of infected patients and animals, thereby providing a high throughput, cost-effective, and accurate tool for surveillance and diagnosis of emerging diseases.
This new tool has been applied to perform a serosurvey of arboviral diseases in horse populations of Mallorca. A total of 167 horses were included in the study. Serum samples were collected before and (59) /or (108) after the summer of 2011, from 8 different areas. The results were as follows for confirmed IgG detection: WNV (3); USUV (2), SLEV (0); TBEV (0); CHIKV (0); EEE (6) and WEE (0). The positive horses for Eastern Equine alphaviruses had history of travel in the new-world. Our results indicated for the first time the circulation of WNV and USUV in the Balearic island of Mallorca (*J. Vanhomwegen et al., Vector Borne Zoonotic Dis. 2017*).

- Task 1.4: Anthropological factors including man-driven movements of reservoir and vector species
Beneficiaries: P1-IP, P7-ANSES, P10-UGOT, P12-INMI, P18-UB - Leader: MR Capobianchi, P12-INMI

HEV seroprevalence study in people attending INMI L Spallanzani Hospital for HIV Ab testing and risk factor analysis
HEV is transmissible through the faecal and oral route; risk factors associated to HEV infection remain to be elucidated. We aimed at describing the HEV incidence (IgM positivity) and the 10-year trend of HEV-IgG seroprevalence among adults asking for voluntary HIV testing in Rome.
Overall, 1200 subjects were selected by random sampling stratified on year of testing. Among them 1191 were tested for IgM and IgG antibodies and 9 were excluded. Given its low frequency (0.59%), the overall 10-year HEV incidence (HEV-IgM positivity) was not analysed further. The overall 10-year prevalence of HEV IgG was 5.21% with weak evidence (p=0.085) that the odds of being HEV-IgG follow an association with the year of testing. Using univariate and multivariate

logistic regression models to assess the association of being HEV-IgG positive and 23 risk factors, the analysis provided very good evidence for a linear association between age and prevalence of HEV-IgG (p=0.003). Referring him/herself as a gay was associated with about 82% increase of the risk of being HEV-IgG positive, and an increased risk of being HEV-IgG positive was found in subjects born outside Italy. These findings underscore the importance of adopting prevention measures, i.e. protected sexual intercourse, not only to prevent HIV and other sexually transmissible infections, but also for HEV (*S. Lanini et al., BMJ Open. 2015*).

HEV in wild boars and domestic pigs in Sweden and France

Our studies showed that Swedish wild boars have a high prevalence of HEV infection (over 25 %) with regional differences between the virus strains infecting wild boars. We have also shown that HEV strains infecting humans are similar to strains that infect wild boars in the same geographic area. Wild boars have spread quite rapidly in the country during the past five years, due to warmer climate and less snow and despite recommendations by the authorities to increase hunting and boar meat consumption. There have been suspicions that hunters have moved young boars from one region to another thereby increasing the risk for human infections in these regions. Sequencing of the viral genomes from wild boars collected in 2009 and from infected humans, allowed to determine if wild boars have been moved by humans or if the spread is a natural movement and if the viral strains of wild boars spread to humans.

Since domestic pigs are a reservoir for HEV, samples were taken from six piglets each in 30 different pig farms in Sweden and the frequency of positive piglets was high: 20 %.

In France**,** as discussed above for Sweden, interactions between domestic pigs and wild boar are more or less frequent depending on pig breeding conditions (open field, closed farms). A study was conducted in France to determine if the presence or the absence of contact with wild boars had an influence on HEV seroprevalence in domestic pigs. Serum samples from 213 individuals were collected at slaughterhouse and tested for anti-HEV antibodies ; a high HEV seroprevalence of 80% was observed but no link was found between high prevalence and close interaction with wild life.

- <u>Task 1.5: Geographic and ecological niche overlaps</u>

Beneficiaries: P1-IP, P7-ANSES, P12-INMI, P16-ISS, P18-UB - Leader: J. Serra-Cobo, P18-UB

This study was started in the precedent European project RABMEDCONTROL and finished in this project. We report the results of the first active survey on lyssaviruses carried out during the same years in bat colonies located in northern Africa and in southern Europe. Twenty-six bat species belonging to 7 families were collected from 2007 through to 2012 in 41 localities from Morocco, Algeria, Egypt, Italy and Spain. Among the 3,051 sera obtained, 15% were positive for EBLV-1. Twenty-four localities (59%) harboured seropositive bats: 1 in Morocco, 3 in Algeria, 1 in Egypt, 2 in Italy and 17 in Spain. EBLV-1 neutralizing antibodies were detected in 16 (62%) bat species belonging to 5 families. It is the first time that EBLV-1–antibodies are detected in bats from Morocco, Algeria, Egypt and Italy. Among 84 sera obtained from *Rousettus aegyptiacus* in Egypt, one was seropositive for LBV. It is the first time that LBV was found in bats from North Africa. Our results show that the circulation of bat lyssaviruses is not negligible in North Africa. In consequence, our findings must serve to alert the sanitary authorities of the health risk, which involves the manipulation of bats.

Work Package 2: "Virus and host evolutionary dynamics"

WP Leader: Philippe LEMEY [Beneficiary 3 – Katholieke Universiteit Leuven (K.U. Leuven]

- Task 2.1: Molecular epidemiology and viral phylodynamics

Beneficiaries: P1-IP, P2-IZSVe, P3-KU Leuven, P4-AMU, P7-ANSES, P10-UGOT, P12-INMI, P13-UEDIN, P16-ISS, P18-UB - Leaders: H. Norder, P10-UGOT/H. Bourhy, P1-IP

. Subtask 2.1.1/HEV evolution and cross-species transmission (Leader: H. Norder, UGOT)

A large screening was performed of different hosts on food items and sewage by Partners 7, 10, 12, and 16 to identify circulating HEV strains. More than 8,600 samples were analysed, and 432 HEV strains were identified. A protocol was developed to sequence two genomic regions, partial polymerase and capsid region. Phylogenetic analysis showed that HEV genotype 3 (HEV3) is common in our countries and that zoonotic spread is frequent, with domestic pigs being the main vector for human infections. Also wild boar HEV3 infected humans. We identified HEV3 transmissions between wild boar and domestic pigs. Different species of deer were also found infected with HEV3. An outbreak of HEV4 was identified in Italy during this project. This strain is prevalent in China, and there were only few previous reports with infected humans from France and Germany. Since these patients were infected locally, HEV4 can already have established in Europe, where there may be an unknown animal reservoir. During the screening of samples from wild animals a new HEV was discovered from moose. It has been classified in Orthohepevirus A as a new genotype. HEV belonging to Orthohepevirus C was identified in rats in Italy.

HEV infections were identified in about 5% - 20% of investigated domestic pigs and wild boars. In both Sweden and France (the Island of Corsica) a high level of interaction occurs between wild or feral and domestic pigs. In four categories of 394 French food products (Figatelli, smoked liver sausage, dried liver, quenelle paste), HEV RNA was detected in 3% dried liver, 25% quenelle paste, 29% smocked liver sausage and 30% figatelli. It was also detected in 22% raw and 4% dry liver sausages from Italy. Clinical cases associated with the consumption of food products containing HEV RNA were identified in France and Italy, showing that this is a common route of infection.

The seroprevalence of HEV was shown to be high in all our countries (>16%). Sewage from Italy and Sweden were therefore analysed for HEV RNA to indicate the frequency of infected persons. It was identified throughout the year, indicating ongoing circulation of HEV in the population. In addition to HEV3 also HEV1 was identified in Italy. This genotype has potential for person-person transmission and a high mortality among pregnant females. These findings warrant further surveillance to inhibit HEV1 spread in Europe.

A clade of HEV strains infecting German wild boars has been observed among clinical cases of hepatitis E in Sweden and isolated from fulminant hepatitis E in Italy. This strain seems to be spreading the last two years and is the most common strain identified from patients with HEV infections that need hospitalization and also from patients with chronic hepatitis E. This strain was identified from blood donors with low level of anti-HEV IgG and lacking anti-HEV IgM.

. Subtask 2.1.2/From local to continental-wide rabies transmission and evolution (Leader: H. Bourhy, P1- IP)

Phylogeography and phylodynamics of dog rabies

Phylodynamics and dispersal of domestic dog rabies in an African city

In this study, we focused on the dog population in the African urban setting of Bangui, the capital of Central African Republic (CAR) that comprises nearly 900,000 inhabitants, where rabies epidemiologic patterns follow periodic waves separated by unexpected extinctions of the chain of transmission. Based on a dense sampling (136 isolates mostly collected between 1990 and 2012) of

near complete genome sequences of dog rabies viruses in Bangui, CAR, and neighbouring countries, we have investigated the small-scale landscape genetics of rabies in its principal reservoir host and provide genetic estimates of the viral transmission dynamics. A combination of phylodynamic analysis and modeling approaches (developed in WP5) clearly indicated that rabies is not sustainable in this urban setting and that the continuous importation of new clusters of virus contributed to periodic epidemiologic waves (*Bourhy et al., PLoS Path, 2016*).

Emergence and Spread of an Arctic-Related Phylogenetic Lineage of Rabies Virus in Nepal.

In this study, we describe for the first time the phylogenetic diversity and evolution of RABV circulating in Nepal, and their geographical relationships within the broader region. Despite Nepal's limited land surface and particular geographical position within the Indian subcontinent, this study revealed the presence of a surprising wide genetic diversity of RABV, with the co-existence of three different phylogenetic groups: an Indian subcontinent clade and two different Arctic-like sub-clades within the Arctic-related clade. This observation suggests at least two independent episodes of rabies introduction from neighbouring countries (*Pant et al., PLoS Negl Trop Dis, 2013*).

Phylogeography of rabies viruses in the red fox population in North-Eastern Italy.

A detailed evolutionary analysis of RABVs circulating in the red fox population in north-eastern Italy identified two viral genetic groups, here referred to as Italy-1 and Italy-2 (Fusaro et al., Infect Genet Evol 2013). Phylogenetic and phylogeographic analyses revealed that both groups had been circulating in the Western Balkans and Slovenia in previous years and were only later introduced into Italy (into the Friuli Venezia Giulia region-FVG), occupying different areas of the Italian territories. Notably, viruses belonging to the Italy-1 group remained confined to the region of introduction and their spread was minimised by the implementation of oral fox vaccination campaigns. In contrast, Italy-2 viruses spread westward over a territory of 100 km from their first identification in FVG, likely crossing the northern territories where surveillance was inadequate.

Phylogeography and phylodynamics of bat rabies and role as RABV reservoir for other hosts.

Phylodynamics and dispersal of European bat lyssavirus in France.

We undertook a comprehensive sequence analysis based on eighty newly obtained EBLV-1 complete genome sequences from nine European countries over a 45-year period to infer selection pressures, rates of nucleotide substitution and evolutionary time scale of two subtypes in Europe. We show that the current lineage of EBLV-1 arose in Europe around 1400 (between 1219 and 1558) and the virus has evolved at a low substitution rate. In parallel, we investigated the genetic structure of French serotine bats at both the nuclear and mitochondrial level and found that this does not account for EBLV-1a and -1b segregation and dispersal in Europe.

Phylogeographic analysis of RABV circulating in bovines in the State of Mato Grosso, Brazil.

We investigated the genetic diversity and the spatial and evolutionary processes of a dataset of 162 RABV cases collected from bovines in the State of Mato Grosso (Brazil) between 2007 and 2011. This study confirmed the role of vampire bats as RABV reservoirs for domestic herbivores in Brazil. Identification of the forest-covered area of Mato Grosso (Northern area) as the main source of the rabies viruses points to the forests/deforestation fronts as one of the potential risk factors for the dissemination of vampire bats and the associated rabies infection in Brazil.

Spatio-temporal dynamics of RABV on global scale and adaptation to new hosts

Using 321 genome sequences spanning an unprecedented diversity of RABV, we compared evolutionary rates and selection pressures in viruses sampled from multiple primary host shifts that occurred on various continents. Strikingly, although dog RABV has jumped to various wildlife species from the order Carnivora, we found no clear evidence that these host-jumping events involved adaptive evolution, with RABV instead characterized by strong purifying selection, suggesting that ecological processes also play an important role in shaping patterns of emergence. However, specific amino acid changes were associated with the parallel emergence of RABV in

ferret-badgers in Asia, and some host shifts were associated with increases in evolutionary rate (*Troupin et al., PLoS Pathog, 2016*).

. Subtask 2.1.3/Phylodynamic comparison of JEV and JEVr (Leader: E. Gould, P4-AMU)

Japanese encephalitis virus: As JEV genotype V became more prominent in Asia, evidence was accumulating to suggest that the current JEV vaccine based on genotype III was not particularly effective against this genotype (Cao et al., PLoS Negl Trop Dis., 2016). Consequently, a novel technique, developed in by P4-AMU (Nougairede et al., PLoS Pathog, 2013), was exploited to produce attenuated encephalitic flaviviruses within days. This novel method proved to be highly successful in generating genetically modified live attenuated flaviviruses. In separate studies but at the same time as those described above, PREDEMICS partners demonstrated that JEV could be transmitted via the oral route in pigs, an important amplification reservoir of JEV (*Ricklin et al., Nat Commun., 2016*). Many recent studies on JEV have contributed to the belief that this virus has a realistic capability of emerging Out of Asia into Africa and Europe and even further afield.
WNV and USUV: while WNV does not appear to be presenting a significantly increasing threat to either humans or avian species in Europe, USUV has gradually increased its geographic range, causing localised avian mortalities and occasional human encephalitic infections. In 2013, PREDEMICS partners published the results of a phylogenetic comparison and genomic sequence characterisation between central European strains of emerging avian USUV and the first isolate of this virus from a case in Italy of human neurovirulent disease (*Gaibani et al., PLoS One, 2013*). More recently, widespread activity of multiple lineages of USUV in Northern Europe, with high rates of avian mortality (*Cadar et al., Euro Surveill. 2017*), support the possibility that USUV could displace WNV in order of its importance as an avian and human pathogen in Europe.
CHIKV and ZIKV: The first report of the global emergence of CHIKV in the Americas (by PREDEMICS partners) (*Leparc-Goffart et al., Lancet 2014*), was followed soon afterwards by the surprising emergence of ZIKV. We performed phylogenetic analysis of the largest collection of ZIKV isolates in 2016 and identified unique amino acid substitutions that were hypothesised to impact ZIKV pathogenesis, transmission and replication efficiency (*Pettersson et al., MBio, 2016*). ZIKV is primarily transmitted by *Ae aegypti* which is rare in Europe and therefore the perceived risk of ZIKV becoming a serious epidemiological threat in Europe seems low at this time.

. Subtasks 2.1.4/The evolution of pathogenicity in avian influenza (Leader: I. Monne,P2-IZSVe)

Through the in depth sequencing and analysis of the whole genome of 232 influenza viruses, this subtask explored i) the evolution of avian influenza viruses pathogenicity, ii) the within and between host diversity existing in the virus population identified during an avian influenza outbreak and iii) the pathways of viral transmission between different geographical regions, holdings and sheds. Our investigations on the emergence of highly pathogenic (HP) viruses of the Eurasian H7N1 and H7N7 subtypes demonstrated the complexity of the evolution of avian influenza viruses in domestic birds, which limits the possibility to make any reliable predictions of the risk for transition from LPAIV to HPAIV (*Monne et al, J Virol., 2014; Fusaro et al., J Virol., 2016*). A comparison of the genetic changes between HPAI viruses and LPAI precursors showed that aside from changes at the cleavage site (CS) of the HA, mutations mainly occurred in the HA in positions adjacent to or apart from the CS, including the receptor-binding site, as well as in the polymerase genes (*Monne et al, 2014*). The evolutionary comparisons between two viral lineages, H7N1 and H7N3, which resulted in a different outcome of the disease (the H7N1 viruses evolved into a HP form, while the H7N3 did not) in similar epidemiological circumstance, revealed that the role of environmental components and their interaction in the evolution of pathogenicity is unclear and that even minute permutations can lead to chaotic and unpredictable effects on virus evolution.
An analysis of 53 HPAI H5Nx belonging to the Gs/Gd lineage (8 H5N8, 2 H5N5 and 43 H5N1) demonstrated that wild birds did play a major role in the multiple and independent introductions of

the HPAI viruses into poultry holdings both in Europe and in Africa (*Fusaro et al., Emerg Infect Dis., 2017, Tassoni et al., Emerg Infect Dis., 2016*). Analyses applied on the whole genome sequences generated from the H5N1 viruses identified in West Africa in 2015, revealed that the viruses introduced in this geographic region possess the genetic constellation of a zoonotic strain which had caused a fatal human infection in 2014 and that their genome contains mutations which have been previously described as being associated with an enhanced binding affinity for α2,6 sialic acid or with an increased virulence in mammalian species (*Tassoni et al 2016*).

- Task 2.2: Within-host viral diversification and adaptation
Beneficiaries: P1-IP, P5-EDI-IVI, P7-ANSES, P12-INMI - Leader: M. Vignuzzi, P1-IP

. Subtask 2.2.1/Genomic analysis of extrinsically (selective pressures) and intrinsically (replication fidelity) manipulated viral mutation distributions (Leader: M. Vignuzzi, P1-IP)

Selection and generation of fidelity variants. In the first years of the program, we tried to isolate high fidelity variants of influenza virus using classic passaging of wild-type in mutagenic conditions. However, of the few candidate mutations identified, none was found to alter fidelity. We thus resorted to forward genetics approaches by error-prone PCR to randomly introduce mutations in the PB1 gene. Libraries of recombinant mutagenized viruses, or corresponding minireplicons, were produced and submitted to serial rounds of selection by passage in mutagenic conditions. These approaches proved successful in selecting for mutagen-resistant PB1 sequences and we identified mutations A401V and N694I for H3N2 and A643G for H1N1pdm09, as the most promising fidelity variant candidates. These will be tested *in vivo* in order to evaluate their potential as basis for the development of attenuated vaccines.
Identification of cellular factors that could alter replication activity, and potentially, fidelity. Since it is possible that cellular factors might also be involved in the modulation of replication fidelity, a set of 98 cellular proteins involved in exo(ribo)nuclease and proofreading activities or part of the cellular exosomes were selected for further screening. Interactions between these cellular proteins and the PB1, PB2, PA, NP and M1 and NS1 proteins were assessed by Protein Complementation Assay (PCA). A comprehensive interaction map and siRNA knockdown identified 12 factors that decreased virus growth. These factors will be further studied to unravel the underlying molecular interactions and the potential modulation of polymerase activity and fidelity.

. Subtask 2.2.2/Intrahost virus population analysis (Leader: M. Vignuzzi, P1-IP)

Development of a bioinformatic pipeline to analyse within-host genetic diversity.
We developed an analysis pipeline VIVAN (**Vi**ral **V**ariance **An**alysis) that allows mapping of the frequency of all minority variants at each nucleotide position and translation of nucleotide to protein sequence. The pipeline identifies all mutations that are statistically significant (above the background error, generally any variant that is >0.1% of the total population) and permits comparative analysis between groups of samples (*Isakov et al, Bioinformatics 2015*).
Characterisation of the genetic diversity of human influenza viruses. We then evaluated the level of intrinsic heterogeneity of the different human influenza virus subtypes that circulated between 2007 and 2012 in France, directly from nasal swab samples. The large set of data obtained from more than 100 samples clearly showed a difference in heterogeneity between the three subtypes, and provided evidence for more diversity within severe versus mild disease. In parallel, the Capobianchi team (INMI, P12) showed that higher genetic heterogeneity was observed in samples from patients hospitalized with severe A(H1N1)pdm09 infection (*Selleri et al., 2012*).
Deep sequencing and characterisation of the genetic diversity of Lyssaviruses. Lyssaviruses occasionally jump to new hosts. However, no strong genetic fingerprints of adaptation could be identified from the few partial genome sequences available. We deep sequenced 170 samples, spanning the 1950-2015 period. Combined with other Genbank sequences, we performed the largest

study of rabies phylogenomics (321 total). Results show that changes in host species are associated with subtle changes in multiple evolutionary pathways, including the occurrence of host-specific parallel evolution (*Troupin et al, Plos Pathogens, 2016*). Preliminary results from experimental passage of rabies virus in dogs and foxes indicate that few consensus changes correlate with cross-species transmission and that complex mechanisms of epistasis may play an important role.

- Task 2.3: Model development and viral evolutionary inference

Beneficiaries: P3-KU Leuven, P13-UEDIN - Leader: P. Lemey, P3-KU Leuven

. Subtask 2.3.1/Testing hypothesis of cross-species transmissions (Leader: P. Lemey, P3-KU Leuven)

A major focus of this subtask was the development and application of an extension of a discrete phylogenetic diffusion approach implemented in a Bayesian statistical framework. This extension parameterizes rates of diffusion as a generalized linear model (GLM) allowing to simultaneously reconstructing diffusion history while testing predictors of the diffusion process (Lemey et al., PloS Path., 2014). Throughout the project we applied this model to address several viral evolutionary and epidemiological questions, including: 1) testing the determinants of both host shifts and cross-species transmissions of rabies virus in North American bats, 2) examining the drivers of global human influenza (H3N2) spread, 3) identifying the predictors of dengue virus spatial dynamics in Brazil, 4) revealing drivers of global migration of influenza A viruses in swine and 5) untangling the patterns of spread of Ebola in the West African epidemic (*Dudas et al., Nature, 2017*).

P13-UEDIN has conducted, and contributed to, several phylodynamic investigations to trace the cross-species transmissions and viral emergence of different viruses into the human population. Applications to influenza involved a host-specific local clock model (Worobey et al., Nature & PNAS, 2014a&b), and comparative analyses of human seasonal influenza A and B variants (*Bedford et al., Nature, 2016*). Applications to other viruses include the spread, circulation, and evolution of the MERS-CoV (*Cotton et al., 2014*) and the emergence and spread of Ebola in West Africa (*Dudas et al., 2014, Gire et al., 2014*).

. Subtask 2.3.2/Integrating stochastic processes of sequence and trait evolution (Leader: P. Lemey, P3-KU Leuven)

As part of this subtask, P3 developed a fast and reliable method capable of estimating site-specific selection in large data sets (*Lemey et al., 2012*). Building on the trait evolutionary models in BEAST, P13-UEDIN and P3-KU Leuven developed an approach to integrate both genetic and antigenic information into a single coherent Bayesian inference framework, and used this to compare the evolutionary dynamics of different influenza variants (*Bedford et al.,eLife, 2014*). P13 and P3 also developed other models or model extensions within the Bayesian evolutionary inference framework to support research within PREDEMICS. This includes for example a method to reconstruct transmission trees for densely-sampled infectious disease outbreaks using phylogenetics (*Hall et al., PLoS Comput Biol, 2015*), a discrete diffusion model extension that incorporates temporal heterogeneity in phylogeographic processes (*Bielejec et al., Sys Bio9, 2014a*), and state-of-the-art marginal likelihood estimators to asses model fit (*Baele et al., 2017*).

Finally, P3 and P13 also developed tools associated to the Bayesian evolutionary inference framework that were used throughout the project, including a software tool, called spreaD3, to visualize phylogeographic estimates (*Bielejec et al., MBE, 2016*). We note that the BEAST methods developed as part of PREDEMICS have also been applied to various other pathogens, including for example HIV-1, salmonid alphavirus, and Foot-and-Mouth Disease Virus.

Work Package 3: "Host-virus interaction and anti-viral response"

WP Leader: Hervé BOURHY [Beneficiary 1 – Institut Pasteur (IP)]

- Task 3.1: Molecular factors conditioning the efficiency of infection

Beneficiaries involved: P1-IP, P2-IZSVe, P4-AMU, P5-EDI-IVI, P7-ANSES, P8-MPG, P11-UNIMAR, P12-INMI - Leader: H. Bourhy, P1-IP

The primary objective of task 3.1, is to investigate the factors involved in the emergence of zoonotic pathogens leading to successful host switching and to understand how viral and host determinants interact to favor/limit the potential for cross-species transmission and adaptation to a new host.

. Subtask 3.1.1/Replication efficiency, pathogenesis and transmissibility in natural hosts (Leader: H. Bourhy, IP)

Replication efficiency, pathogenesis and transmissibility in natural hosts have been studied in the different virus models chosen by PREDEMICS: Influenza virus, Lyssavirus, hepatitis E virus and Japanese encephalitis and related viruses.

A: *Influenza virus* *(P11-UNIMAR, P1a-IP and P5-EDI-IVI)*

Restriction on avian viruses replication in human airway epithelium

Results revealed that i) multiple selective pressures and many viral genes restrict replication of avian viruses in human airway epithelium, ii)15 passages in HTBE cells were not sufficient to fully overcome these pressures, iii) all viruses acquired mutations in the HA and NP, and all 3 variants of Mallard/Alberta/98 acquired mutations in the same region of the PA suggesting particularly important role of these proteins in the avian-to-human adaptation.

Role of the avian PB1 gene segment in the emergence of 1968 pandemic virus

This study demonstrated for the first time that the avian PB1 segment of the 1968 pandemic virus served to facilitate viral growth and transmissibility by enhancing activity of the viral polymerase complex. Thus, in addition to the acquisition of antigenically novel HA (i.e., antigenic shift), enhanced viral polymerase activity is required for the emergence of pandemic influenza viruses from their seasonal human precursors. (*Wendel I et al. J Virol 2015, 89:4170-9*).

Adaptive changes in the HA that were critical for the emergence of the H3N2/1968 pandemic influenza virus from its avian precursor

It was found that avian-like mutation N81D significantly decreased viral fitness in HTBE cultures, suggesting that substitution D81N in the avian HA was critical for virus adaptation from birds to humans. These findings improved knowledge on phenotypic and genotypic markers of emerging zoonotic and pandemic viruses.

Characterization of emerging MERS-CoV, A/H7N9 IAV and Ebola virus

In September 2012, a novel human coronavirus (MERS-CoV) was isolated in association with cases of an acute, rapidly deteriorating respiratory illness. P11 participated in the study on characterization of this virus. These studies revealed that MERS-CoV resembles SARS CoV by its ability to replicate in primary, continuous and differentiated cells of the human airway epithelium and to prevent induction of IRF-3-mediated antiviral interferon responses. However, MERS-CoV was markedly more sensitive to the antiviral state established by ectopic IFN, suggesting that contemporary variant of this virus may not spread with the same speed and scale as did SARS-CoV. (*Zielecki F et al. J Virol 2013, 87:5300-4*).

An avian influenza A(H7N9) virus has emerged in south eastern China in March 2013 causing occasional human infections with about 20% mortality. P11 participated in the development of

rapid PCR diagnostic test for the new virus (collaboration between partners from PREDEMICS and ANTIGONE). (*Corman VM et al. Euro Surveill 2013, 18:20461*).
The Ebola virus (EBOV) outbreak in West Africa started in December 2013 and claimed more than 11,000 lives. P11 collaborated with Stephan Becker's team at UNIMAR in the studies on characterization of this virus. These studies demonstrated emergence of adaptive changes in the Ebola virus genome during virus circulation in humans and opened avenues for further studies on their role in virus transmissibility and pathogenicity. (*Dietzel E et al. J Virol 2017, 91: e01913-16*).

Effects of membrane fusion characteristics of IAVs on viral sensitivity to interferon (IFN) and IFITM proteins

The replication and pathogenicity of influenza viruses critically depend on their ability to tolerate the antiviral IFN response. To determine a potential role for the IAV HA in viral sensitivity to IFN, P11 studied the restriction of IAV infection in IFN-beta-treated human epithelial cells by using 2:6 recombinant IAVs that shared six gene segments of A/Puerto Rico/8/1934 virus (PR8) and contained HAs and NAs of representative avian, human, and zoonotic H5N1 and H7N9 viruses. The results showed for the first time that the sensitivity of IAVs to the IFN-induced antiviral state and IFITM2 and IFITM3 proteins depends on the pH value at which the viral HA undergoes a conformational transition and mediates membrane fusion. These findings imply that the high pH optimum of membrane fusion typical of zoonotic IAVs of gallinaceous poultry, such as H5N1 and H7N9, may contribute to their enhanced virulence in humans. (*Baumann J et al. J Virol 2015, 90:1569-77*).

Importance of PB2 mutations for avian influenza viruses to adapt to pigs *(P5-EDI-IVI)*

Partner 5-EDI-IVI has identified that avian influenza virus PB2 mediates enhanced antiviral responses in porcine dendritic cells when compared to a PB2 of swine influenza origin. The data indicate that for avian viruses to adapt to pigs, mutations in PB2 might also be necessary to afford reduced antiviral responses. (*Manuela Ocaña-Macchi et al. Virology 2012, May 25;427(1):1-9*).

B. Lyssavirus (P7b-ANSES; P1a-IP PVP; P1b-IP DyLAH; P8-MPG)

Molecular drivers of cross species transmission

The natural evolution of RABV provides a potent example of multiple host shifts and an important opportunity to determine the mechanisms that underpin viral emergence. The main objectives of this study was (i) to analyse the genetic diversity of rabies virus (RABV) populations, (ii) to identify the molecular signatures of RABV adaptation to a new host and (iii) to characterize the intrinsic diversity during cross-species transmissions in order to build hypothesis on the potential role of some of these sub-populations in the adaptation mechanisms. This was achieved by determining and comparing genome sequences spanning an unprecedented diversity of RABV observed *in natura* and by performing homologous and heterologous experimental passages in dog and fox using various wild lyssavirus isolates specifically adapted to one of these two species. The same type of passages was performed in primary neuronal and muscular cells obtained from dogs and foxes. Altogether, these results indicated that there are very few mutations that could be easily correlated with the ability of the virus for cross-species transmission and that complex mechanisms of epistasis could play a role. Further investigations looking for the role of minor variants present in intrinsic diversity of each isolate during adaptation to new species are presently ongoing. **(***Troupin C et al., PLoS Pathog. 2016 Dec 15;12(12):e1006041*).

Molecular drivers of replication and transcription efficiency: how mutations of rabies virus phosphoprotein affect replication complex activity and induce the emergence of new viral populations

The viral nucleoprotein (N), phosphoprotein (P), and viral polymerase (L) participate to the replication complex of RABV. This study suggests that mutations at several sites of the P protein can influence the formation of the N-RNA-P complex and affect the phenotype of viral recombinant

populations. It also reveals the complex epistasis relationship between some of these mutations located on the P protein and the N and L proteins.

C. Hepatitis E virus (P7a-ANSES)

A quantitative proteomic analysis was carried out to identify cellular factors and pathways modulated during acute HEV infection of swine. Liver samples were analysed by a differential proteomic approach, two-dimensional difference in gel electrophoresis, and 61 modulated proteins were identified by mass spectroscopy. A comparative analysis of the liver proteome modulated during infection with three different strains of HEV genotype 3 provides an important basis for further investigations on the factors involved in HEV replication and the mechanism of HEV pathogenesis. (*Rogée S et al. J Virol. 2014 Oct 15. pii: JVI.02208-14*).

D. Japanese encephalitis virus and other related arboviruses (e.g. Zika virus) (P1a-IP-ERI-GA); P5-EDI-IVI, P12-INMI)

Pathogenic properties of JEV and ZIKV

A full characterization of an understudied JEV genotype 5 (g5) strain was performed and its virological and infectious properties were compared to those of a widely studied JEV genotype 3 strain (g3). While the 2 strains behaved similarly when infecting cell lines *in vitro*, we observed that g5 was highly pathogenic *in vivo* in a mouse model for encephalitis, while the mice were resistant to g3 infection. We constructed chimeric viruses from the strains infectious clones and further demonstrated that the structural region of JEV genome was responsible for g5 poor clearance in mice. (*de Wispelaere M et al. J Virol. 2015 Jun;89(11):5862-75*).

The flavivirus genome encodes 3 structural and 7 non-structural proteins. One of the structural proteins, the membrane (M) protein, is synthesized as a precursor prM and is cleaved into pr and M during viral secretion. We studied the role of the M protein of JEV and WNV in the viral infectious cycle and showed that a single substitution at position 36 in this protein impairs JEV and WNV infectious cycle in mammalian cells, but not in mosquito cells. Furthermore, we demonstrate that the mutant JEV and WNV viruses are completely attenuated *in vivo* in a mouse model for flaviviral encephalitis, exemplifying the efficiency of a vaccination strategy approach that relies on the production of a strongly attenuated flavivirus affected in the late stages of the infectious cycle. (*de Wispelaere M et al. J Virol. 2015 Dec 9;90(5):2676-89*).

The recent ZIKV outbreak in South America and French Polynesia was associated with an epidemic of severe congenital malformations including microcephaly, a disease characterized by a reduced size of the cerebral cortex. While WNV and JEV can cause encephalitis, they are not associated with microcephaly, suggesting different cell targets in the CNS. We compared the cell populations infected by ZIKV or WNV in slices of mouse developing neocortex and demonstrated a remarkable tropism of ZIKV infection for neural stem cells, while WNV targeted mainly the mature neurons. We further showed that ZIKV infection, but not WNV infection, impairs cell cycle progression of neural stem cells, while both viruses inhibited apoptosis at early stages of infection. **(***Brault JB et al. EBioMedicine. 2016 Aug;10:71-6*).

Potential of emergence of vector-borne diseases in Europe

Developments in transportation drive the rapid, global dispersion of pathogens and their vectors. In the framework of PREDEMICS, we focused our studies on the potential transmission of JEV and Zika virus (ZIKV) by European mosquitoes.

We aimed to study the vector competence of European mosquito populations, such as *Culex pipiens* and *Aedes albopictus* for JEV genotypes 3 and 5. We also wanted to assess whether the virus transmitted by European mosquitoes was capable of developing a pathogenic infection in mammalian hosts.

A second approach has consisted in comparing transmission of ZIKV by *Ae. aegypti* and a French population of *Ae. albopictus* in order to better define their competence as well as the potential risk of emergence of ZIKV in Europe. Interestingly, whereas both vector's salivary glands are highly infected, transmission rates of this virus to the saliva remain relatively low. Proteomic analyses are underway in order to better understand vector/virus interaction in these two mosquito species. (*de Wispelaere M et al. PLoS Negl Trop Dis. 2017 Jan 13;11(1):e0005294*).

Tropism of Japanese encephalitis virus and other related arboviruses to pigs and porcine lymphoid tissues, macrophages and monocyte-derived dendritic cells

Partner P5-EDI-IVI has identified that JEV in addition to its neurotropism also has a high level of replication in various secondary lymphoid tissues, in particular the tonsils. In this organ, the virus can persist for several months even after vaccination (*Ricklin ME et al. Vet Res. 2016 Feb 24;47:34 ; García-Nicolás O et al. Viruses. 2017 May 22;9(5)*).

. Another important finding was that pigs were highly susceptible to oronasal infection and that contact transmission does occur between animals. Altogether, these *in vivo* findings show that pigs are not only an amplification host for JEV but may also act as maintenance host. Furthermore, the virus may transmit and spread in this species in absence of arthropod vectors, facilitating its spread and overwintering in regions with temperate climate (*Ricklin ME et al. Nature Commun 2016 Feb 23;7:10832*). We also think that these finding are relevant for other flaviviruses as vector-free transmission could be more common than expected. Knowledge on these events, even if rare are of high relevance for public health. P5-EDI-IVI has also investigated in detail the comparative tropism of various flaviviruses for human and porcine cells, with a focus on monocytic cells. The viruses included in our analyses were mostly of high public health relevance including JEV, ZIKV, USUV, WNV and DENV. We found that for porcine cells (macrophages and monocyte-derived dendritic cells) JEV had by far the highest tropism followed by WNV, USUV, while ZIKV and DENV had only a limited ability to infect and replicate. Surprisingly, also with human macrophages and monocyte-derived dendritic cells JEV was found to be the virus with the highest infection and replication rates. Nevertheless, this was followed by USUV, ZIKV, DENV and WNV. The surprisingly high tropism of USUV for human cells is a concern, considering the presence of USUV in various regions in Europe. A publication describing these results is currently in preparation.

. Subtask 3.1.2/Identification of HEV mechanism of entry (Leader: N. Pavio, P7a-ANSES)

To characterise the internalisation pathway(s) involved in HEV entry in hepatocytes *in vitro*, we used human bipotent progenitor HepaRG cells that are able to differentiate into cholangiocytes and hepatocytes. Protocols using drugs inhibiting clathrin-dependent endocytosis, caveolin- or lipid raft-dependent endocytosis and macropinocytosis were set up in this model. HepaRG cell lines in which specific endocytic pathways are inhibited were also developed using lentiviruses. An assay was then developed to quantify HEV internalisation into HepaRG using the different tools developed in the lab. The protocol was optimised in order to quantify HEV entry into cells in a sensitive and reproducible manner using real-time RT-qPCR. Unfortunately, although many different conditions were tested, non-specific absorption of the virus to the cell surface could not be prevented, leading to high background and inconsistent results.

. Subtask 3.1.3/Identification of cellular factors promoting IAV polymerase activity (Leader: N. Naffakh, P1a-IP)

Partner P1a-IP developed an original infectious protein complementation assay for the detection of influenza A virus (IAV) polymerase-host protein-protein interactions throughout the viral cycle. This assay was validated using a random set of human proteins and a literature-curated set of human proteins already described as interactors of the polymerase of IAV. It was also used to screen a set of 26 human proteins that are involved in intracellular transport and had not been documented with

respect to their interaction with IAV polymerase. Eight of them were found positive. (*Munier S et al. Molecular and Cellular Proteomics, 2013 oct, 12: 2845-55*).
Further investigations were carried out to characterize some of the 8 IAV polymerase partners that we identified through our protein-protein interaction screen, using co-purification and si-RNA mediated knock-down experiments. P1a-IP particularly focused on DDX19, an RNA helicase of the DEAD-box family (Factor). P1a-IP showed that i) the human RNA helicase DDX19 associates with intronless, unspliced and spliced influenza A virus (IAV) mRNAs and promotes their nuclear export, and ii) the viral polymerase associates with DDX19 in an RNA-independent way. Overall, these results provide a model in which DDX19 is recruited by the IAV polymerase to viral mRNAs in the nucleus of infected cells to enhance their nuclear export. (*Diot C et al. Sci Rep. 2016 Sep 22;6:33763*).
A comparative interactomics approach was developed to analyse the interplay between 558 factors of the human Ubiquitin-Proteasome System (UPS) and the PB2 polymerase subunit derived from seasonal H3N2 and H1N1 viruses, an H1N1pdm virus, or highly pathogenic H1N1 (from 1918) and H7N9 viruses. 80 out of 558 factors of the UPS were selected as potential partners and 42 were further validated, and showed differential interaction with PB2 proteins of distinct origins. These findings demonstrated that our comparative interactomic approach could be used as a proxy to evaluate the potential of highly pathogenic avian IAVs to cause human infection. The functional validation of the UPS factors targeted by PB2, achieved using three strains including the currently circulating $H1N1_{pdm09}$ and H3N2 ones, identified 36 novel host factors regulating IAV infection in a general or a strain-specific manner.
In the frame of a collaboration with Dr Stephen Cusack (EMBL Grenoble), who solved the co-crystal structure of a FluPol bound to a peptide-mimic of the C-terminal domain of the cellular RNA polymerase II (PolII-CTD), P1a-IP used reverse genetics to test the mutations of four PolII-CTD-contacting residues in the PA subunit of the FluPol, in an infectious context. We showed that the mutant viruses were severely attenuated and genetically unstable. Indeed, next generation sequencing of plaque-purified viruses revealed non-synonymous neo-mutations on the PB2 and/or the PB1, NP and M segments. (*Lukarska M et al. Nature, 541, 117–121*).
Finally, in collaboration with Dr. Thibaut Crépin and Rob Ruigrok (IBS, Grenoble), P1a-IP used cell-based and *in vitro* assays to achieve a precise mapping of the protein-protein interaction domains between IAV polymerase and the human RED-SMU1 splicing complex, and the structure of a minimal RED-SMU1 complex was solved. These data open the way to host-directed anti-IAV therapeutic approaches. A manuscript is currently in preparation.

- Task 3.2: Role of the anti-viral innate immunity
Beneficiaries: P1-IP, P5-EDI-IVI, P7-ANSES, P12-INMI, P17-UNIBO - Leader: MR Capobianchi, P12-INMI

Innate immunity plays a very important role in virus-host interaction by modulating the chance of success of the infection and therefore crossing of the species barrier. Several viruses have developed mechanisms able to counteract the innate immunity mechanisms of defence.

. Subtask 3.2.1/Evasion of the host innate immunity by modulation of the type-I IFN pathway (Leader: H. Bourhy, P1b-IP)

A: *Influenza virus* *(P1a-IP and P5-FDEA-IVI)*

Type-I IFN pathway in transfected human cells with IAV variants including H1N1pdm09
Current evidences indicate that the regulatory property of the non-structural NS1 (NS1) protein of influenza A, which modulates via multiple molecular mechanisms both the induction of IFN expression and the function of IFN-activated antiviral effectors, is strain specific. Moreover, its

regulatory feature is also partially responsible for the ease of influenza virus to adapt to a new host and to infect multiple animal species in order to study the capability of different variants of NS1 gene to counteract the interferon system an experimental combined geno-phenotypic approach was developed. It consists to investigate the genetic characteristic of the NS1 coding region of influenza H1N1pdm09 strains isolated from human infections in Rome and analyse the phylogenetic relatedness of sequences obtained and then to develop a phenotypic assay which allows to test the biological effects on the IFN response of those variants related with severe infection. The inhibitory effects displayed by different sub-types of pandemic NS1 protein on the IFN system were evaluated analysing the mRNA expression of several IFN stimulated genes.

B. Lyssavirus (P7b-ANSES; P1a-IP PVP; P1b-IP DyLAH)

A key feature of rabies virus is its stealth, allowing it to spread within the host and escape the immune response. Several mechanisms can be distinguished.

P1a-IP DyLAH identified a novel member of the human NF-κB family, denoted RelAp43, the nucleotide sequence of which contains several exons as well as an intron of the RelA gene. RelAp43 is expressed in all cell lines and tissues tested and exhibits all the properties of a NF-κB protein. it is a class I member of the NF-κB family, able to potentiate RelA-mediated transactivation and stabilize dimers comprising p50. It is also targeted by the matrix (M) protein of lyssaviruses, the agents of rabies, resulting in an inhibition of the NF-κB pathway. The mechanisms involved in RelAp43-M protein interaction. were studied. (*Luco S, PLoS Pathog 2012, 8(12): e1003060; Wiltzer L et al. J Virol. 2012 Sep;86(18):10194-9 ; Ben Khalifa Y et al. Sci Rep. 2016 Dec 21;6:39420*).

Further, monitoring close range interactions, P1b-IP found that RelAp43 plays an important role in the stabilization of the p105-ABIN2-TPL2 complex, which is essential in the regulation of both NF-kB and MAPK pathways, bringing a new insight on the dynamics within the host protein complex. These results were confirmed in living cells and in mice. Overall, these data suggest that rabies virus interference with the p105-ABIN2-TPL2 complex is a cornerstone of its stealth strategy to escape the immune response. A manuscript has been recently submitted on this topic.

The evasion of host innate immunity by RABV, the prototype of the genus *Lyssavirus*, also depends on a unique mechanism of selective targeting of interferon-activated STAT proteins by the viral P-protein. However, the immune evasion strategies of other lyssaviruses, including several lethal human pathogens, was unresolved. We have shown that this mechanism is conserved between the most distantly related members of the genus, providing important insights into the pathogenesis and potential therapeutic targeting of lyssaviruses. P1a in collaboration with external collaborators of PREDEMICS (D. Blondel, N. Ito and G. Moseley) identified a hydrophobic pocket of the P-protein C-terminal domain as critical to STAT-binding/antagonism. This interface was found to be functionally and spatially independent of the region responsible for N-protein interaction, which is critical to genome replication. Based on these findings, the first mutant RABV lacking STAT-association was generated. This mutant was strongly attenuated in brains of infected mice, producing no major neurological symptoms, compared with the invariably lethal wild-type virus. These data represent direct evidence that P-protein-STAT interaction is critical to rabies. (*Wiltzer L et al. J Infect Dis. 2014 Jun 1;209(11):1744-53*).

We then used P and M defective RABV mutants to quantify the immunosubversive effect of both proteins during the infection. All the data collected contribute to understand that the role of P and M proteins is not restricted to one immune pathway and that viral proteins of RABV cooperate to restrain in parallel, as well as sequentially, several signaling pathways involved in innate immune response. A manuscript on this last topic is in preparation.

C. Hepatitis E virus (P7a-ANSES)

In this sub-task, P7a-ANSES aimed to better understand the ability of the polyprotein encoded by ORF1 of HEV to interfere with the type-I IFN system. ORF1 protein consists of several putative functional domains including a methyltransferase (MT), a domain of unknown function (Y), a

papain-like cysteine protease (PLP) and a macro domain. Preliminary results showed that following IFN-β treatment, MT-PLP is able to inhibit the phosphorylation of STAT1 but not STAT2 and that MT-PLP interferes with the nuclear translocation of STAT1. We are now investigating further the mechanisms involved in such inhibition. In parallel, P7a-ANSES is investigating whether the inhibitory effect on the IFN-I response pathway of the different domains of ORF-1 depends on the HEV genotype involved (HEV-1 vs HEV-3). In the future, this study might then contribute to identify viral and cellular factors involved in the interspecies transmission of HEV.

D. Japanese encephalitis virus and other related arboviruses (e.g. Zika virus) (P1a-IP-ERI-GA); P5-EDI-IVI, P12-INMI)

Vγ9Vδ2 T cells inhibit WNV by cytolytic and non-cytolytic mechanisms

West Nile virus (WNV) causes a severe central nervous system infection in humans, primarily in the elderly and immunocompromised subjects. Human γδ T-cells play a critical role in the immune response against viruses, and studies of WNV meningoencephalitis in laboratory mice described a role of γδ T-cells in the protective immune response. Aim of this study was to analyze the cytolytic and non-cytolytic antiviral activity of human Vδ2 T-cells against WNV replication. Altogether, our results provide insight into the effector functions of human Vδ2 T-cells against WNV. The possibility to target these cells by zoledronic acid, a commercially available drug used in humans, could potentially offer a new immunotherapeutic strategy for WNV infection. (*Agrati C et al. New Microbiol. 2016 Apr;39(2):139-42*).

West Nile Virus and the innate immunity

Type III interferons (IFN-lambda) are the most recently discovered members of IFN family. Synergism between different IFN types is well established, but for type I and type III IFNs no conclusive evidence has been reported so far. Possible synergism/antagonism between IFN-alpha and IFN-lambda in the inhibition of virus replication (EMCV, WNV lineage 1 and 2, CHIKV and HSV-1), and in the activation of intracellular pathways of IFN response (MxA and 2'-5' OAS) was evaluated in different cell lines (Vero E6, A549 and Wish cells). Elucidating the interplay between IFN-alpha and -lambda may help to better understand innate defence mechanisms against viral infections, including the molecular mechanisms underlying the influence of IL-28B polymorphisms in the response to HCV and other viral infections. (*Bordi L et al. J Biol Regul Homeost Agents. 2013 Oct-Dec;27(4):1001-9*).

Human Endometrial stromal cells and Zika replication

Zika virus (ZIKV) is a recently re-emerged flavivirus transmitted to humans by mosquito bites but also from mother to fetus and by sexual intercourse. P12-INMI here showed that primary human endometrial stromal cells (HESC) are highly permissive to ZIKV infection and support its in vitro replication. This study showed that endometrial stromal cells, particularly if decidualized, likely represent a crucial cell target of ZIKV reaching them, either via the uterine vasculature in the viremic phase of the infection or by sexual viral transmission, and a potential source of virus spreading to placental trophoblasts during pregnancy. (*Pagani I et al. Sci Rep. 2017 Mar 10;7:44286*).

Human Zika infection induces a reduction of IFN-γ producing CD4 T cells and a parallel expansion of effector Vδ2 T cells

The definition of the immunological response to Zika (ZIKV) infection in humans represents a key issue to identify protective profile useful for vaccine development and for pathogenesis studies. We studied phenotype and functionality of T-cells in 7 patients with acute ZIKV infection and compared them with Dengue (DENV)-patients and healthy donors. This study provides new knowledge on the immune response profile during self-limited infection that may help in vaccine efficacy definition, and in identifying possible immuno-pathogenetic mechanisms of severe infection.

E. Ebola virus (P12-INMI)

Subversion of immune response during EBOV infection
Data on immune responses during human Ebola virus disease (EVD) are scanty, due to limitations imposed by biosafety requirements and logistical issues to perform studies on sequential samples during the course of disease. A sustained activation of T-cells was recently described but functional studies during the acute phase of human EVD are still missing. Two EVD patients were sampled sequentially from soon after symptom onset until recovery. T-cell subsets kinetics, activation, exhaustion markers and functionality were analyzed by flow cytometry and ELISpot assay. An early and sustained decrease of CD4 T-cells was seen in both patients, with an inversion of the CD4/CD8 ratio that was reverted during the recovery period. In parallel with the CD4-T-cell depletion, a massive T-cell activation occurred. Functional studies showed a dramatic T cell anergy and an increased expression of exhaustion markers. The immunosuppression was paralleled by EBV reactivation in both patients. **(***Agrati C et al. Cell Death Dis. 2016 Mar 31;7:e2164*).

. Subtask 3.2.2/Role of innate immunity receptor polymorphisms in restricting the host range (Leader: V. Sambri, P17-UNIBO)

West Nile virus (WNV) is a mosquito-borne, RNA virus belonging to the genus Flavivirus. Despite the majority of WNV infections in humans are asymptomatic (80%), some infected patients develop West Nile fever (WNF, 20%) and a small subset (<1%) develop severe neuroinvasive disease (WNND), which may include meningitis, meningoencephalitis and acute flaccid paralysis. Considering that only a small proportion of people infected with WNV develops severe disease and that risk factors, besides older age, are not well defined, there is a strong rationale to suspect a genetic predisposition to severe clinical forms of WNV infection.The results obtained, even if preliminary, suggest that TLR8 variants could have functional relevance in the setting of WNV infection for assessing individual patient's risk profiles, could help to elucidate pathogenetic pathways of viral infection, and could have relevance for a future potential use of TLR ligands in the course of WNV infection.

- Task 3.3: Mechanisms of broad adaptive protection
Beneficiaries involved: P1-IP, P2-IZSVe, P4-AMU, P5-EDI-IVI, P7-ANSES, P12-INMI
Leader: A. Summerfield, P5-EDI-IVI

. Subtask 3.3.1/Impact of host immune response on virus evolution (Leader: A. Summerfield, P5-EDI-IVI)

Evolution of an H3N6 low pathogenicity avian influenza (LPAI) virus isolated from waterfowl, after its replication in either naïve or immunized ferrets
P2-IZSVe contributed to the implementation of a whole-genome sequencing approach to study the evolution of an H3N6 low pathogenicity avian influenza (LPAI) virus isolated from waterfowl, after its replication in either naïve or immunized ferrets. In the study design, the replication of a H3-subtype avian strain in ferrets previously immunized with a homosubtypic seasonal H3N2 virus had the goal to reproduce the emergence and circulation of a virus in the human population, to understand whether pre-existing immunity can drive and facilitate the selection of adaptive mutations. The results suggested that pre-existing immunity to a homologous subtype might facilitate the adaptation of an emerging avian influenza virus to the ferret host. In light of these results, avoiding contact with poultry and wild birds is advisable as ferrets are highly susceptible to wholly AIV and can select for adaptive mutations to humans and swine, like the G228S. These results require further investigation to understand whether this rapid adaptation is strain specific or it might occur also for other H3-subtype avian strains. Unraveling the mechanism behind this phenomenon could help design and assessing better pre-pandemic AIV vaccines.

Cross-reactive humoral and cell-mediated immunity to H3N2v influenza strain
Human cases of infection due to a novel swine-origin variant of influenza A virus subtype H3N2 (H3N2v) have recently been identified in the United States. Aim of this study was to assess humoral and cell-mediated cross immune responses to H3N2v in immuno-competent (healthy donors, HD) and immuno-compromised hosts (HIV-infected subjects) never exposed to H3N2v influenza strain. Overall, a high prevalence of HD and HIV patients showing cross-reactive immunity against two H3N2v strains was observed, with a slightly lower proportion in HIV persons. Other studies focused on HIV subjects at different stages of diseases are needed in order to define how cross immunity can be affected by advanced immunosuppression. **(***Agrati C et al. PLoS One. 2014 Aug 27;9(8):e105651*).

. *Subtask 3.3.2/Contribution of opsonising antibodies to (cross)-protection and identify the viral targets and cellular and humoral components (IAV, JEV and related flavivirus in the pig model) (Leader: A. Summerfield, P5-EDI-IVI)*

Partner P5-EDI-IVI has investigated in detail if opsonizing but not neutralizing antibodies contribute to protective immunity or could rather enhanced disease in the pig model. Our data show no evidence for disease-enhancing activities with the vaccine/challenge virus pair selected and using virus replicon particles as a vaccine delivery system. It rather appeared that the virus specific multifunctional CD4+ T helper cells alone or combined with opsonizing antibodies mediate protective effects. These results are relevant for the design of broadly reactive influenza virus vaccines. Using virus replicon particles, we also evaluated the protective value of nucleoprotein in the swine model and to our surprise found that immunity against this protein was not protective but rather enhanced inflammatory responses in the lung without preventing virus replication. In the frame of this work, we also identified a novel functional property of antibodies against NP. Anti-NP antibodies are able to mediate potent antiviral interferon type I responses by complexing ribonucleoproteins for delivery to plasmacytoid dendritic cells. It is currently unclear if such responses are for the benefit of the host. At least our pig model implies rather negative effects of such responses during early phases of the infection. (*Ricklin ME et al. Front Immunol. 2016 Jun 30;7:253*).
P5-EDI-IVI also found a JEV vaccine based on a lentiviral vector expressing prM and E of JEV to induce high levels of opsonizing antibodies that strongly enhanced macrophage infection rather than neutralizing the virus. Considering these findings, we evaluated the protective value of such as vaccine and found it to confer protection against heterologous challenge. These results are good news has they imply that at least in this model such antibodies do not appear to be a concern. (*García-Nicolás O et al. Viruses. 2017 May 22;9(5)*)
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. *Subtask 3.3.3/Development of intervention strategies against JEV (Leader: R. Charrel, P4-AMU)*

Two novel intervention strategies against JEV were developed to be applied in pigs. One is the abovementioned lentivirus-based vaccine that was found to induced high levels of neutralising and opsonizing antibodies and mediate protection against heterologous challenge. It was developed in a cooperation between IP and EDI-IVI. A utility patent application entitled « *lentiviral vector-based japanese encephalitis immunogenic composition* » has been submitted for an international protection of the data under the ownership of the Institut Pasteur.
The second vaccine developed used a re-encoding strategy to attenuate JEV. This vaccine also induced protective immunity against heterologous challenge with a JEV from a different genotype. Further experimentations are required to determine if the degree of attenuation is sufficient. Considering the importance of pigs as amplifying host and possibly also maintenance host (see Subtask 3.1.), such vaccines might be very valuable to control JEV in future. (*de Wispelaere M et al. PLoS Negl Trop Dis. 2015 Oct 5;9(10):e0004081*).

Work Package 4: "Sharing platforms"

WP Leader: Alan HAY [Beneficiary 19 – Freunde von GISAID e.V. (GISAID)]

The objective of this WP was to apply the highly successful mechanism developed by GISAID (the Global Initiative on Sharing All Influenza Data) for influenza more broadly to other viruses, in particular those of special interest to the current programme, Flaviviruses, Lyssaviruses and Hepatitis E viruses, in addition to influenza viruses.
This has been accomplished by developing a software application to accommodate an increased quantity and variety of data, compared to the original EpiFlu^{TM} database, readily amenable for adaptation to data of the different types of viruses and linking of advanced analytical tools. To this end an advanced version (v2.0) of the EpiFlu database was built as the basis for subsequent development of the other databases.
The v2.0 platform incorporates several features, developed in collaboration with researchers outside the PREDEMICS project, superior to the original database, including: an automatic annotation (and curation) pipeline to facilitate uploading and standardisation of the genetic sequence data, and import of data from other databases, e.g. INSDC; a tool to automatically designate clades of H5 viruses; a phylogenetic tool incorporating identification and annotation of the amino acid sequence changes occurring during virus evolution; a *FluSurver* tool which assists interpretation of the significance of such changes, in terms of their functional consequences, the frequency and geographical distribution of viruses with the mutations, together with links to published data.
This system will be used as the basis for developing software to link mutations of three non-influenza virus groups to phenotypic data in the literature. While most features of the platform are functional and a developmental version has been used for hands-on training in bioinformatics workshops and demonstrations, some essential features are still undergoing tests before the platform goes online for general use. A further feature will be added to allow for a temporary publishing embargo on unpublished data to encourage more rapid sharing of data.

Concomitant with development of the database software, schemes for software-based curation and annotation of genetic sequence data for the other three viruses, Flaviviruses, Lyssaviruses (extended to include Ebola virus) and Hepatitis E viruses, were developed together with other partners, based on the concepts used previously for curation and annotation of influenza data. A server was provided for annotation of the sequences, as a web service available for all PREDEMICS members.

Having demonstrated the functionality of the software on the v2.0 influenza platform, copies of the software were uploaded to the Oracle Cloud under an agreement with Institut Pasteur for development of the other three individual virus databases.

Work Package 5: "Modelling of ecological, genetic and anthropological data"

WP Leader: Christl DONNELLY [Beneficiary 6 – Imperial College of Science, Technology and Medicine (IMPERIAL)]

A wide range of research has been undertaken within WP5. It would be impossible to describe it all here. Thus, we have chosen to highlight a few of the more prominent themes relying to the modelling and analysis of Ebola, Middle East Respiratory Syndrome (MERS), Yellow Fever (YF) and rabies. Much of this work was responsive, to the largest Ebola epidemic ever observed, to recent large Yellow Fever epidemics, and to the newly identified MERS coronavirus (CoV). The modelling work also addressed high priority ongoing threats, including rabies and influenza.

Case Study: Outbreak of Ebola in West Africa

On March 23, 2014, the World Health Organization (WHO) was notified of an outbreak of Ebola virus disease (EVD) in Guinea. On August 8, the WHO declared the epidemic to be a "public health emergency of international concern." The epidemic was focussed mainly in Guinea, Liberia and Sierra Leone and peaked in these countries in early autumn 2014. As part of the WHO Ebola Response Team, from August 2014 Imperial College London team members regularly received line-list data describing the outbreak in West Africa. We provided the WHO and partners with 37 reports exploring the data, on topics such as Case Fatality Ratio across different age groups, to exposure risks from Ebola. We followed up our initial analysis of the epidemic with an update paper and an analysis of Ebola among children. We also compared Ebola among men and women and undertook a detailed analysis of the exposure patterns of Ebola cases. A modelling analysis of the impact that rapid diagnostic tests might have in managing Ebola epidemics was published in a special *Nature* supplement on rapid diagnostics. Also published is a review of epidemiological parameters from past Ebola outbreaks which collates these details in one place for the first time.

For the first time, large urban centres such as Conakry, the capital of Guinea, were affected. An observational study of patients with Ebola virus disease in three regions of Guinea, including Conakry, aimed to map the routes of transmission and assess the effect of interventions. Between Feb 10, 2014, and Aug 25, 2014, we obtained data from the linelist of all confirmed and probable cases in Guinea (as of Sept 16, 2014), a laboratory database of information about patients, and interviews with patients and their families and neighbours. With this, we mapped chains of transmission, identified which setting infections most probably originated from (community, hospitals, or funerals), and computed the context-specific and overall R_0s.

Of 193 confirmed and probable cases of Ebola virus disease reported in Conakry, Boffa, and Télimélé, 152 (79%) were positioned in chains of transmission. Health-care workers contributed little to transmission. In March, 2014, individuals with Ebola virus disease who were not health-care workers infected a mean of 2.3 people (95% CI 1.6–3.2): 1.4 (0.9–2.2) in the community, 0.4 (0.1–0.9) in hospitals, and 0.5 (0.2–1.0) at funerals. After the implementation of infection control in April, the R_0 in hospitals and at funerals reduced to lower than 0.1. In the community, the R_0 dropped by 50% for patients that were admitted to hospital, but remained unchanged for those that were not. In March, hospital transmissions constituted 35% (seven of 20) of all transmissions and funeral transmissions 15% (three); but from April to the end of the study period, they constituted only 9% (11 of 128) and 4% (five), respectively. 82% (119 of 145) of transmission occurred in the community and 72% (105) between family members.

In Conakry, interventions had the potential to stop the epidemic, but reintroductions of the disease and poor cooperation of a few families led to prolonged low-level spread, showing the challenges of Ebola virus disease control in large urban centres. Monitoring of chains of transmission is crucial to assess and optimise local control strategies for Ebola virus disease.

The case fatality ratio (CFR) of Ebola virus disease (EVD) can vary over time and space for reasons that are not fully understood. We investigated whether viremia in EVD patients may be used to

evaluate baseline EVD CFRs. We analysed the laboratory and epidemiological records of patients with EVD confirmed by reverse transcription PCR hospitalized in the Conakry area, Guinea, between 1 March 2014 and 28 February 2015. We used viremia and other variables to model the CFR. Viremia in EVD patients was a strong predictor of death that partly explained variations in CFR in the study population.

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Case Study: Middle East Respiratory Syndrome [MERS]

The continuing circulation of MERS-CoV in the Middle East makes the international dissemination of the disease an ongoing threat. To inform risk assessment, we investigated the spatiotemporal pattern of MERS global dissemination and looked for factors explaining the heterogeneity observed in transmission events following importation. We reviewed imported MERS cases worldwide. We modelled importations in time based on air travel combined with incidence in Middle East. We used the detailed history of MERS case management after importation (time to hospitalization and isolation, number of hospitals visited,…) in logistic regression to identify risk factors for secondary transmission. We assessed changes in time to hospitalization and isolation in relation to collective

and public health attention to the epidemic, measured by three indicators (Google Trends, ProMED-mail, Disease Outbreak News). Modelled importation events were found to reproduce both the temporal and geographical structure of those observed – the Pearson correlation coefficient between predicted and observed monthly time series was large ($r = 0.78$, $p < 0.001$). The risk of secondary transmission following importation increased with the time to case isolation or death (OR = 1.7 p = 0.04) and more precisely with the duration of hospitalization (OR = 1.7, p = 0.02). The average daily number of secondary cases was 0.02 [0.0,0.12] in the community and 0.20 [0.03,9.0] in the hospital. Time from hospitalisation to isolation decreased in periods of high public health attention (2.33 ± 0.34 vs. 6.44 ± 0.97 days during baseline attention). Countries at risk of importation should focus their resources on strict infection control measures for the management of potential cases in healthcare settings and on prompt MERS cases identification. Individual and collective awareness are key to substantially improve such preparedness.

With more than 1,700 laboratory infections reported to the WHO, MERS-CoV remains a significant threat for public health. However, the lack of detailed understanding of the modes of transmission from the animal reservoir and the paradoxical and sometimes inconsistent data on human-to-human transmission mean that key drivers of MERS-CoV epidemics remain difficult to assess. In collaboration with the Ministry of Health of the Kingdom of Saudi Arabia (KSA) and Johns Hopkins University, we quantified the determinants of MERS-CoV transmission from the detailed epidemiological records of 681 MERS-CoV cases detected by KSA, January 2013 to July 2014. We developed mathematical and statistical models to assess how infections from the animal reservoir, different levels of mixing and heterogeneities in transmission contributed to the build-up of MERS-CoV epidemics in KSA. We estimate that 12% (95% CI: 9%, 15%) of cases were infected from the reservoir, the rest via human-to-human transmission in clusters (60%; CI: 57%, 63%), within (23%; CI: 20%, 27%), or between (5%; CI: 2%, 8%) regions. The reproduction number at the start of a cluster was 0.45 (CI: 0.33, 0.58) on average, but with large SD (0.53; CI: 0.35, 0.78). It was >1 in 12% (CI: 6%, 18%) of clusters but fell by approximately one-half (47% CI: 34%, 63%) its original value after 10 cases on average. The ongoing exposure of humans to MERS-CoV from the reservoir is of major concern, given the continued risk of substantial outbreaks in health care systems.

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Case study: Yellow Fever

Yellow fever is a vector-borne disease affecting humans and non-human primates in tropical areas of Africa and South America. While eradication is not feasible due to the wildlife reservoir, large scale vaccination activities in Africa during the 1940s to 1960s reduced yellow fever incidence for several decades. However, after a period of low vaccination coverage, yellow fever has resurged in the continent. The method described in Garske et al. (2014) has been used by the Global Alliance for Vaccines and Immunization (GAVI Alliance) to estimate the potential impact of future vaccination campaigns.

Since late 2015, an epidemic of yellow fever has caused more than 7334 suspected cases in Angola and the Democratic Republic of the Congo, including 393 deaths. We sought to understand the spatial spread of this outbreak to optimise the use of the limited available vaccine stock. We jointly analysed datasets describing the epidemic of yellow fever, vector suitability, human demography, and mobility in central Africa to understand and predict the spread of yellow fever virus. We used a standard logistic model to infer the district-specific yellow fever virus infection risk during the course of the epidemic in the region. The early spread of yellow fever virus was characterised by fast exponential growth (doubling time of 5–7 days) and fast spatial expansion (49 districts reported cases after only 3 months) from Luanda, the capital of Angola. Early invasion was positively correlated with high population density (Pearson's *r* 0·52, 95% CI 0·34–0·66). The further away locations were from Luanda, the later the date of invasion (Pearson's *r* 0·60, 95% CI 0·52–0·66). In a Cox model, we noted that districts with higher population densities also had higher risks of sustained transmission (the hazard ratio for cases ceasing was 0·74, 95% CI 0·13–0·92 per log-unit increase in the population size of a district). A model that captured human mobility and vector suitability successfully discriminated districts with high risk of invasion from others with a lower risk (area under the curve 0·94, 95% CI 0·92–0·97). If at the start of the epidemic, sufficient vaccines had been available to target 50 out of 313 districts in the area, our model would have correctly identified 27 (84%) of the 32 districts that were eventually affected. Our findings show the contributions of ecological and demographic factors to the ongoing spread of the yellow fever outbreak and provide estimates of the areas that could be prioritised for vaccination, although other constraints such as vaccine supply and delivery need to be accounted for before such insights can be translated into policy.

Our activities in yellow fever have expanded recently, in part thanks to additional funding from the Gates foundation catalysed by our earlier work. We continue to work closely with WHO and Gavi, the Global Vaccine Alliance, on estimating the burden of YF across Africa and the impact of vaccination. Furthermore, we have been supporting WHO in the response to the 2016 YF outbreak in Angola and the DRC, performing real-time epidemiological analyses and developing frameworks for prioritising districts for vaccination in the context of global vaccine shortages, to inform novel dose-sparing strategies. As part of this support we have provided 7 reports to WHO between April and June, and staff have visited WHO in June 2016 for two weeks to provide further in-depth support and inform the vaccination strategies to curb the ongoing outbreak. We are also informing the EYE (Elimination of Yellow Fever epidemics) strategy, which is a long-term plan to increase YF vaccination coverage across the endemic areas in Africa, by analysing YF transmission intensity and current population-level vaccination coverage to estimate populations at risk and prioritise districts and countries for vaccination. As part of this we have developed an online tool to visualise the vaccination coverage across Africa (https://polici.shinyapps.io/yellow_fever_africa/).

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Case study: Rabies

Rabies is a fatal zoonosis characterized by a complex epidemiological situation which remains a serious public health problem in developing countries. Rabies incidence is largely attributed to the growth of domestic dog population considered as the most important vector for human exposure. Phylogenetic and virological analysis of isolates collected in Bangui, the capital city of the Central

African Republic, indicate the presence of sequentially circulating subtypes and a reproductive number R_0 close to one. None of these subtypes seems to persist in the sole population of Bangui and mechanisms underlying the virus persistence are still unknown. Here we investigate two factors: spatial fragmentation of the host population and heterogeneous incubation period. To study the role and interplay of these factors on the rabies virus epidemiology, we introduced a stochastic metapopulation epidemic model, where dog settlements are inferred from human demographic data (see mapping method in Task 5.2) incubation and infectious periods follow realistic data-driven distributions. By exploring different epidemic scenarios, we found that the virus can persist even for very low transmissibilities maintaining a stable dog population, and producing invasion cycles in agreement with empirical observations. Interestingly, no persistence would be observed for the same parameters once an exponentially distributed incubation periods is considered. In addition, no persistence is observed if a spatial explicit structure for the domestic dog population is not considered.
In order to validate the framework developed we compared the dominant oscillation periods obtained empirically in Bangui with the corresponding result obtained in our simulations. Our findings show that the best agreement with data is obtained for an R_0 value 1.03 with an annual birth rate of the domestic dog population equal to 1.22 dogs/year.

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Case study: Influenza
We developed an approach to obtain unbiased estimates of R_0, even when the temporal trend in spillover (animal-to-human) exposure was not fully known, so long as the serial interval of the infection and the timing of a sudden drop in spillover exposure were known (e.g. day of market closure). Applying our method to data from the three largest outbreaks of influenza A/H7N9 outbreak in China in 2013, we found evidence that human-to-human transmission accounted for 13% (95% credible interval 1%–32%) of cases overall. We estimated R_0 for the three clusters to be: 0.19 in Shanghai, 0.29 in Jiangsu and 0.03 in Zhejiang. If a reliable temporal trend for the spillover hazard could be estimated, for example by implementing widespread routine sampling in sentinel markets, it should be possible to estimate sub-critical values of R_0 even more accurately. Should a similar strain emerge with $R_0>1$, these methods could give a real-time indication that sustained transmission is occurring with well-characterised uncertainty.
It is important to elucidate the stringency of bottlenecks during influenza transmission to shed light on mechanisms that underlie the evolution and propagation of antigenic drift, host range switching or drug resistance. The virus spreads between people by different routes, including through the air in droplets and aerosols, and by direct contact. Transmission through the air imposed a tight bottleneck since most recipient animals became infected by only one virus. In contrast, a direct contact transmission chain propagated a mixture of viruses suggesting the dose transferred by this route was higher. These data imply that transmission events with a looser bottleneck can propagate minority variants and may be an important route for influenza evolution.

- Kucharski A, Mills H, Pinsent A, Fraser C, Van Kerkhove M, Donnelly CA, Riley S. *PLOS Currents Outbreaks*. 2014 Mar 7. Edition 1.
- Frise R, Bradley K, van Doremalen N, Galiano M, Elderfield RA, Stilwell P, Ashcroft JW, Fernandez-Alonso M, Miah S, Lackenby A, Roberts KL, Donnelly CA, and Barclay WS. Scientfic Reports 6 article number 29793, 2016. doi: 10.1038/srep29793

These case studies demonstrate the breadth and depth of the work undertaken. Many publications resulted, often in the most prestigious journals. The results obtained gave biological and epidemiological insights relevant to control, improved situational awareness and tested hypotheses.

Work Package 6: "Training"

WP Leader: Jean-Pierre KRAEHENBUHL [Beneficiary 15 – Fondation Health Sciences e-Training (HSeT)]

The training program, dedicated to Isabel Minguez-Tudela, aimed at fostering exchange of ideas, information and staff members with an interdisciplinary approach, involving scientists from both animal and human health sectors, and promote links with international organizations in Europe.
It comprised a **Scientists exchange program** that proved a very efficient means for transfer of methodolologies and exchange of ideas. Thirteen scientists, including one from Uganda, completed their training in 5 PREDEMICS partners' laboratories and two laboratories outside the consortium.
PREDEMICS provided **training opportunities** with the organization of two training courses directly related to zoonotic features of viruses studied in PREDEMICS.
The first one on *zoonotic features of viral infections* (April 8th-11th, 2014, Rome, Italy) was attended by 26 trainees from 16 countries. Pre-workshop activities, including individual and team activities, had to be completed before the face-to-face course. All trainees passed a final exam.
The second one on *integrated view of emerging zoonotic viral infections* (September 19th-22nd, 2016, Paris, France) was attended by 9 trainees, of which 5 were from the ANTIGONE consortium. Before the face-to-face meeting the trainees had to i/ learn how to write a grant following an online training application, and ii/ prepare a poster on their work.
The PREDEMICS consortium also sponsored and organized the 17th (27-31 August 2012; 50 attendees) and 19th (7-12 September, 2014; 75 attendees) *International BioInformatics Workshop on Virus Evolution and Molecular Epidemiology*, in Belgrade, Serbia and Rome, Italy, respectively. These workshops, included two parallel modules ('Phylogenetic inference' and 'Evolutionary Hypothesis Testing'), an additional two modules of choice, and keynote lectures. The workshop in Rome was extended with a module on Big data analysis / Next Generation Sequencing analysis.
In addition, four **hands-on courses and workshops** were organized.
Three workshops on the control and surveillance of rabies were organized in close collaboration with WHO, the University of Lausanne, Switzerland, and the International network of Institut Pasteur, and with the participation of FAO and Global Alliance for Rabies Control (GARC). They took place in: Dakar, Senegal (3-14 December 2013; 31 trainees from 16 African countries); Phnom Penh, Cambodia (26 October-7 November 2015; 19 trainees from 9 Asian countries); Yaoundé, Cameroun (25 October-5 November 2016; 26 trainees from 12 English speaking African countries).
For these courses, we used an approach called customized online training, (COLT) described in (Bourhy et al. *Bull World Health Organ. 2015 Jul 1; 93(7): 503–506),* which focuses on small sets of trainees and is designed for situations where acquisition of skills and direct training by experts are needed. Pre-workshop activities took place during 4 months and the 2-week face-to-face workshops were followed by post-workshop activities that resulted in the production of guidelines on the control and surveillance of rabies adopted by many countries.
A fourth workshop focused on Zoonotic disease at the human-wildlife-livestock interface was organised by (P6-IMPERIAL) in Tanzania (2-5 February 2016; 25 participants from 9 African countries. Attendees included academics, veterinarians, and individuals from NGOs, research institutes, WHO, CDC and government units. The aim was to strengthen strategic interdisciplinary partnerships to improve the understanding and control of zoonotic diseases. The course included plenary talks and breakout group activities to burning research questions and control needs for Rabies, Livestock Zoonoses, Zoonoses & the Environment and Vector-Borne Diseases.
Training and dissemination was also fostered through the **PREDEMICS website**. (http://predemics.biomedtrain.eu) created by P15-HSeT and equipped with a learning management system to follow the activities of the trainees, to record the self-assessment and the exams, and to animate individual and team activities via a forum. The data management system, also developed by HSeT, made available content prepared with other HSeT partners.

1.4 – The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

Work Package 1: "Environment, ecological and anthropological factors"
WP Leader: Jean-Claude MANUGUERRA [Beneficiary 1 – Institut Pasteur (IP)]

For the whole duration of the PREDEMICS project, infectious diseases have continued to emerge or re-emerge: Ebola Virus Disease in 2013/2015 spread in 3 countries in West Africa and Chikungunya in 2013/2014, then Zika in 2013/2016 caused outbreaks in the Pacific Ocean and then in Latin America including territories belonging to EU member states. These emerging infectious diseases of humans had a zoonotic origin as anticipated when designing this project. During the same time, major outbreaks of highly pathogenic avian influenza struck some EU countries, sometimes very severely and repeatedly as in France where, in 2016 alone, 5 episodes due to H5N1, H5N2, H5N9 and H5N8 viruses were recorded. This had a very high economic burden and the threat is persistent in spite of the biosecurity measures taken.

Through this project, the factors involved in the emergence of human pathogens were studied for influenza A virus, Japanese encephalitis virus and related flaviviruses such as Zika and West Nile viruses, hepatitis E virus and lyssavirus in different types of animal hosts (pigs, birds, canids and even horses). The impact of PREDEMICS in this regard might become considerable in Europe. For WP1, the impact is related to the development of a novel, high throughput serological platform for flaviviruses (more than 10 viruses) and alphaviruses such as Chikungunya virus. This serological platform proved very useful during the Zika outbreak for the follow up of pregnant women and is the basis of current research projects such as one in Columbia and one in 10 countries around the globe for mathematical modelling of the epidemiology of Zika and other arboviruses in humans.

Also, the development of serological and molecular assays for the detection of HEV in humans, animals and the environment considerably advanced our understanding of the prevalence and circulation of HEV in Europe and underlined the surveillance of human wastewaters as effective means of evaluation of the circulation of HEV in humans.

WP1 also brought fundamental knowledge by identifying molecular determinants of influenza virus survival outside the host, by determining the factors involved in the persistence of viruses within the hosts (individuals, colonies…) as found for EBLV-1 lyssavirus in bats, or by highlighting the role of geographically-based genetic differentiation of animal populations (e.g. foxes) in the spread of infectious diseases. Such knowledge will be useful to implement more effective control measures.

The data generated through WP1 are or will be made available to the scientific community by their publication in peer reviewed scientific journals, scientific conferences and seminars. They should also reach the general public by dedicated conferences or through the media (radio, television, web…)

The results of WP1 should benefit to the general public through advice and recommendations to public health managers and government policy makers, such as those presented in deliverables (e.g. D1.4 *Strategies for the management of host populations* or D1.10 *Identification of risk factors for human infection, maps of health risk assessment and updated maps of emerging infectious diseases "hotspot" by disease*).

As in the other WPs, the diversity of specialists among the collaborators is remarkable since veterinarians, physicians, ecologists, statisticians, computational biologists, and specialists in RNA viruses worked together. WP1 results combined the environmental, humans and animal factors illustrating the One Health Approach of the whole project.

Work Package 2: "Virus and host evolutionary dynamics"

WP Leader: Philippe LEMEY [Beneficiary 3 – Katholieke Universiteit Leuven (K.U. Leuven]

WP2 has contributed to the overarching objective of PREDEMICS through the achievement of three main goals: 1) characterizing the molecular epidemiology of IAV, HEV, LYS, JEV and related flaviviruses (JEVr), 2) elucidating the patterns and variation of within-host viral diversification according to host species and upon cross-species transmission or during the process of adaptation to a new target host, and 3) developing new molecular models and inference tools to study viral evolution and epidemiology.

Molecular epidemiological investigations are crucial to characterize the emergence and spread of new pathogens and to assess the risk of cross-species transmission of pathogens from animal reservoirs to humans as well as between different animal species. This is elegantly illustrated by the work on HEV, which revealed genotype 3 as a commonly circulating variant in Europe with frequent zoonotic spread and with domestic pigs representing the main vector for human infections. This highlights the need for a `One Health' approach to prevent HEV circulation. Transmission through food products was revealed to be a common route of infection, which can have important repercussions for the food production industry. The work also highlights the need for future studies to identify possible virulence factors and host determinants of circulating HEV lineages.

The molecular epidemiological work on rabies has also resulted in important epidemiological insights with practical public health implications. For example, the finding of metapopulation dynamics that maintain periodic epidemic waves on a restricted spatial scale are key to informing the design of effective control measures, which could massively reduce costs related to human bites management and deaths. By merging epidemiological time series analysis and phylogenetics, this work made an interdisciplinary connecting with the dynamical modelling research in WP5. Other findings for fox rabies for example highlighted the need for vigilance and early warning systems to react to trans-boundary diseases.

The research on JEV and JEVr has identified important factors leading to emergence and dispersal of these viruses and provided an assessment of the likelihood of their emergence and establishment in Europe. For JEV specifically, an oral transmission route in pigs was demonstrated which needs to be taken into account in risk assessment of JEV emergence in Europe, where pigs are now an important part of the economy. Other research showed the potential of concrete action based on molecular epidemiological insights. Specifically, the increasing circulation of JEV genotype 5 motivated the production of genetically modified live attenuated flaviviruses that could be used for vaccine production. JEV vaccines may prove an important instrument in reducing the epidemic threat of JEV in Europe. In terms of risk of emergence in Europe, WNV has shown the capability of causing epidemics but has not proven to be a major avian pathogen in Europe. In contrast, increasing USUV infections have been reported and this virus could effectively displace WNV in order of importance in the context of pathogenicity in Europe. The approaches used to gain insights into JEVr were also deployed to study two human pathogenic arboviruses, chikungunya (CHIKV) and Zika (ZIKV) virus, which emerged in the Americas during the project. Such research is needed in an immediate response to a public health threat with severe socio-economic implications.

The molecular epidemiological research on avian influenza illustrates the efforts of PREDEMICS to develop more efficient monitoring and control programs that are crucial to mitigate the economic burden of such outbreaks. It was for example demonstrated that a delay of even a few days in disease detection and stamping out in poultry farms could lead to a chaotic evolution of the virus population and to the emergence of viruses with devastating pathogenic properties for poultry,

highlighting the need to implement prompt and strict control strategies. Our evolutionary analyses showed that the sudden appearance of a highly pathogenic influenza epidemic in poultry was very likely the consequence of a direct or indirect contact with wild birds migrating from regions where highly pathogenic influenza viruses are endemic, proving the need to strengthen the existing biosecurity measures in poultry farms and to increase our knowledge about waterfowl annual migration. Thanks to the project, a wet and dry laboratory strategy was developed to implement a real time and in-depth characterization of the evolutionary dynamics of avian influenza viruses. This provided European and international decision-making organizations involved in animal and public health with the necessary support to reconstruct the transmission dynamics of the recent AI outbreaks and to define their zoonotic potential. Genome sequences of more than 200 viruses were generated and made available in public databases. In this respect, a strong interaction was established through the GISAID platform within the WP7 activities. Results obtained from our work were disseminated to the scientific community and policy makers through several oral and poster presentations to national and international conferences and workshops and 7 publications in international peer-reviewed journals, 6 of them freely accessible on-line.

In addition to ZIKV and CHIKV, on which PREDEMICS has focused despite the fact they were not part of the original objective, PREDEMICS researchers have also performed crucial molecular epidemiological research on the MERS-CoV and Ebola outbreaks, further illustrating important contributions in response to both global and regional public health threats.

Although epidemiological analyses capture long-term evolutionary dynamics of viral pathogens and identify the displacement of genotypes by new variants, they do not necessarily identify the evolutionary intermediates occurring within or between hosts. Elucidating such 'missing links' that help explain the circulating strains, has been the focus of another task on within-host viral diversification and adaptation. This work has applied next generation sequencing (NGS) to influenza and lyssaviruses to explore the mutational distribution of the mutant spectrum and to identify the most prominent minority genotypes that could affect the evolutionary trajectory of a virus, e.g. following transmission to new hosts. Unravelling the molecular determinants of mutational spectra and viral fitness in various environmental conditions is crucial for surveillance. Further work on this is needed in support of efforts to predict strain emergence dominance in epidemiological settings. Having evolved into a widely adopted technology that can readily generate within-host diversity, the work in this task is now stimulating model development (cf. task described below) to integrate such information in epidemiological reconstructions.

The final WP2 task consisted of developing molecular evolutionary models and inference tools in support of the previous tasks and in support of the research community in general. While the focus was on testing hypothesis of cross-species transmissions and integrating stochastic processes of sequence and trait evolution, many of the developments transcended these objectives and applications have been explored beyond the viruses we focused on in PREDEMICS and even beyond the field of life sciences. Moreover, a specific application of phylogenetic diffusion models to influenza has reached out to WP5, and further reinforced the connection between both these WPs. All the developments have been implemented in a freely available, flexible, cross-platform program for Bayesian analysis of molecular sequences (BEAST: http://beast.bio.ed.ac.uk). We have made use of several dissemination opportunities, including one workshop specifically organized under the PREDEMICS auspices in Belgrade in 2012 (http://regaweb.med.kuleuven.be/veme-workshop/2012/), to instruct stakeholders in the use of this software. Through the application of BEAST in outbreak situations, specifically during the West African Ebola epidemic, we have gained considerable expertise on the computational restrictions in rapidly generating actionable knowledge. Therefore, more efforts will be invested in making a version of the software that can support real-time epidemiological analyses in future outbreaks.

Work Package 3: "Host-virus interaction and anti-viral response"

WP Leader: Hervé BOURHY [Beneficiary 1 – Institut Pasteur (IP)]

Emerging infectious diseases have arisen significantly over time and also recently during the period of the PREDEMICS program. Therefore there is a need for understanding factors involved in emergence of human pathogens for a better prediction of the risks of emergence in order to build intervention strategies. WP3 studied these factors in humans and in different types of animal hosts (pigs, birds, canids) for some of the most important zoonotic RNA viruses (Influenza virus, Japanese encephalitis virus and related flaviviruses, hepatitis E virus and lyssavirus). These studies using these viruses as models were also extended to some related viruses of interest such as Middle-East Respiratory Syndrome coronavirus, Ebola virus, West Nile virus and Zika virus that emerged and became of public health concern during the time frame of PREDEMICS.

Molecular factors involved in the emergence of several of these highly pathogen viruses leading to tropism for new target cells and successful host switching were extensively studied in several relevant cellular and animal models. Therefore WP3 lead to an unprecedented amount of information and data concerning how viral and host determinants interact to favor/limit the potential for cross-species transmission and adaptation to a new host. Potential of emergence of some of the most fearsome vector borne disease in Europe and the potential transmission of JEV and Zika virus by European mosquitoes were also investigated leading to important conclusions in terms of public health and control strategies. Further, several new mechanisms developed by these viruses to counteract the innate immunity mechanisms of defense were also evidenced, in particular mechanisms of evasion of the host innate immunity by modulation of the type-I IFN pathway. This significantly contributes to shed new light on the complexity and very important role played by innate immunity in virus-host interaction by modulating the chance of success of the infection and therefore crossing of the species barrier.

Finally mechanisms of broad adaptive protection were thoroughly investigated in particular the impact of host immune response on virus evolution and the contribution of opsonising antibodies to (cross)-protection. As in many cases, the discovery of factors involved in emergence throw a very interesting light on fundamental aspects of the Influenza virus, HEV, JEV, Zika virus and lyssavirus reproductive and dissemination process and vice versa.
All this information contributes to the design of appropriate disease control and preventive measures. In particular, specific and innovative intervention strategies were designed and successfully tested against JEV.

All together the data accumulated by WP3 in collaboration with the other WP will enhance the success of prediction, surveillance and control of future emerging zoonotic viral infections and will contribute to help health officers, to respond more adequately and rapidly to these newly emerging viruses and the future ones. The data generated through WP3 has also been made available to the scientific community by their publication in open access peer reviewed scientific journals, scientific conferences, seminars and teaching and training activities (link with WP6). This fulfills the general public demands that science should be ready to help to respond in the fastest possible manner.

Work Package 4: "Sharing platforms"
WP Leader: Alan HAY [Beneficiary 19 – Freunde von GISAID e.V. (GISAID)]

The advent of the GISAID sharing mechanism ten years ago transformed the sharing of influenza genetic sequence data, removing impediments to scientists and governments for sharing data pre-publication and has made the rapid, timely sharing of influenza data of immediate public health concern commonplace. The user-friendly advanced tools on the new v2.0 platform will enable many labs lacking in-house computing expertise, especially in developing countries, to conduct their own analyses of genetic data on influenza viruses from their country/region and appreciate more readily the significance and impact of influenza on local health services. Such empowerment enhances the shared benefits accruing from participation in international networks to combat emerging and re-emerging infectious disease, as in the case of the WHO Global Influenza Surveillance and Response System (GISRS).

Providing the technical basis for extending the principles of the GISAID Initiative to other emerging threats, including those encompassed by PREDEMICS, should assist in meeting an imperative recently highlighted in various Editorials and Commentaries regarding the lack of data sharing in hindering effective outbreak response to e.g. MERS CoV, Ebola and Zika viruses.

While use of GISAID's EpiFlu^{TM} database is freely available, GISAID has co-organised, often in association with international meetings arranged by other organisations, such as WHO, PAHO, WHO EURO, ECDC or isirv, numerous bioinformatics training workshops, demonstrations and lectures in various parts of the world, to enable network participants, medical scientists and public health experts to benefit from the wealth of genetic and related information available on influenza. Data shared via the EpiFlu^{TM} database is used and acknowledged in a vast number of papers published on influenza.

Work Package 5: "Modelling of ecological, genetic and anthropological data"

WP Leader: Christl DONNELLY [Beneficiary 6 – Imperial College of Science, Technology and Medicine (IMPERIAL)]

The largest and most immediate socio-economic impact of analytical work of WP5 was a result of the analyses of the West African Ebola epidemic. PREDEMICS-funded Imperial College London researchers were part of the World Health Organization (WHO) Collaborating Centre on Infectious Disease Modelling http://apps.who.int/whocc/Detail.aspx?cc_ref=UNK-226&cc_city=london& (designated in 2010). Through this mechanism, PREDEMICS-funded researchers became part of the WHO Ebola Response Team (also referred to in one publication as the International Ebola Response Team).

This involved intensive collaboration from 2014 onward with Imperial staff and provided numerous epidemiological reports to collaborators in WHO and in the affected countries (Guinea, Liberia and Sierra Leone). This work directly informed the priorities of ongoing control efforts, improving situational awareness and providing immediate feedback on the impacts of control policies. The collaboration was with senior staff up to and including Dr Bruce Aylward (WHO Deputy Director General) and Dr Chris Dye (Director of Strategy in the Office of the **Director** General at the WHO).

As well as direct reports to WHO and staff within affected countries, results were disseminated to the wider public health and academic communities through peer-reviewed publications in top journals including *New England Journal of Medicine:*
https://www.ncbi.nlm.nih.gov/pubmed/25244186 [WHO Ebola Response Team]
https://www.ncbi.nlm.nih.gov/pubmed/25539446 [WHO Ebola Response Team]
https://www.ncbi.nlm.nih.gov/pubmed/25806936 [WHO Ebola Response Team]
https://www.ncbi.nlm.nih.gov/pubmed/26736011 [WHO Ebola Response Team]
https://www.ncbi.nlm.nih.gov/pubmed/27509108 [WHO Ebola Response Team]
https://www.ncbi.nlm.nih.gov/pubmed/27846234 [International Ebola Response Team]
https://www.ncbi.nlm.nih.gov/pubmed/28396480 [Cori *et al*.]
https://www.ncbi.nlm.nih.gov/pubmed/28396479 [Garske *et al*.]
https://www.ncbi.nlm.nih.gov/pubmed/26181387 [Lipsitch *et al*.]
https://www.ncbi.nlm.nih.gov/pubmed/28351674 [Nouvellet *et al*.]
https://www.ncbi.nlm.nih.gov/pubmed/26633764 [Nouvellet *et al*.]
https://www.ncbi.nlm.nih.gov/pubmed/26029377 [Van Kerkhove *et al*.]
In addition to these publications, and their often extensive online supplements, the researchers made extra efforts to ensure that the tools they developed were both available and well documented, using Recon (the R Epidemics Consortium http://www.repidemicsconsortium.org/) to disseminate computer code for others to use and develop further.
To facilitate this work, members of the team repeatedly travelled to WHO headquarters in Geneva and to Sierra Leone over the course of the Ebola epidemic, although the vast majority of the Imperial College London Ebola-related work was undertaken in London.

PREDEMICS-funded researchers also worked closely with WHO and Gavi, the Global Vaccine Alliance, on estimating the burden of yellow fever (YF) across Africa and the impact of vaccination. In particular, they supported WHO in the response to the 2016 YF outbreak in Angola and the Democratic Republic of Congo, performing real-time epidemiological analyses and developing frameworks for prioritising districts for vaccination in the context of global vaccine shortages, to inform novel dose-sparing strategies. As part of this support they have provided several reports to WHO between April and June 2016, and researchers visited WHO in June 2016

for two weeks to provide further in-depth support and inform the vaccination strategies to curb the ongoing outbreak. They also informed the EYE (Elimination of Yellow Fever Epidemics) strategy, which is a long-term plan to increase YF vaccination coverage across the endemic areas in Africa, by analysing YF transmission intensity and current population-level vaccination coverage to estimate populations at risk and prioritise districts and countries for vaccination. As part of this they have developed an online tool to visualise the vaccination coverage across Africa (https://polici.shinyapps.io/yellow_fever_africa/).

Middle Eastern Respiratory Syndrome (MERS) is another example where PREDEMICS-funded researchers undertook real-time analyses to understand the transmission dynamics of an emerging infectious disease and provided insights which were used by public health collaborators to inform control efforts in affected areas. The research is most comprehensively described in these publications which were published jointly with staff from Saudi Arabia's Ministry of Health:
https://www.ncbi.nlm.nih.gov/pubmed/27457935 [Cauchemez *et al*.]
https://www.ncbi.nlm.nih.gov/pubmed/26851269 [Lessler *et al*.]
PREDEMICS-funded researchers also looked more broadly bringing together data from across the Middle East to estimate the risks of exported cases:
https://www.ncbi.nlm.nih.gov/pubmed/24239323 [Cauchemez *et al*.]

Vittoria Colizza's group has worked more generally on the use of realistic networks to improve epidemics models, e.g. https://www.ncbi.nlm.nih.gov/pubmed/27842507 [Bioglio *et al*.]. It was announced in June 2017 that she was awarded the 2017 Erdős–Rényi Prize in recognition of her "contributions to fundamental and data-driven network-based modelling of epidemic processes, including seminal studies on metapopulation systems, the impact of air transportation, and the predictability of epidemic outbreaks"
https://isi.it/en/news-events/vittoria-colizza-receives-prestigious-erd-s-r-nyi-prize-2017-in-network-science.
Methodological research is crucial to underpin applied epidemiological studies to allow them to have the greatest relevance and impact.

Work Package 6: "Training"

WP Leader: Jean-Pierre KRAEHENBUHL [Beneficiary 15 – Fondation Health Sciences e-Training (HSeT)]

A new training concept, that we named COLT for Customized OnLine Training, was developed during the granting period. COLT provides online tailored learning experiences based on the requests of the training organizers. COLT allows mapping student's individual progress at every step using a Learning Management System (LMS) developed by HseT. COLT is well suited to build skills and competence, as well as acquisition of knowledge. COLT tailors features to each individual student in a way that would be impractical in a system designed for mass audiences. Therefore the different processes in selecting and organizing activities before, during, and after the face-to-face sessions provide a paradigm shift for a more sustainable knowledge uptake and translation into everyday practice.

Work Package 7: "Project Management"

WP Leader: Sylvie VAN DER WERF [Beneficiary 1 – Institut Pasteur (IP)]

An excellence-oriented management system based on a two-level structure was put in place from the very beginning of the project in order to ensure the successful achievement of the PREDEMICS ambitious objectives described in Annex I-Description of Work.

i) *Decision-making/Strategic Management* involved the Project/Scientific Coordinator, Prof. Sylvie van der Werf (IP), and Steering Committee, composed of the Scientific Coordinator (Chair) and all Work Package Leaders, officially appointed at the PREDEMICS Kick-Off Meeting held on 22-23 November 2011 in Paris, France.

ii) *Day-to-Day operational management* was led by the Project Coordinator, Prof. Sylvie van der Werf, with the support of Sophie Ablott and Soizic Sergeant, PREDEMICS Project Managers within the Grants Office of Institut Pasteur, and associated departments in the execution of all management-related, financial, legal and administrative tasks.

All project meetings, along with regular internal communication, were organised to discuss and ensure the scientific coherence and excellence of the project, and to follow its dissemination and communication activities:

- The Kick-Off meeting was held on 22nd-23rd November 2011, in Paris, France;
- The 2nd Annual meeting was held on 3rd-5th October 2012, in Palma de Mallorca, Spain;
- The 3rd Annual meeting was organised on 6th-8th November 2013, in Paris, France;
- The 4th Annual meeting was held on 6th-7th October 2014, in Rome, Italy;
- The 5th Annual meeting took place on 26th-27th November 2015, in Rotterdam, Netherlands:
- The 6th Annual meeting took place on 10th-11th October 2016 in Paris, France;
- The Final meeting was organised on 18th-19th April 2017 in Paris, France.

The Scientific and Steering Committee met during each of these meetings to monitor the advancement of the project and prepare the next scientific and dissemination activities, including preparing post-grant activities in order to maintain and build on the project's results. The Scientific Advisory Board also attended these meetings; their expertise and insight was fruitful, and their support for post-grant communication activities is encouraging.

Aware of the EU Open Science policy, PREDEMICS actively disseminated and communicated on its activities and results to ensure the full exploitation of data generated from the project, widen the

knowledge of the EU scientific community working on potentially pandemic diseases, and participate in developing a critical mass of EU scientists and EU scientific leadership. The consortium generated more than 200 high-ranking scientific publications in peer-reviewed journals, the majority of them having Open Access, and around 500 dissemination activities (including presentations at wide-audience scientific meetings, publications in lay press and production of dissemination documentation targeting different audiences--the international scientific community, the veterinarian and medical community, stakeholders and policy decision makers in the field of animal and human health as well as the general public).

The PREDEMICS consortium established strong interactions with other EU-funded projects that share the same context (dealing with emergence of zoonotic pathogens), similar approaches or objectives (environment, ecology, anthropology; virus and host evolutionary) and study on same or related viruses (IAV, HEV, JEVr, LYS), to focus on the same relevant impact:
- promote awareness on emerging diseases,
- increase visibility of research on emerging diseases in Europe,
- increase capacity-building potential, and
- promote data-sharing.

Joint meetings and workshops with ANTIGONE and/or EMPERIE projects were organised on a regular basis in an effort to extend dissemination opportunities and stimulate European cooperation beyond teams used to be working together, thus participating in the realisation of the European Research Area.

In addition, the PREDEMICS consortium promoted links with leading international organisations, such as OIE, FAO, WHO, ECDC, EFSA, ISARIC to target the major stakeholders and decision makers in the fields of animal and human Health. Among other activities, this was mostly done through the organisation of a major workshop specifically tailored to target this audience, which was organised at the end of the project. The meeting focused on strategies, prediction for the future and implementation of the findings of PREDEMICS, and fuelled current reflections on health sciences communication towards policy makers and citizens, and on the One Health approach. The event opened a significant opportunity to the PREDEMICS partners for providing evidence-based data to policy makers that could translate PREDEMICS results into the implementation of adequate control strategies contributing to prevention of emergence events and increased preparedness. A very positive and tangible impact of this stakeholder meeting is the opportunity to write a position paper for consideration for publication in Eurosurveillance.

The management of Intellectual Property has been coordinated and supported with the legal advice of the Institut Pasteur's Management Team. All institutions involved in the PREDEMICS project employed individuals highly trained in all aspects of the legal procedures for IPR management and exploitation of results. The PREDEMICS Consortium took all necessary measures to protect all results (patentable or not) arising from the project. Whenever possible, the Consortium favoured the sharing of biological materials, reagents or other products resulting from the project in order to optimize the use of any knowledge by the research community. Among exploitable foreground, three patents were registered by researchers from the PREDEMICS consortium. These discoveries constitute a key allowing further innovation in epidemics surveillance, likely to be exploited for industrial or commercial use.

Finally, implementing a specific ethics reporting procedure (including a periodic follow-up on ethics--human sample and personal data, animal issues, and dual use) and promoting gender equality aspects as the project unfolded encouraged good practices in the participating organisations and will have a lasting impact on each participant’s daily management practice.

1.5 - Address of the project public website, if applicable as well as relevant contact details

http://predemics.biomedtrain.eu/cms/default.aspx

2. Use and dissemination of foreground

See EC portal

2.1 – Section A (public)

- Template A1: List of all scientific (peer reviewed) publications relating to the foreground of the project.

See EC portal

- Template A2: List of all dissemination activities (publications, conferences, workshops, web sites/applications, press releases, flyers, articles published in the popular press, videos, media briefings, presentations, exhibitions, thesis, interviews, films, TV clips, posters).

See EC portal

2.2 – Section B (public or confidential: confidential information to be marked clearly)

Part B1

See EC portal

Part B2

See EC portal

3. Report on societal implications

See EC portal