Esempi di progetto di ricerca

Project title

New indicators and on-farm practices to improve honeybee health in the *Aethina tumida* era in Europe

Version 2

Honeybee health improved through new indicators and on-farm practices in the *Aethina tumida* era in Europe

Version 3

Improving honeybee health in the *Aethina tumida* era in Europe through new indicators and on-farm practices

Summary

The estimated value of pollination service is between 13.5 and 21.5 billion dollars (FAO, 2006). European beekeeping suffers significant regional differences in colony losses due to external impacts on beekeeping, including climate and prevalence of diseases (EPILOBEE, 2014). This situation is likely to worsen with the spread of *Aethina tumida* (Small Hive Beetle – SHB), a new parasite affecting honeybee, found for the first time in the EU in Italy in 2014. Together with other bee diseases (American Foulbrood - AFB, European Foulbrood – EFB and *Nosemosis),* SHB may play an important role in colony losses and to beekeeping economy.

The **BPRACTICES** project aims to develop new management practices (Good Beekeeping Practices - GBPs) adopting new clinical methods, biomechanical and innovative biomolecular techniques respecting the natural behaviour of bees. The research activities will focus on developing new biosensors from honey to monitor SHB presence and PCR techniques to diagnose in advance honeybee diseases (AFB, EFB, SHB) from debris. Another goal will be to accelerate and to raise efficiency of the clinical inspection of the hives to detect SHB. At the apiary level we will indicate a proper bee-friendly management (e.g. traps for SHB, honeybee queen-cages for varroa control, powder sugar method to assess varroa infestation level) to monitor and control the honeybee diseases, protecting their health and avoiding the application of chemical treatments guaranteeing quality and safety of hive products.

The innovations will be validated in the daily apiary activities and disseminated internationally in collaboration with the International Federation of Beekeepers' Associations (Apimondia). Economical impact on beekeeping industry will be quantified.

Consumers will be aware of the positive environmental impact of beekeeping and the ecosystem services provided, thanks to a cutting-edge traceability system using the QR-code/RFID technology.

La popolazione delle api europee sta declinando [è decimata, falcidiata] per l'aggressione concentrica degli insetticidi, dell'inquinamento atmosferico e di diverse malattie…

Project aims

Colony losses in Europe are strongly related to the prevalence of honeybee diseases in the different Countries. The spread of the exotic parasite of the hive *Aethina tumida* (Small Hive Beetle – SHB) from Italy will get even worse the bee mortality affecting also the pollination service, the environmental biodiversity granted by bees and the beekeeping economy.

The **BPRACTICES** project aims to develop new management practices (Good Beekeeping Practices - GBPs) adopting new clinical methods, biomechanical and innovative biomolecular techniques respecting the natural behaviour of bees. **BPRACTICES** project has an innovative approach oriented to the diagnostic and prevention of the majors honeybee diseases (*Varroa destructor* and associated viruses, American and European Foulbrood, *Nosema* spp., *Aethina tumida*) adopting the identification and validation of proper Good Beekeeping Practices. These will include the application of new and revolutionary diagnostic techniques like biosensors from honey and PCR analyses from hive debris, protecting the honeybee health and avoiding in the meantime the application of chemical treatments guaranteeing quality and safety of hive products. This sustainable production system, that stimulates the natural behaviour of honeybees to increase their health, will be communicated to the consumers through to an innovative informative technology (QRCode/RFID system) that will allow to know all the production details.

All those objectives are achieved by using multidisciplinary strategies: with the combination of scientific research, on-field experience for the validation of the methods, food safety control and economic, societal and commercial analysis. This wide approach is possible thanks to the multi-actor involvement in specific Work Packages (WPs). It includes the participation of an expert team integrated by researchers that combine different specialities and abilities, with the practical and useful experience of the beekeepers (Apimondia).

The increase in productivity, resilience and competitiveness of European Animal Production (Research Area 1) will be reached with the improvement of apiary management in order to prevent the spread of the main honeybee diseases in the EU. This will increase quantity, quality and safety of hive productions and reduce to a bare the economic losses related to diseases and sub-optimal hive management in the EU. It will be carried out with a country-adapted approach of bee diseases able to prevent future threats to European beekeeping studying the main problems and comparing the most effective containment methods adopted in different countries and reaching common solutions. The external collaboration with the University of Mississippi (USA) will guarantee the possibility to compare different methods to diagnose and control honeybee diseases, and to obtain positive samples (e.g. honey to test with biosensors) for Aethina tumida that is

The management of resources use to reduce waste and enhancement of the environmental sustainability of European Animal Production (Research Area 2) will be achieved by avoiding the beekeeping sanitary problems, reducing the costs (treatments, colony losses, production reduction) and increasing the outputs by improving the efficiency of their management. It is closely related to the environmental sustainability of this production, reached by using zero environmental impact treatments that also avoid pathogen resistance and implement biodiversity thanks to the pollination service. These facts are linked to the increase of the economic competitiveness of European apiculture. The economic impact and competitiveness of European beekeeping with the new breeding system and innovative technologies will be quantified (quality/safety/value of hive products). Similarly, its resilience and adaptation to market fluctuations will be evaluated analysing the real cost of production for the beekeepers in the different regions of Europe.

In order to achieve better on-farm practices to enhance consumer acceptability and address societal challenges associated with animal welfare, product quality and safety, biodiversity and provision of ecosystem services (Research Area 3), an on-farm bee-friendly management will enhance high quality hive products. This sustainable production system, that stimulates the natural behaviour of honeybees to increase their health, will be transferred to consumers with an innovative traceability system (QRCode/RFID system), in order to make them aware of the quality and safety aspects of hive products.

The implementation of this innovative production system will be applicable to every European country and it will be applied to beekeeping at all levels, from the ecological aspect to the economic elements.

Thanks to the collaboration with the European Union Reference Laboratory for Bee Health (ANSES) and an extra-EU collaboration with the Mississippi State University (USA) and the involvement of the International Federation of Beekeepers' Associations (Apimondia), the innovative production system will benefit from international collaboration enlarging the successful solutions available for each disease.

Implementation plan

WP 1 - "Varroosis and virosis".Lead: Partner 3

Varroosis is one of the major honey bee disease responsible for colony losses worldwide. The WP 1 of the **BPRACTICES** project will identify all the GBPs to be applied at the apiary level to properly assess the infestation rate of the parasite *Varroa destructor* adopting the same method for all the European project partecipating countries (standardization of the powder sugar techniques);reduce varroa mite population avoiding the application of high environmental impact chemical treatments and increasing the efficacy of organic treatments using innovative non-residual bio-mechanical methods like the brood-removal, the temporary queen caging techniques; prevent bee virus infections.

Duration: 30 months (01/02/2017-01/08/2019)

WP 2 - "American Foulbrood and European Foulbrood".Lead: Partner 5

American Foulbrood (AFB) and European Foulbrood (EFB) are the most widespread and damaging of the honeybee brood diseases. The WP 2 of the **BPRACTICES** project will identify all the GBPs to be applied at the apiary level to foresee symptomatic presence in hives of the responsible bacteria. New methods (e.g. PCR analyses of debris), in association with probiotics (e.g. *Lactobacillus* spp.) treatments and shook swarm techniques to prevent and control those diseases will be standardized and validated at the apiary level.

Duration: 30 months (01/02/2017-01/08/2019)

WP 3 - "Nosema". Lead: Partner 4

Nosemosis is the most common pathology affecting adult honeybee and it is associated to a reduced lifespan and winter mortality. The WP 3 of the **BPRACTICES** project will develop new GBPs to prevent and control nosemosis at the apiary level with the goal to enhance colony development and reduce colony infection. These GBPs will include: samplings and quantification of nosema spores in adult honey bees; laboratory analysis (optical microscopy counts or PCR); treatments with organic compounds (e.g. plant extracts)

Duration: 30 months (01/02/2017-01/08/2019)

Background

STATE OF THE ART

Honeybee colonies are in decline in Europe (Potts et al., 2010) and the causes of such losses are multifactorial (McMenamin and Genersch, 2015). The impact of the reduction of managed honeybees is enormous considering that honeybees are the single most important global commercial insect pollinator (Garibaldi et al., 2013). They contribute, with other pollinators, at least 22 billion EUR each year to the European agriculture industry and ensure biodiversity with the pollination service on over 80% of crops and wild plants (European Commission, 2016). Moreover, EU is the largest global consumer of honey (more than 20% of total worldwide consumption) but around 40% of Europe's consumption needs are met through honey imports (FAOSTAT, 2016; CBI, 2015). The largest supplier of honey to the European market is China, which represents 26% of total honey imports directed to the EU despite some quality issues concerning residues (CBI, 2015). Globalization leads to the risk for consumers of losing the awareness to differentiate products according to their quality. High-quality products obtained by a production system based on the three pillars of sustainability (economy, environment and society) should be promoted.

The European EPILOBEE project (Laurent et al. 2015, Chauzat et al., 2014) underlined the lack of explanatory studies about risk factors affecting colony health like disease prevalence, environment condition and farming practices adopted by beekeepers to detect and control the major honeybee diseases: *Varroa destructor* and associated viruses, American Foulbrood (AFB), European Foulbrood (EFB) and *Nosema* spp.

Varroa destructor is the most widespread and hard to control disease (Rosenkranz et al., 2010). Quite all non-organic "hard treatments" produced resistant mites (Maggi et al., 2010; Kanga et al., 2010; Pettis, 2004) and reduced the quality and safety of hive products (Rosenkranz et al., 2010). American and European Foulbrood cause considerable economic losses (Forsgren, 2010; Genersch, 2010) and the use of antibiotics is still considered an illegal possible solution to pursue with the subsequent risk of residues in hive products and bacterial resistance. *Nosema* spp. (especially *N. ceranae*) is an emerging pathogen affecting adult honeybees and it is associated to a reduced lifespan and increase of winter mortality (Higes et al., 2010). Furthermore, with the spread of the exotic parasite *Aethina tumida* (*A. tumida* or Small Hive Beetle – SHB) from Italy (Neumann et al., 2016; Mutinelli et al., 2014) beekeeping trade in EU is facing a great risk of productivity reduction and exports halt. Moreover, the association between *A. tumida* and *Kodamaea ohmeri* (Hayes et al., 2015; Brenda et al., 2008) a yeast able to cause septicemia in immunocompromised persons (Fernández-Ruiz, M., 2016; Distasi M. A., 2015) has been recently demonstrated. Today, good beekeeping management at the apiary level is a crucial point to maintain a healthy bee population (ANSES, 2015). Cross-valuable methods or guidelines internationally adopted to prevent and control the above-mentioned honeybee diseases in a sustainable way, including harmonized methods and analytical techniques for laboratory diagnosis, at the EU level, have not been adopted so far (Laurent et al., 2015; Chauzat et al., 2014; Chauzat et al., 2013), leading to a very variable quality and quantity of EU hive productions.

The **BPRACTICES** project will answer to the above-mentioned needs with an improvement of EU beekeeping production system towards the development of an innovative holistic approach (from apiary to jar) considering the good beekeeping practices application as the best and systemic tool (considering breeding, housing, welfare and nutrition aspects) to prevent and reduce the honey bee diseases, enhance quality and quantity of hive products, guaranteeing consumers awareness.

The project is focused on providing a cross-EU stakeholders debate on good beekeeping practices. Multidisciplinary strategies will be adopted combining the on-field experience of beekeepers, honey traders and retailers with the latest findings of scientific research, food safety, social and economic aspects to obtain shared and harmonized practices.

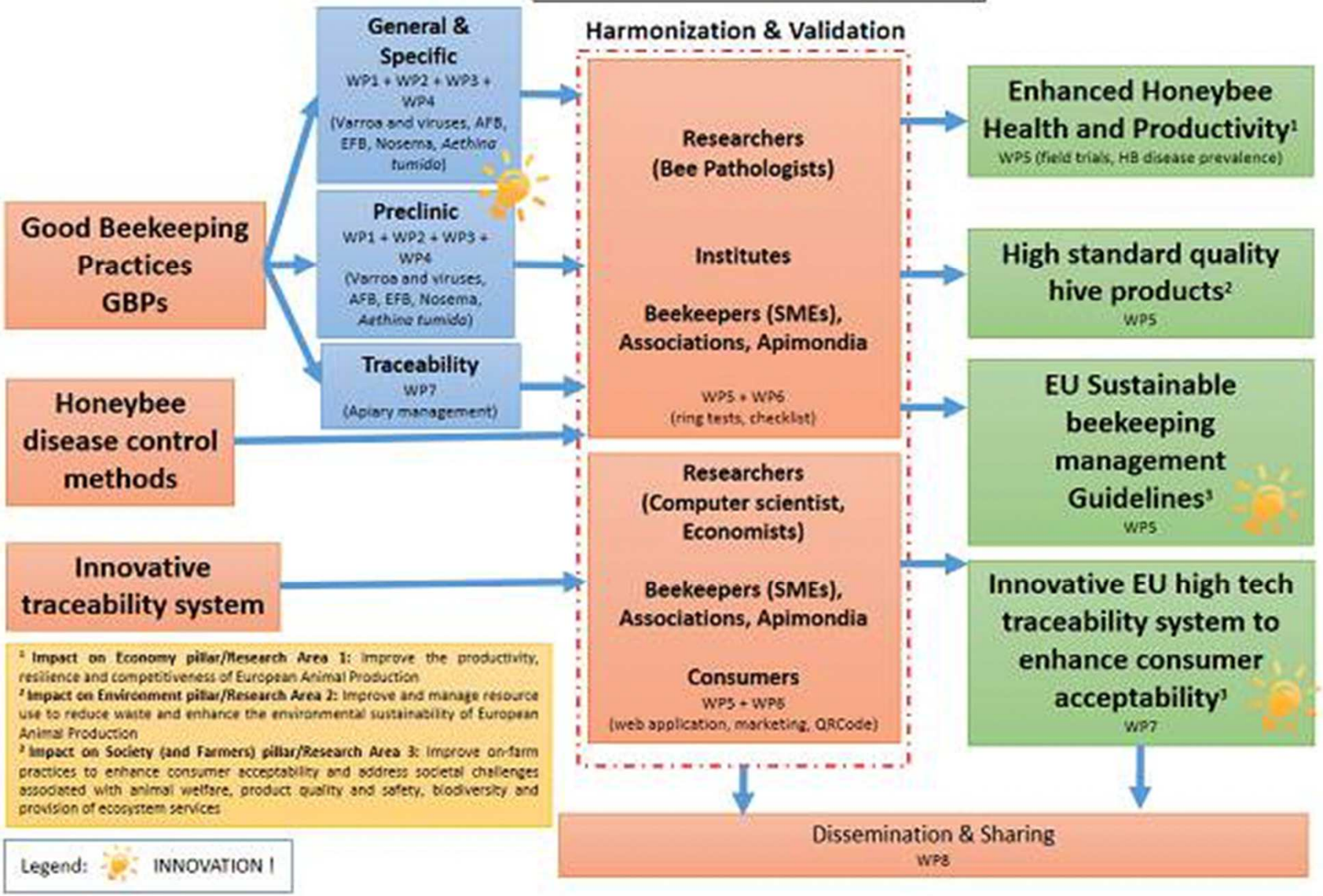
The outputs of the project will be:

1. Good Beekeeping Practices (GBPs) guidelines, harmonized within partner countries in the project;
2. guidelines on innovative laboratory diagnostic methods, harmonized among project partners, with the collaboration of the European Union Reference Laboratory for Bee Health (ANSES);
3. sustainable honeybee diseases control guidelines in respect of bee welfare and hive products quality;
4. an economic study concerning the impact of the innovative GBPs system application;
5. dissemination of results and technical assistance/training, that will benefit from the transnational participation of Apimondia (http://apimondia.com/) and FAO TECA platform (http://teca.fao.org/) and the release of a free web-application to act as a dynamic surveillance system on colony health status and a on-going training and up-skilling for beekeepers.

All the studies will be carried out considering the ethical aspect of refinement (methods that avoid suffering and improve animal welfare).

The **BPRACTICES** project will develop a transnational European new system for Bee Healthcare focused on: preclinical disease approach, prevention, surveillance and control adopting a sustainable low-environmental impact approach respecting the product's quality and consumer's safety. Moreover, **BPRACTICES** include an innovative traceability system (QR Code/RFID based) applied for the first time throughout the entire hive production chain (from the hive to the jar) to the advantage of beekeepers and consumers. **BPRACTICES** will impact on: the economy pillar (Research Area 1) improving the productivity, resilience and competitiveness of European production from the hives; the environment pillar (Research Area 2) promoting a sustainable and environment-friendly bee management; the society and farmers' pillar (Research Area 3) developing an innovative traceability system to share and disseminate innovative on-farm practices and enhance consumer acceptability and awareness of high-quality products coming from a sector respectful of animal welfare, able to improve biodiversity and provision of ecosystem services.

Figure 1. BPRACTICES project organization



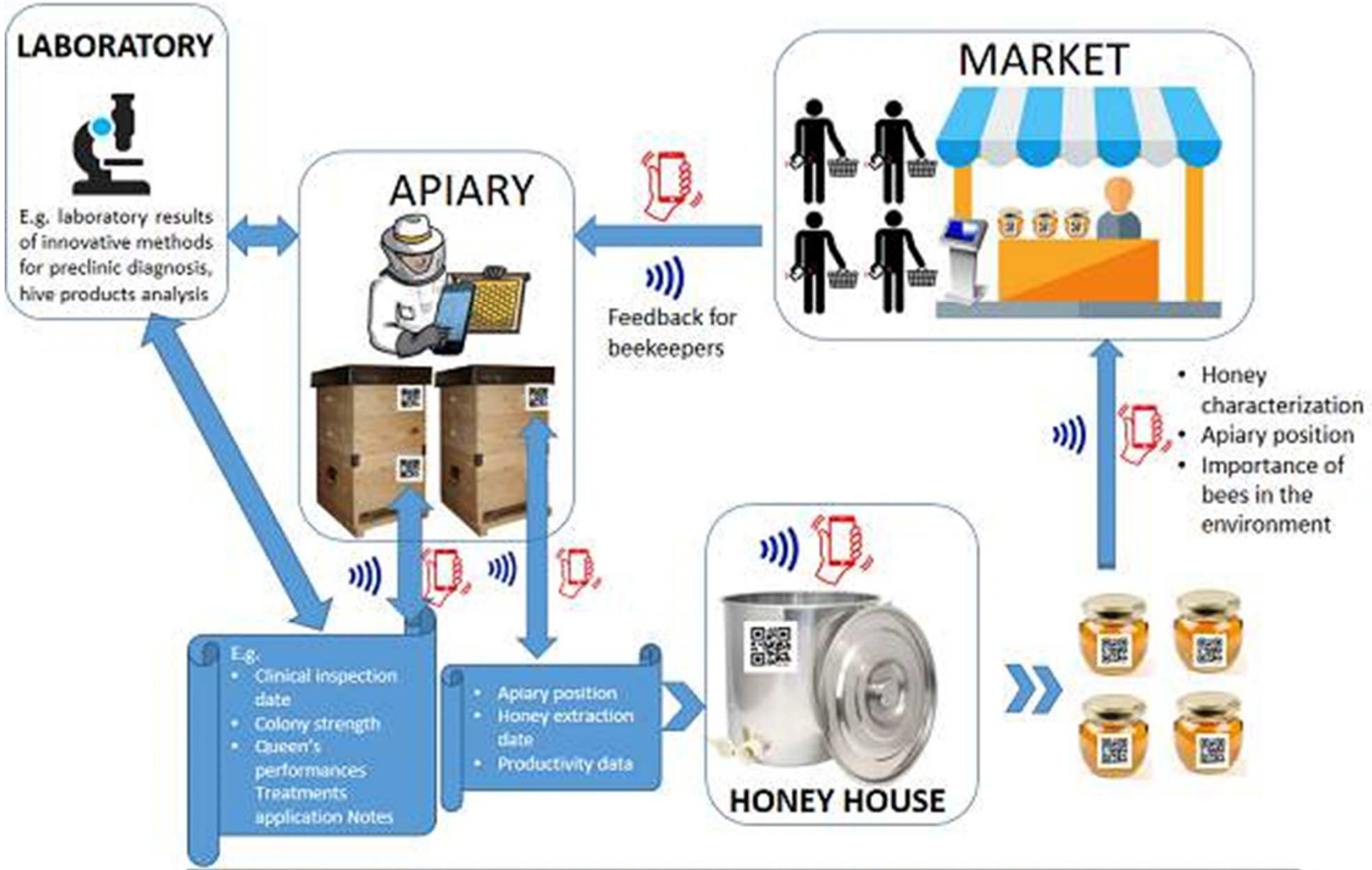
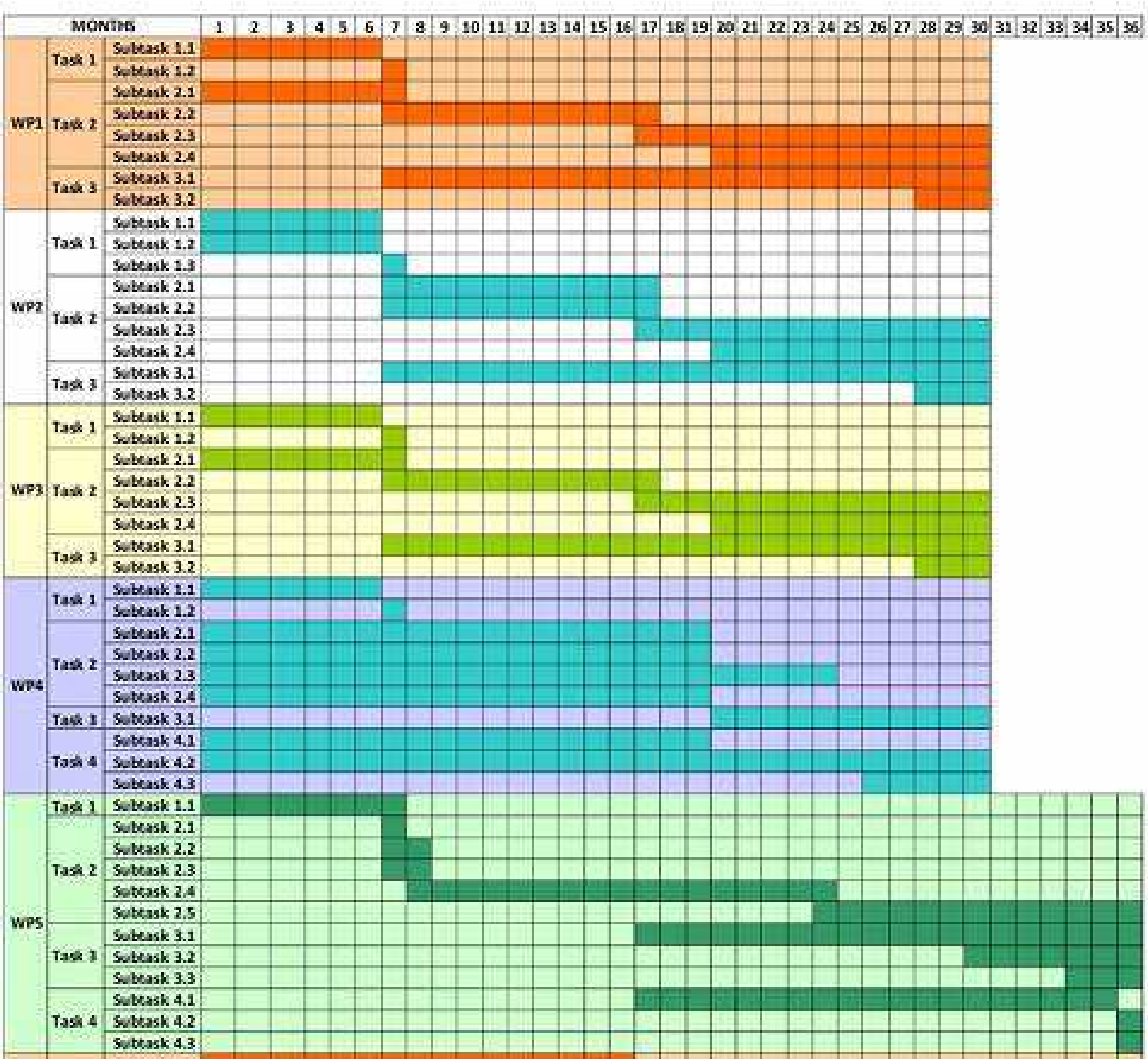


Figure 2. Innovative traceability system

FINANCES

Requested funding **[in k€]**

Organisation name	Person costs	Travel	Consumables	Subcontracting	Requested Funding	Total Own Contribution	Total Costs
Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M.Aleandri"	112	10	13	75	224	68	292
Overhead	11	1	2				
University of Namik Kemal	18	8	18	18	62	9	71
Overhead							
Agricultural Institute of Slovenia	34	5	18		69	34	103
Overhead	6	2	4				
Centro de Investigación Apícola y Agroambiental de Marchamalo (CIAPA)	58	6	11		94	53	147
Overhead	14	2	3				
Austrian Agency for Health & Food Safety	152	24	20		196	65	261
Overhead							
Mississippi State University					0	6	6
Overhead							
Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe)	16	6	4	4	33	38	71
Overhead	2	1					


MONTHS
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WP1
Task 1
Subtask 1.1
Subtask 1.2
Task 2
Subtask 2.1
Subtask 2.2
Subtask 2.3
Subtask 2.4
Task 3
Subtask 3.1
Subtask 3.2
WP2
Task 1
Subtask 1.1
Subtask 1.2
Subtask 1.3
Task 2
Subtask 2.1
Subtask 2.2
Subtask 2.3
Subtask 2.4
Task 3
Subtask 3.1
Subtask 3.2
WP3
Task 1
Subtask 1.1
Subtask 1.2
Task 2
Subtask 2.1
Subtask 2.2
Subtask 2.3
Subtask 2.4
Task 3
Subtask 3.1
Subtask 3.2
WP4
Task 1
Subtask 1.1
Subtask 1.2
Task 2
Subtask 2.1
Subtask 2.2
Subtask 2.3
Subtask 2.4
Task 3
Subtask 3.1
Task 4
Subtask 4.1
Subtask 4.2
Subtask 4.3
WP5
Task 1
Subtask 1.1
Task 2
Subtask 2.1
Subtask 2.2
Subtask 2.3
Subtask 2.4
Subtask 2.5
Task 3
Subtask 3.1
Subtask 3.2
Subtask 3.3
Task 4
Subtask 4.1
Subtask 4.2
Subtask 4.3

Secondo progetto di ricerca

Title

Identification and characterization of oligopeptides with anti-aggregation activity in normal cerebrospinal fluid. In vitro and in vivo experimental studies for the development of novel strategies to prevent prion and other protein-misfolding neurodegenerative diseases

Alternative title

Exploiting an anti-aggregation activity in normal cerebrospinal fluid to design novel strategies for preventing protein-misfolding neurodegenerative diseases

A.1 summary description

Many neurodegenerative diseases share a common pathogenic mechanism, which triggers an irreversible, self-sustained cascade of protein misfolding and aggregation. An in vitro aggregation reaction, the RT-QuIC assay, has been recently developed to help in diagnosing these diseases. Using this assay, we have discovered that “normal” cerebrospinal fluid (CSF) carries a powerful activity that prevents prion protein (PrP) aggregation and co-elutes with PrP peptides naturally present in CSF. Such activity is also shared by proteolytic products of recombinant PrP.

In this project we will identify and characterize natural and synthetic peptides endowed with anti-aggregation activity. The most potent peptides will be evaluated in tissue culture and animal studies, to determine whether their activity is conserved in biological settings and whether they can prevent or even treat protein misfolding diseases.

B.1 Hypothesis and significance

We have learned that normal proteins expressed in the CNS carry the potential to trigger dreadful diseases by undergoing pathogenic conformation changes. This astonishing discovery suggests that there should exist mechanisms to keep this devastating potential tightly in check. Here, we propose that PrP, whose aggregation is responsible for CJD, is endowed with an inherent safety mechanism. Indeed, we have found that several peptides derived from PrP inhibit the seeding effect of the pathogenically conformed protein. Furthermore, an anti-aggregation activity (AAA) is found in normal CSF along with PrP-derived short peptides. Our preliminary data suggest that such peptides are responsible for AAA. Confirmation of these results would suggest critical pathogenic mechanisms and point at new strategies to prevent and possibly treat the currently incurable CJD.

B.2 Preliminary data

While preparing rPrP as the substrate for diagnostic RT-QuIC, we noticed that occasional batches aggregated spontaneously (autoaggregating PrP, AAPrP), independent of seeding (Fig. 1A), due to minor variations in pH and temperature during preparation. As expected, CSF from confirmed CJD patients induced PrP aggregation in the RT-QuIC test and even accelerated aggregation of AAPrP (Fig. 1B). Startlingly, non-CJD CSF completely inhibited AAPrP aggregation (Fig. 1B). After confirming that the AAA of non-CJD CSF was consistently detected using different batches of AAPrP and a large number of CSF samples, we digested CSF fractions with different enzymes. We found that AAA was proteinase K (PK)-sensitive and thus associated with proteins. Ultrafiltration of non-CJD CSF showed that AAA peaked in a fraction comprising MW between ca. 3 and 10 kDa (Fig. 1C).
Unexpectedly, we detected a powerful AAA in PK-digests of purified rPrP, indicating that in these preparations the activity was probably carried by PrP peptides (Fig. 1D). This AAA peaked in the 1-3 kDa MW fraction (Fig. 1C). As a specificity control, four other proteins (G3PD, carbonic anhydrase, trypsinogen, and BSA), identically digested, showed no AAA activity, but actually accelerated AAPrP aggregation (Fig. 1E).
We analyzed the 1-3 kDa fraction of PK-digested rPrP by high-resolution tandem mass spectrometry (MS) and detected three most abundant peptides. A comparison with a PrP model (1) showed that they map to the outer loops of the protein (Fig. 1F), in line with a previous PrP proteolysis study (2). Synthetic peptides largely overlapping those identified in PrP digests showed similar AAA (Fig. 1G). Intriguingly, MS identified a longer peptide, encompassing two of those just described, in the 3-10 kDa fraction of undigested non-CJD CSF (Fig. 1H). This result suggests that the AAA identified in CSF might be mediated by natural PrP peptides.
LATE BREAKING RESULTS: while submitting this project, we have obtained evidence that products of rPrP digestion with PK also inhibit alpha-synuclein aggregation in an RT-QuIC assay (Fig. 1I). These data suggest that PrP peptides have a broader AAA and deserve urgent and thorough investigation. Although we don’t have time to fully integrate the new results into the project, we have amended Aim 1 to take them into account.

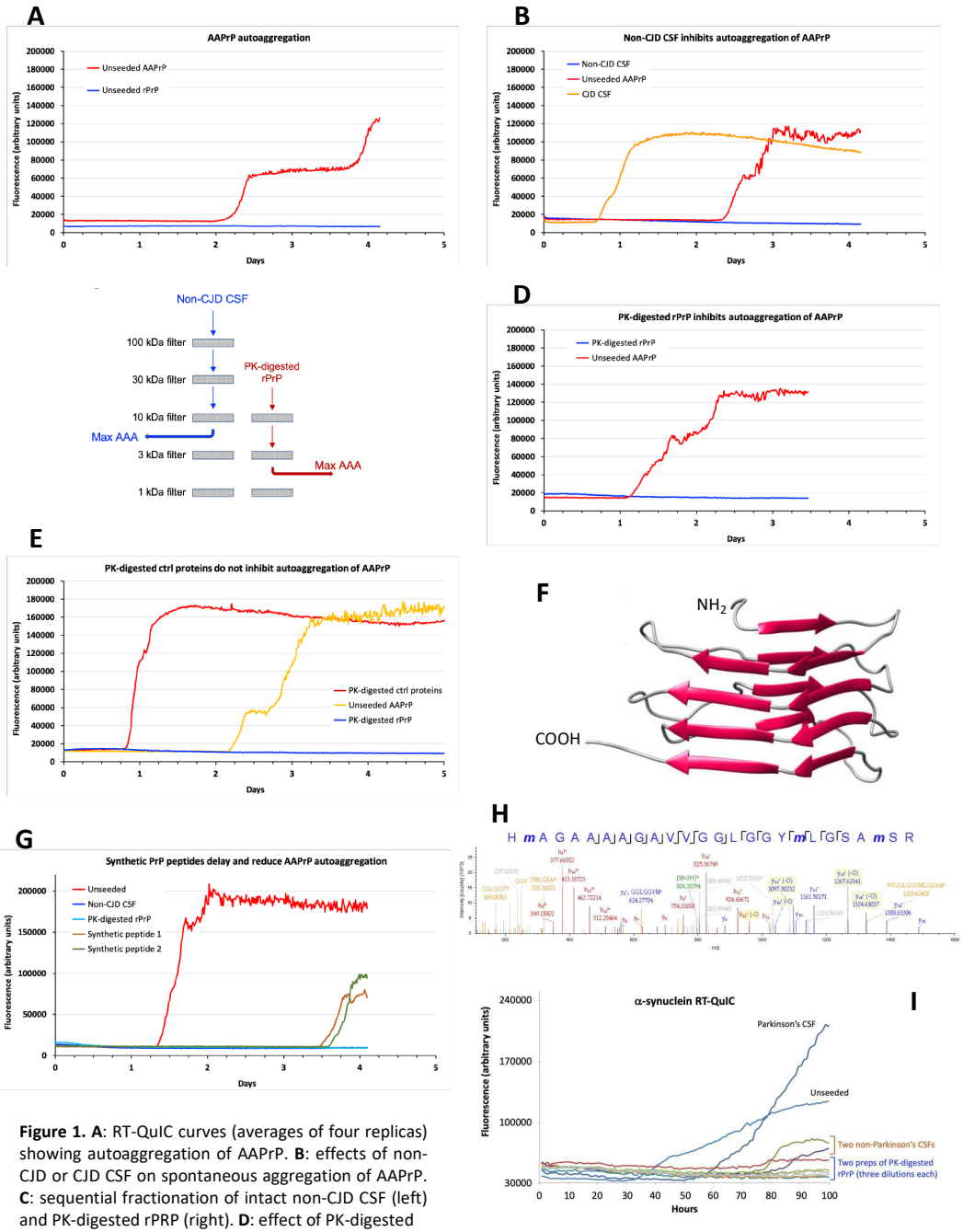

Figure 1. A: RT-QuIC curves (averages of four replicas) showing autoaggregation of AAPrP. **B**: effects of non-CJD or CJD CSF on spontaneous aggregation of AAPrP. **C**: sequential fractionation of intact non-CJD CSF (left) and PK-digested rPRP (right). **D**: effect of PK-digested rPrP (1-3 kDa fraction) on spontaneous aggregation of AAPrP. **E**: PK-digested control proteins do not prevent AAPrP autoaggregation, but actually accelerate it. **F**: schematic of the human PrP (Spagnolli, 2019), comprised of rungs (red) and loops (gray). **G**: effects of two synthetic PrP peptides on AAPrP autoaggre gation. **H**: MS/MS spectrum of an endogenous PrP peptide identified in the 3-10 kDa fraction of human non-CJD CSF; red and blue peaks: b and y fragment ions, respectively; orange peaks: internal fragments; m: oxidized methionine. **I**: effects of non-Parkinson's CSFs and three dilutions of each of two preparations of PK-digested rPrP on α-synuclein aggregation in RT-QuIC.

B.3 Specific aims 1

In vitro functional and molecular characterization of AAA in CSF and in PK digests of rPrP

B.4 Specific aims 2

Assessment and characterization of the AAA activities defined in Aim 1 in a *cellular* model system. Focus on the ability of AAA to prevent and/or revert PrP aggregate formation

B.5 Specific aims 3

Test of AAA as a potential preventive and curative strategy in an established *mouse* model

E. RISK ANALYSIS, POSSIBLE PROBLEMS AND SOLUTIONS

Aim 1

Though our preliminary results suggest that PrP-derived peptides are responsible for AAA in non-CJD CSF, it is possible that natural AAA is due to different proteins/peptides. If evidence indicates that natural AAA is due to non-PrP polypeptides, we will investigate all the proteins represented in the CSF 3-10 kDa fraction. This approach is feasible since a very sensitive MS analysis of such fraction has shown that it contains only a few hundred peptides, from just several tens of proteins. Any peptide in the 3-10 kDa fraction that can be plausibly linked to AAA will be synthesized and tested by RT-QuIC.

Aim 2

AAA peptides might be degraded by proteases in tissue culture medium. MS will be used to measure their half-life in culture and they will be added to medium as frequently as needed to stabilize their concentration. Should peptides display extremely short half-lives, we will use chemical modification strategies to stabilize them (8,9).

We anticipate that our synthetic peptides will not need to enter the cells to exert AAA. Yet, should they be inactive in the Scrapie Cell Assay, we will determine if membrane-permeable peptide variants carrying cell-penetrating peptides (5-20 AA) at their termini (10) display AAA in this test. Intracellular delivery will be quantitatively determined by MS.

Aim 3

AAA peptide solutions will have to dwell in the infusion pumps for up to 40 days. In simulations performed beforehand, peptide stability will be assessed by MS as described above. If necessary, DMSO will be added to the solvent, to prevent aggregation or precipitation. A DMSO concentration up to 25% is tolerated in low-rate intracisternal infusion (11).