FINAL PUBLISHABLE SUMMARY REPORT

- **Executive summary**

Inflammation is a fundamental protective mechanism and at the same time the driving force of a variety of major diseases in humans. Indeed, acute self-resolving inflammation usually plays a positive role for the host, as exemplified by infectious diseases where its positive role is well established and testified by its perception as innate immunity. On the other hand, non-resolving inflammation and its chronic evolution is a key determinant of immunopathology and clinical manifestations of most major diseases in humans, including autoimmune diseases, asthma, chronic obstructive pulmonary disease, obesity, neurodegenerative processes, atherosclerosis complications, and tumours. As a consequence, it is increasingly appreciated that the problem with inflammation is not how often it starts, but how often it fails to resolve. Appropriate resolution of inflammatory responses, which also drives activation of tissue damage repair mechanisms and return of local tissues to homeostasis, is a necessary process for maintaining health. Interestingly, cells sustaining these processes are also key to the pro-inflammatory responses, and the underlying "pro-resolving" molecular pathways are triggered as part of the pro-inflammatory response. This clearly indicates resolution of inflammation as an active process requiring functional repolarization of inflammatory cells that calls our attention on the underlying molecular mechanisms.
"Pushing for" inflammation resolution by exploiting active naturally-occurring pro-resolving processes may have significant advantages over the attempt to simply "push back" inflammation by passive blockade of proinflammatory mediators. The TIMER project has successfully investigated and clarified the activity of molecules involved in resolution of inflammation, which can be further exploited for novel therapeutic approaches.

- **Summary description of project context and objectives**

The general objective of the **TIMER** Consortium has been to identify and validate new molecules involved in the resolution of inflammation as a basis for the development of innovative therapeutic strategies in chronic inflammatory and autoimmune diseases. The project has fostered the discovery of new natural or synthetic "pro-resolving" molecules from plant and animals, and the validation of endogenous inflammation "pro-resolving" mechanisms, including atypical chemokine receptors, decoy receptors, and microRNA.

Efforts were mainly focused on the regulation by "pro-resolving" agents in two molecular systems of key relevance in inflammation: **the chemokine system**, which regulates recruitment, permanence and egress of leukocyte in tissues; and **the Toll Like Receptor (TLR)/IL-1R system**, which is central for the activation of infiltrating leukocytes.

- **Main S & T results/foregrounds**

The main S&T results are described below for each RTD WPs:

WP1 Discovery. We have used natural resources and chemical strategies in an attempt to define novel anti-inflammatory drugs which control inflammation. Several extracts and fractions were characterized and subsequently tested. We have synthesized two new partial agonists of TLR7 and produced novel constructs of Evasins, chemokines inhibitors characterized by members of the consortium in a previous ì project funded by European Commission (INNOCHEM). In addition, we have unravelled three novel mechanisms which control inflammation. First, TIR8 acts on members of the IL-1 receptor family and TLR as negative regulator of the TLR/IL-1R pathway in acute lung inflammation and as promoter of resolution. Second, we found a series of microRNAs expressed in human primary monocytes in an IL-10-dependent manner. Third we found that succinate is a novel metabolism-derived regulator of inflammation.

WP2 *In vitro* pharmacology. A series of studies characterized novel chemical entities, including the flavonoid manzoin A and derivatives, able to inhibit LPS-induced TNF production. A partial agonist of TLR7, TMX-306, was developed and tested on human leukocytes. We have provided the first evidence that the atypical chemokine receptor ACKR2 (formerly known as D6) is a natural arrestin-biased 7TM receptor, and found that a ACKR2 N-terminal peptide can block inflammatory chemokine activity. Extensive *in vitro* characterization of Evasin constructs and fusion proteins was carried out and we made novel pH-dependent

anti-chemokine antibodies. We found that TLR signalling is a prominent target of the IL-10-dependent anti-inflammatory microRNA in monocyte/macrophages.

WP3 *In vivo* validation. We have shown the potential anti-inflammatory and analgesic activities of *Aedes aegypti* and *L. longipalpis* saliva. TMX-302 and TMX-306, partial TLR7 agonists developed in the frame of the consortium activities, have shown promising effects in inhibiting lung inflammation and airway hyper-reactivity *in vivo*. Different systems to study resolution of inflammation have been developed and are being used to screen for multiple for plant extracts, their fractions, and purified molecules. ACKR2 was found to control mobilization of $Ly6C^{high}$ immunosuppressive monocytes and to play an important role in the pathogenesis of graft-versus-host disease, myocardial infarction, wound healing and COPD. ACKR2 tunes chemokine levels in tumours and its suppression operates as an oncogenic event unleashing tumour promotion via an inadequate intratumoural control of inflammatory chemokines. Results obtained in models of hepatocarcinoma and breast cancer suggest that TIR8 plays a negative regulatory role in the development and activation of anti-tumour immune responses, in particular by tuning NK cell activity. miR-135b plays a key role in gout by direct targeting the IL-1 signalling pathway and regulating the intensity of inflammation. We found a crucial role of H_2O_2 for resolution of allergic inflammation and the novel pro-resolution effects of angiotensin 1-7 in the context of neutrophilic inflammation. For the first time, the pro-resolving effects of Tat-GILZ were described and the cross-talk between this protein and Annexin-A1. In addition, we found that protease processing of Annexin A1 to be very relevant in the context of resolution. We have identified the relevance of the molecule survivin and sphingosine pathway in maintaining inflammation in vivo.

WP4 Early clinical development. A phase I study for intravesical delivery of TMX-101, a TLR7 agonist, has demonstrated safety of the compound. Phase II data suggest TMX-101 is also efficacious in humans. A clinical development plan was drafted and discussed with FDA in order to obtain marketing approval in 4 years. Two pH dependent anti-CXCL10 antibody candidates were identified, tested in preclinical models of inflammation and are being evaluated further for trials in humans. The design of a clinical study evaluating the effects of chemokine receptor antagonists (CXCR1/2 allosteric inhibitors) in palmoplantar pustolosis has been complete and is due to start in the Spring of 2016.

- **Potential impact and main dissemination activities and exploitation of results**

Potential Impact

The overreaching objective of TIMER was to orchestrate the activities of leaders in the fields of inflammation, resolution of inflammation, animal models of infectious, inflammatory disease, and pharmacology with cutting-edge laboratory instrumentation. The project has capitalized on the strong background of productive collaborative interactions among European and Brazilian participants. Strategic strength of the consortium was represented by the proven capacity to identify new molecules of synthetic or natural origin with a potential for application in humans, testified by the activities performed by 6 members of the consortium that had been part of the European Commission-funded project INNOCHEM, which also included one participant from Brazil (UFMG). The INNOCHEM Consortium led to 3 early clinical trials with simple chemicals and antibodies targeting chemokines and their receptors.

A major theme in TIMER was to challenge existing theories with new perspectives and bring forward possible new players leading to innovative therapeutic strategies targeting diseases sustained by uncontrolled activation of innate immunity and inflammation to be tested in relevant preclinical models and/or in humans.

Through an integrated interdisciplinary approach, the TIMER Consortium has successfully investigated complex disease processes and produced translatable clinical data for novel therapeutics. Of special relevance, we have developed novel systems to study inflammation resolution and identified several key molecular control points in inflammation resolution that may be used for the development of novel therapies. In particular, we have uncovered a crucial role of TIR8 acts as negative regulator of the TLR/IL-1R pathway in acute lung inflammation and as promoter of resolution. Similarly, several novel unique pathways capable of controlling inflammation have been discovered, including angiotensin 1-7, Tat-GILZ, Annexin-A1, survivin and the sphingosine pathway. Further studies should define the potential of modifiers of these pathways in the treatment of human diseases. Fundamental biology has been discovered in the context of inflammation resolution and control, including the mechanisms by which the atypical chemokine receptor ACKR2 may control chemokine levels and how it may interfere with chronic inflammation and cancer growth. The fundamental role of several mRNAs and metabolism in the regulation of inflammation has been

studied. The latter are fundamental findings in inflammation biology that may have substantial impact in the way we understand inflammation and its regulation. We have discovered novel natural molecules and have found several novel activities in insect saliva that modulate inflammation and its resolution. These molecules should be developed further for their potential as novel treatments for chronic inflammation. A series of TLR7 agonists have been developed and studied for their effects on inflammation and cancer. Clinical phase I and II data on TMX-101 show this compound to be safe and potentially effective in humans and is currently being tested further in humans. Novel anti-chemokine antibodies with novel mechanism of action (ie. pH dependency) have been selected, tested in preclinical models and are now being evaluated in humans. And finally, a chemokine receptor antagonist is now ready to start for study in palmoplantar pustolosis. Overall, we have made significant progress into the many phases of novel drug development for the treatment of inflammation: novel biology and concepts have been identified, novel molecules developed and studied, preclinical proof of concept studies carried out and initial clinical studies carried out. The relevance of resolution for chronic inflammation has only started to be understood. Our studies provide novel biology, targets and initial clinical studies to move the field forward.

Dissemination activities

Since the beginning of the project, the dissemination activities were aimed at enhancing visibility of the consortium and of the project, mainly for informing the scientific community and stakeholders and for communicating the value of the research funded by the European Community through FP7.

The main dissemination approaches from the project start included:

- **Development of a Project website**

The first edition of the portal on TIMER and TARKINAID, a "sister" project financed in the same period and assessing different aspect of inflammation, has been completed at Month 3 and the portal website (**EUmBRella website:** http://www.eumbrella.org) officially launched. The first edition of the portal has been revised and improved in the course of the second reporting period. The website has been set up and developed by beneficiary 10 ALTA, who has been in charge for updating it until the end of the project. The portal website includes also the individual TIMER website (http://www.eumbrella.org/timer.html).
The portal EUmBRella website includes:

- Public content for TIMER project
- Public content for TARKINAID project
- Public content focused on the EU-Brazil Research Partnership in Chronic Inflammatory and Autoimmune Diseases
- Dissemination and training
- Link to TRIAD (independent website)
- Link to PODIO social network (restricted area for the participants of TIMER, TARKINAID and TRIAD)
- **Development of Project information material**

A Brochure of the project was prepared by beneficiary 10-ALTA, which was distributed at the 15th International Congress of Immunology (August 22-27, 2013, Milan, Italy), at the 11th World Congress on Inflammation (September 21-25, 2013, Natal, Brazil), and in occasion of project meetings.
ALTA has also designed the logo of the EUmBRella website and of the TIMER website (see below) that visually identifies the project and that has been used in occasion of meetings, posters, and communication activities.

Project logos

- **Participation at scientific events**
  see list in A2

- **Press releases**

The activation of the TIMER project has been communicated to the media via publication on the Istituto Clinico Humanitas website, on the on-line journal HumanitasSalute.it (http://www.humanitasalute.it/i-piu-letti/5677-un-timer-contro-linfiammazione), and on the ISSUU-Scientific report 2011/2012 by Humanitas Research Hospital (http://issuu.com/hsalute/docs/scientificreport2012).

On Monday, January 23, 2012 the starting of the TIMER project has been communicated also via the Institute for Research in Biomedicine website (http://www.irb.ch/eu-invests-research-brazil-fight-inflammatory-disorders-irb-one-grantees), and via a press release of the University of the Italian Switzerland on the same day. Furthermore, an article was published by the newspaper "Il Corriere del Ticino" on January 24th.

On February 4th, 2012 the Institute for Research in Biomedicine, after the successful publication in the Journal of Experimental Medicine of the research conducted in the frame of TIMER, has published a press release via the University of the Italian Switzerland, underling the importance of the European Commission contribution to the research.

- **On line articles**

An article on TIMER project has been published on 28/09/2015 on Horizon 2020 website, in the Projects' stories section:
http://ec.europa.eu/programmes/horizon2020/en/news/inflammation-needs-closure

- **Publications/chapter of books/reviews**

See A1 for publications.

A Research Topic issue in *Frontiers in Immunology* on "Inflammation, its resolution and therapeutic targeting" has been launched. Deadlines for manuscript submission, **February 29th, 2016**.

List of contributors

The papers in bold are joint publications among Timer partners.

1. "Regulatory role of IL-1R8 in immunity and cancer" – Cecilia Garlanda, Martina Molgora, Isabella Barajon, Alberto Mantovani
   Submitted - by Independent reviewers
2. **"Pyruvate kinase M2: a potential target for resolution of inflammation" - Eva M. Palsson-McDermott, Jose Carlos Alves-Filho**
   Submitted - Interacting review
3. "Evasins: therapeutic potential of a new family of chemokine binding proteins from ticks" – Amanda E. Proudfoot1, Christine A. Power, Pauline Bonvin
   Submitted
4. **"Atypical chemokine receptors for resolution of inflammation"- Gerry Graham, Raffaella Bonecchi**
   Submitted
5. "Resolution of Inflammation: What Controls its Onset?" - Michelle A. Sugimoto, Lirlândia P. Sousa, Vanessa Pinho, Mauro Perretti, Mauro M. Teixeira
   Submitted - Interacting review
6. "Paradoxical roles of the neutrophil in sepsis: protective and deleterious" - Fabiane Sônego, Fernanda V. Castanheira, Raphael G. Ferreira, Alexandre Kanashiro,
   Caio A. Leite, Daniele C. Nascimento, David F. Colón, Vanessa F. Borges, José C. Alves-Filho, Fernando Q. Cunha
   Submitted - by Independent reviewers

7. **"Potential of PEGylated toll-like receptor 7 ligands for controlling inflammation and functional changes in mouse models of asthma and silicosis" - Tatiana Paula T. Ferreira, Livia L. Mariano, Roberta G. Bortolini, Ana Carolina S. Arantes, Andrey Fernandes, Michelle Berni, Valentina Cecchinato, Mariagrazia Uguccioni, Roberto Maj, Alcide Barberis, Patricia M. Silva, Marco A. Martins**
   Published March 11th, 2016
8. **"Modulation of the chemokine responses" - Mariagrazia Uguccioni, Amanda E. Proudfoot**
   Submitted- by Independent reviewers
9. **"Allosteric modulation of chemoattractant receptors" - Marcello Allegretti, Maria C. Cesta, Massimo Locati**
   Submitted - by Independent reviewers

Topic Editors: Mariagrazia Uguccioni, Mauro Teixeira, Massimo Locati, and Alberto Mantovani

All papers will be sent to the Timer EU project officer once they will be published.

Exploitation activities

Several of TIMER deliverables lend themselves clearly to successful exploitation. Namely:
1. Knowledge base of TLR signalling pathways and atypical chemokine receptors will become an invaluable resource to researchers in the field of inflammation. This deliverable has the capacity to become a central point within the research community, and will have high dissemination impact.
2. Development of novel therapeutics derived from a systems biology approach in experimental and preclinical studies.

A major theme in TIMER was to identify and validate new molecules involved in the resolution of inflammation as a basis for the development of innovative therapeutic strategies in chronic inflammatory and autoimmune diseases. The project involved discovery of new natural or synthetic "pro-resolving" molecules from plant and animals and investigation on endogenous inflammation "pro-resolving" mechanisms identified by various partners of the Consortium, including atypical chemokine receptors, decoy receptors, and microRNA. Efforts have been mainly focused on the regulation by "pro-resolving" agents on two molecular systems of key relevance in inflammation: the chemokine system, which regulates recruitment, permanence and egress of leukocyte in tissues; and the Toll Like Receptor (TLR)/IL-1R system, which is central for the activation of infiltrating leukocytes.

We have obtained important results which can be applied to develop strategies promoting resolution of inflammation by targeting prime movers of the inflammatory reaction: inflammatory chemokines and their receptors, which are key elements in dictating leukocyte infiltration, and the members of the TLR/IL-1R superfamily, which are major leukocyte activators.

A patent has been produced as detailed below:

Type of IP Rigths	**Application reference (e.g EP123456)**	**Intellectual Property Organization**	**Subject or title of application**	**Confidential (Y/N)**	**Foreseen embargo date**	**Applicant(s) (as on the application**
Patent	62/139,597	NovImmune	Methods to increase the concentartion of circulating factors using conditional binding antibodies	Y	Filed on 27.03.15 – so 18 months until publication	Nicolas Fischer, Pauline Bonvin, Marie Kosco-Vilbois

Project website and relevant contact details

The project website has the address: http://www.eumbrella.org/timer.html. The TIMER website is included within the Portal: **EUmBRella website:** http://www.eumbrella.org, including the 3 EU research projects (TIMER, TARKINAID, TRIAD) funded under the 7th European Framework Programme (Health 2012 Programme) for Research in the field of chronic inflammatory/autoimmune diseases, within the bilateral S&T cooperation between Europe and Brazil.

Contractors involved:

- Fondazione Humanitas per la Ricerca (ITALY): Alberto Mantovani
- Universidade Federal de Minas Gerais (BRAZIL): Mauro Teixeira
- Universidade de Sao Paulo (BRAZIL): Fernando Queiroz Chuna
- Fundacaov Oswaldo Cruz (BRAZIL): Marco Aurelio Martins
- Istituto di Ricerca in Biomedicina (SWITZERLAND): Mariagrazia Uguccioni
- University of Glasgow (UK): Gerry Graham
- Telormedix SA (SWITZERLAND): Robero May- out from the project 15th May 2015
- The Provost Fellows, Foundation Scholars & the Other Members of Board of the College of the Holy & Undivided Trinity of Queen Elizabeth near Dublin (IRELAND): Luke O'Neil
- ALTA Ricerca e Svluppo in Biotecnologie S.r.l.u. (ITALY): Paola Cesaroni
- NovImmune SA (SWITZERLAND): Marie Kosko-Vilbois
- Dompè Farmaceutici Spa (ITALY): Marcello Allegretti

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