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REPORT OF MEETING

XXIst scientific meeting of the Italian Association of Developmental and Comparative Immunobiology (IADCI), 12 - 14 February 2020, Department of Biotechnology and Life sciences (DBSV), University of Insubria, Varese, Italy

Organizers: **Annalisa Grimaldi, Magda de Eguileor, Gianluca Tettamanti, Roberto Valvassori, Nicolò Baranzini, Daniele Bruno, Aurora Montali, Laura Pulze**

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Session 1. Chairmen: Maria Rosaria Coscia, CNR of Naples, Naples, Italy and Piero G. Giulianini, University of Trieste, Trieste, Italy

Fish lymphocytes as an equivalent of mammalian innate-type lymphocytes?

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The immune defence system of vertebrates in its molecular and cellular components is remarkably conserved from teleost fish, the more ancient extant representatives at the base of the evolutionary lineage that brings to mammals. Multiple observations support the hypothesis that a layered system of immune responses accumulated among vertebrates over evolution, and lower layers behave as the immune system actually present in extant fish species. In this view, lymphocytes are classified as responsible of acquired responses, but recent evidences show that mammalian lymphocyte subpopulations may behave as innate cells, engaging non-self rapidly and without antigen presentation.

Innate-like lymphocytes i) maintain gut homeostasis and provide early responses to intestinal infections, ii) are involved in autoimmune diseases and cancer iii) are able to combat non-self in a MHC-independent fashion, iv) produce unbiased natural polyreactive antibodies, v) are associated to typical cytokine patterns. The main lymphocyte subpopulations displaying innate-like activities in mammals have been identified as B1-B cells, γδ-T cells, MAIT cells, and NKT cells.

Our research focuses on the spatial and temporal origin of fish lymphocyte populations, on their *in vitro* activities, and on the molecular and morphological signatures of fish lymphocytes. The aim is to predict knowledge related to mammalian innate lymphocytes from fish lymphocytes and to model human diseases. This review will present evidences suggesting the similarities between fish lymphocytes and mammalian innate-like lymphocytes.

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T lymphocytes in the sea bass (*Dicentrarchus labrax*) intestine

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The European sea bass *Dicentrarchus labrax* is a marine species in which lymphocyte distribution in the digestive tract (DT) was first described, and much knowledge on DT immuno-physiology is available, including gene expression profiling that revealed functional specialization along the DT.

Anatomically, sea bass does not contain lymphoid aggregates in the intestinal mucosa, however, its gut harbors high numbers of CD3ε$^+$, TCRβ$^+$ and CD8α$^+$ cells and rare CD4$^+$ cells. Among the intestinal T lymphocytes, a large population expresses γδ-T receptors. These cells play an essential role in intestinal cell-mediated immunity, and it is postulated in teleosts they are important in tolerance or in attack against the microbiota. In sea bass, TCRγ mRNA was constitutively expressed at high levels in DT, and intestinal leukocytes stimulated *in vitro* by poly I:C showed an increase in TCRγ transcript levels. In addition, we showed that the TCRγ chain undergoes spontaneous somatic recombination in the DT, together with an increase of RAG-1 transcripts in its posterior segment. When juveniles were infected *in vivo* with a retrovirus (*Betanodavirus*), expression of TCRγ was down-regulated. Recently, a polyclonal antiserum was obtained against peptides deduced from the sea bass TCRγ full length sequence. IHC showed the presence of TCRγ-bearing cells in the intestinal mucosa, mainly located in the lamina propria and rarely in the epithelium. Immunogold in pre-embedding of intestinal leukocytes revealed the ultrastructural characteristics of TCRγ$^+$ cells, which fit with the typical lymphocyte morphology. In mammals γδ-T cells possess unique features; for instance, they do not display MHC restriction, and they distinguish unconventional antigens, such as phosphorylated microbial metabolites and lipid antigens. Sea bass intestinal mucosa responds to different diets and although the interplay between nutrition and the immune system is well recognised in teleosts, the link between diet, gut microbiota and health in sea bass is only in its infancy.

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Evolution, adaptation and immune functions of fish F-type lectins. The novelty of FBL from *Trematomus bernacchii* (Boulenger, 1902)

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Lectins are a protein family, present in almost all living organisms and involved in different biological pathways, such as immune responses.

The Fucose Binding Lectin (FBL), constitute the latest lectin family identified and characterized in fishes. The FBL family is constituted by a large number of proteins exhibiting multiples of the F-type motif, either tandemly arrayed or in mosaic combinations with other domains.

In an early step a FBL has been isolated and characterized from serum of the Antarctic fish *Trematomus bernacchii* by affinity chromatography on fucose-agarose column.

A clear Bacterial agglutinating activity (BA) towards different bacteria strains (*Escherichia coli*, *Kokuria rhizophyla* and *Bacillus subtilis*) and Hemagglutinating activity (HA) toward rabbit erythrocytes was induced from the serum as well from the purified protein and thus confirm its involvement in host pathogen interactions.

In SDS-PAGE analysis, the FBL exhibited an apparent molecular weight of 30 kDa. This data is confirmed from the sequence of the F lectin recognised on the *T. bernacchii* transcriptome. The sequence shows a similar and coherent structure with a supposed Mw 32.16 kDa and an isoelectropoint of 5.21. Furthermore, sequencing the N-terminus confirmed the identity of the sequence runned on SDS PAGE and blotted on PVDF membrane.

The HA activity was analyzed at different temperatures and it was maintained also at the physiological living low temperatures of this fish habitat (close to 0 °C).

Therefore, in order to identify trends linked to cold adaptation in Antarctic fish, we present our hypothesis on the conformational change determined by the aminoacidic substitutions respect the others fish fucolectins living in warmer water on the light of the general phylogenetic scenario including the new preliminary data on sharks FBL.

Investigations on *IgT* genes of sub-Antarctic fish shed light on the CH2 exon loss

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The Antarctic fish belong to the Perciform suborder Notothenioidei, which constitutes the major ichthyofauna living in the freezing waters surrounding the Antarctic continent and represent one of the most successful examples of adaptive radiation in a marine environment. The development of the Antarctic Polar Front, a thermal barrier that inhibits species from migrating between sub-Antarctic and Antarctic waters, has contributed as an important biological boundary to the diversification of Notothenioidei into eight different families: five are mainly Antarctic and three occur in the coastal waters of New Zealand, Australia, and high-latitude South America. In the past few years, we have isolated and characterized the □ gene, encoding the IgT heavy chain constant region, from one or more members of each Antarctic notothenioid family, disclosing that all of them share the loss of most heavy chain second constant domain (CH2). This finding prompted us to go backwards through the phylogeny of Notothenioidei in an attempt to reconstruct the loss of the CH2 exon. To this end, we have focused on some species each representative of one of the three sub-Antarctic notothenioid families: *Bovicthus diacanthus* and *Cottoperca gobio* (family

Bovichtidae), *Eleginops maclovinus* (family Eleginopsioidae), *Pseudaphritis urvillii* (family Pseudaphritioidea). These species have proved to be crucial in our investigations on the molecular changes occurred in exon–intron regions through evolution of Notothenioidei. Interestingly, based on a comparative analysis at genomic level, we highlighted that *P. urvillii* and *G. gobio* retained the whole CH2 domain like *B. diacanthus*. However, both species showed also some deletions in close proximity to the CH2 exon similarly to *E. maclovinus* that we had previously found to be devoid of almost the entire CH2 domain. Overall, our studies have shed light on key steps of genome modifications, occurred during notothenioid IgT evolutionary history.

Too warm or not too warm… Is the antioxidant system of Antarctic fish ready to face climate changes?

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Antarctic fish are considered highly stenothermal with limited evolutionary potential to cope with climate changes, due to the short- and long-term stability of thermal conditions in the Southern Ocean over the last million years. Recently, warm acclimation experiments performed on some notothenioid species have indicated a significant capacity for these fish to elevate their heat tolerance, over the environmental critical thermal maximum (CTmax), through acclimation. This result suggests that thermal plasticity may be universal throughout the Antarctic ichthyofauna. However, the physiological and genetic bases of their heat tolerance has been poorly studied. In the present work we described the molecular characterization of mitochondrial peroxiredoxins (Prdx) in the Antarctic emerald rockcod *Trematomus bernacchii* and gene expression of these antioxidant enzymes in various tissues, in response to short-term thermal stress. The obtained data are the first on the molecular and functional characterization of the genes encoding Prdx3 and Prdx5 of Antarctic fish, and constitute a further contribution to study these enzymes in specific ecological contexts such as Antarctica. Our expression analyses may be important for predicting climate change responses in this organism. In fact, the obtained results revealed rapid and specific responses of the Antarctic emerald rockcod to wormer temperature. The presence of a Prdx (*prdx3* gene) whose expression is activated by increased temperatures, may be a condition that limits the stenothermy of *T. bernacchii*, making this species less vulnerable to moderate environmental temperature changes and other environmental perturbations associated with global climate changes. (Supported by P.N.R.A. and M.I.U.R. grants)

Heat stress in *Trematomus bernacchii:* bias of experimental design

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The studies on *Trematomus bernacchii* suggest that this shallow water benthic species has reduced ability to acclimate and tolerate warmer conditions, since like the other Antarctic marine organisms it adapted to the extreme and stable Antarctic sea.

Understanding the effects of exposure of stenothermic species to prolonged sub-lethal heat stress is of extreme interest in the scenario of rising polar sea temperatures presented by climate change.

This study evaluated the impact of a 1.5 °C temperature increase over 19 days on some hematological and transcriptomic parameters. Blood samples were obtained from heart, fixed and embedded in either acrylic or epoxy resin for ultrastructural analysis. The morphology of erythrocytes in semithin section was evaluated through the ImageJ shape descriptors. The mean erythrocytes circularity in control animals decreased from 0.810 ± 0.034 to 0.672 ± 0.079 after 19 days. The mean circularity in experimental ones changed from 0.820 ± 0.030 to 0.626 ± 0.051. The erythrocytes became more elliptical over the time during the experiment, regardless of the temperature of the water. The pairwise control-experimental comparisons of cells circularity are all not significant ($p > 0.05$), whilst difference of cells circularity is already significant ($p < 0.05$) by comparing freshly caught animals (0.911 ± 0.018) and the control ones after 1 week of acclimation at the beginning of the experiment.

Samples of gills, brain and muscle were also collected from specimens in all conditions in order to perform RNAseq analysis and study the response of *T. bernacchii* to temperature increase also from a transcriptomic point of view. A reference transcriptome was assembled with sequencing reads obtained from Short Read Archive, yielding promising results as quality and completeness were high: N50 = 1650 and BUSCO score against the Actinopterygii ortholog database (82.9 % complete, 10 % fragmented and 7 % missing). Differential expression analysis are now in progress.

These results highlight how biases in experimental design can affect the study of sensitive polar species physiology. Therefore, in order to better understand the physiological responses to challenges of these species it is advisable to adopt an eco-physiological approach minimizing the effects of confinement during experiment. Morevover, the awareness of these limits will be of great help in the analysis of transcriptomic data.

Glutathione peroxidases in the striped rockcod *Trematomus hansoni*

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A large number of special physiological features that allow the life in their extreme environment characterizes Antarctic species. In particular, the low temperature and salt concentration are chemiophysical conditions that increase oxygen solubility and, consequently, the pO_2 and the rate of ROS formation. With the aim to study the components of the antioxidant defense system in the Antarctic teleosts, we have characterized three genes codifying glutathione peroxidases (GPxs) in the striped rockcod *Trematomus hansoni*. GPxs are a family of antioxidant enzymes that are able to reduce hydrogen peroxide and organic hydroperoxides, thus representing an important protection against the risk of oxidative stress. In the genome of *T. hansoni* we have verified the presence of three of the six GPxs isoforms known in vertebrates: GPx1, GPx3, GPx4. Multi-alignment analysis, performed with fish orthologous sequences, demonstrated high conservation of the amino acids involved in catalytic activity of the GPx isoforms of *T. hansoni*. However, some substitutions with polar amino acids are characteristics of GPx1 of Antarctic species, probably related to a thermal adaptation. The gene transcriptions of GPx1 from various tissues (gills, heart, liver, spleen, and skeletal muscle) of *T. hansoni* has been measured by Real-Time qPCR. The gills are the organ in which the highest levels of GPx1 mRNA accumulation is present. GPx activity have been measured in the same organs. The highest levels are present in heart and liver. The tissue-specific differences in the mRNA and active protein accumulations are probably related to a peculiar regulation of gene expression and the physiological function characteristic of these organs. (Supported by P.N.R.A. and M.I.U.R. grants).

Assessing immunological memory in the solitary ascidian *Ciona robusta*

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Available information on the immune defensive mechanisms active in the solitary ascidian *Ciona robusta* includes the functional evaluation of phagocytic and encapsulating activity, largely brought about by phagocytic cells within the haemocyte population, the molecular and functional definition of complement components, and the genome-based identification of a number of immune-related genes and pathways, homologous to vertebrate counterparts. Since *C. robusta* only displays highly conserved innate immune mechanisms, being devoid of an adaptive immune system, this organism is an excellent model for studying the features of innate memory, *i.e.*, the capacity of the innate immune system to re-programming its responsiveness to potentially dangerous agents upon repeated exposure. In this study, we have developed an *in vivo* model for assessing the establishment and molecular/functional features of innate memory, by sequentially exposing *C. robusta* to a priming stimulus (the gram-negative bacterial agent LPS, or the gram-positive molecule LTA), followed by a period of resting to return to basal conditions, and a challenge with the same agents in homologous or cross-stimulation. The endpoints of immune activation were a functional activity (phagocytosis) and the molecular profiles of immune-related gene expression. The results show that exposure of *C. robusta* to bacterial agents induces a reaction that primes animals for developing a different (expectedly more protective) response to subsequent microbial challenges, showing the effective establishment of an immune memory. This immune memory relies on the modulation of a number of different mechanisms, some of which are priming-specific, others that are challenge-specific, and others that are completely non-specific, *i.e.*, are common to all priming/challenge combinations (*e.g.*, up-regulation of the *Tnf* and *Lbp* genes).

Memory-dependent expression of the humoral immunity-related gene *C3ar* inversely correlates with memory-dependent variations of phagocytic rate, suggesting that complement activation and phagocytosis are alternative defensive mechanisms in *C. robusta*. Conversely, memory-dependent expression of the cellular immunity-related gene *Cd36* directly correlates with variations of phagocytic rate, suggesting a direct involvement of this gene in the functional regulation of phagocytosis.

Stress granules in C*iona robusta*: molecular evolution of TIAR and TTP and early evidence of their gene expression under stress conditions induced by metals

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Stress granules are non-membranous cytoplasmic foci composed of messengers (not translated), ribonucleoproteins, translation initiation components and other additional proteins, that represent a primary mechanism by which gene expression is rapidly modulated when cells are

subjected to adverse environmental conditions (KEDERSHA *et al.*, 2002; ANDERSON *et al.*, 2009; LAVUT *et al.*, 2012; WARIS *et al.*, 2014). Very few works have been devoted to study the presence of molecular components of stress granules in invertebrate animals. In this work, we characterized, for the first time in the solitary ascidian *Ciona robusta*, the genetic sequences of two important protein components of stress granules, TIAR (TIA-1 related to proteins) and TTP (tristetraprolin), and carried out the first studies on their gene expression. The sequences characterized for *tiar* and *ttp* genes have allowed to start a study on the molecular evolution of these proteins in animals: for TIAR the obtained results are consistent with recent phylogenetic analysis that place tunicates as sister group of vertebrates, whereas the phylogenetic position of TTP remains still uncertain. The data on mRNA expression, provided by qRT-PCR analysis, are absolutely the first obtained in non-mammalian animals. As expected, the exposure to each metal (Cu, Zn and Cd) led to a generalized decrease in mRNA expression levels for both TIAR and TTP, suggesting that the metal accumulation induce acute stress and the inhibition of the transcription of *tiar* and *ttp* genes. The data presented here improved our knowledge about the molecular evolution anti-stress proteins in metazoans and emphasize the importance of the transcription of *tiar* and *ttp* genes, which represents an efficient physiological response allowing *C. robusta* to survive in the presence of metals in the marine environment (Supported by M.I.U.R. grant).

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Cocktails and long lasting immunity: evolution of innate defenses and bacterial resistance

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Upon infection, insects produce a cocktail of immune effectors to combat pathogens. This result of natural selection is in contrast to medical applications of antimicrobials, where often only one drug is employed.

Here, I will first discuss interactions between antimicrobial peptides as a main group of immune effectors and what the evolutionary consequences are for both, host and pathogen. Antimicrobial peptides are usually synergistic when tested in vitro, in vivo the situation is more complex and yields results that cannot be predicted from in vitro studies. Also, I will report on how antimicrobial peptide resistant bacteria fare when exposed to an insect immune system. Based on these findings I will then ask, how to explain the evolution of complex immune systems. Finally, I will ponder if and how research on insect AMPs and bacterial resistance evolution against them can inform medical applications of AMPs and of antimicrobials more generally.

Horizontal gene transfer can drive the evolution of insect immune defences

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Horizontal gene transfer (HGT), the accidental acquisition of genetic material by an organism, is extremely common in prokaryotes but represents an important mechanism for genome evolution and acquisition of new traits also in eukaryotes. In multicellular organisms this phenomenon is often mediated by transposable elements or symbionts and parasites.

Recently, an original mechanism of HGT has been described in insects. In particular, the analysis of genomes of different lepidopteran species revealed multiple DNA sequences derived from polydnaviruses (PDV). PDV are symbionts of parasitic wasps attacking lepidopteran larvae, which are injected during the oviposition and trigger metabolic and physiological alterations in the host, including immune suppression, to ensure progeny survival and development. Despite the frequency of this peculiar HGT, an in depth characterization of the function of acquired sequences in recipient lepidopterans is almost lacking. This work elucidates the functional role of *Sl gasmin*, a PDV gene that has been transferred to an ancestor of the moth species *Spodoptera littoralis* and domesticated.

Sl gasmin is highly expressed in circulating immune cells, the hemocytes, of *S. littoralis* larvae and *Sl gasmin* transcripts significantly increase upon pathogen challenge. The functional characterization of *Sl gasmin*, performed by RNA interference, has elucidated its fundamental role in the phagocytosis of bacteria by hemocytes. Moreover, proteomic analyses have allowed to clarify that Sl gasmin protein is secreted by hemocyes into insect hemolymph and acts as an opsonization factor. Indeed, the protein binds on bacteria surface and mediates their recognition by hemocytes.

This study demonstrates that in insects, important immune functions do not necessarily originate from evolution of existing genes but can be acquired by HGT events, paradoxically mediated by their natural enemies that are specialized in evading insect immune responses.

Analysis of cellular and humoral immune response in *Hermetia illucens* larvae

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The larvae of the black soldier fly, *Hermetia illucens*, are well known bioconverters due to their ability to grow and develop on a wide range of organic waste substrates. Despite the great interest on the use of these larvae for bioconversion, there is a lack of knowledge about their biology, in particular regarding their immune system.

In the present study we investigated the mechanisms involved in cellular and humoral response in *H. illucens* larvae. First, we performed a morphological characterization of immune circulating cells (i.e., hemocytes), before and after the immune challenge, and evaluated their phagocytosis and encapsulation capacity. Moreover, we analyzed the activity of key components of the humoral immune response, such as phenoloxidase, lysozyme, and antimicrobial peptides (AMPs).

Our results clearly show that *H. illucens* larvae are able to mount efficient immune responses, both at cellular and humoral level. Hemocytes proliferation and responses are rapidly activated after infection and the humoral response mainly involves the increase in lysozyme activity and the recruitment of AMPs, while phenoloxidase seems to be inhibited in infected larvae.

This study provides basic information about the *H. illucens* larval immune system and opens up the possibility to improve the quality of the larvae during mass rearing through a modulation of the immune response.

Innate immune response and antimicrobial peptides in the silkworm *Bombyx mori*

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The domesticated silkworm *Bombyx mori* has been extensively studied as a model organism for Lepidoptera genetics and for its economic value in silk production. Since silkworm infectious diseases can cause crop losses of ~27–35 %, a great attention has been posed on the study of *B. mori* immune mechanisms. *B. mori* has an innate immune system, whose main effectors are the antimicrobial peptides (AMPs). Silkworm strains are commonly grouped into four geographical types (Japanese, Chinese, European and Tropical), characterised by a variable susceptibility to infections. Recently we showed that silkworm strains originating from the four geographical areas were characterised by a different susceptibility to *Serratia marcescens* and *Enterococcus mundtii* bacteria, with the Indian strain displaying the lowest sensitivity to *S. marcescens* and the European one to *E. mundtii* (Romoli *et al*. 2017). The resistance of the Indian strain to *S. marcescens* seemed to be associated with its capability to recognise the pathogen and promptly activate the systemic AMP transcription. On the other hand, all the strains were able to activate the AMP response against *E. mundtii*. However, the highest resistance of the European strain appeared to be related to the specific composition of its AMP cocktail, containing more effective variants such as a peculiar Cecropin B isoform, named Q53 CecB.

A further characterisation of the Q53 CecB antimicrobial property and action mechanism showed that the peptide exerted an efficient membranolytic activity also against relevant human pathogens, such as *Pseudomonas aeruginosa.* In addition, Q53 CecB did not show any cytotoxic activity or haemolytic effects against human cell lines or erythrocytes (Romoli *et al.* 2019;).

Taken together our data suggest that *B. mori* strains with distinct genetic backgrounds employ different strategies to counteract bacterial infections, whose efficacy appears to be pathogen dependent. The highest antimicrobial activity of the European Q53 CecB variant against clinically relevant microorganisms represents an interesting feature for possible biomedical applications.

Use of silkworm as infection model for novel antibiotic screening and development

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The development of new drugs requires a series of preclinical tests followed by the clinical trials needed for assessing the safety and efficacy of the compound. Commonly, mammalian models (e.g., mice and rats) are used in preclinical tests. Due to the bioethical issue, the time required for the analyses, and the high costs, in

the last few years, alternative animal models, as Lepidoptera, have been proposed for the initial screening phase.

In this study, we evaluated the response of the silkworm *Bombyx mori*, infected with *Staphylococcus aureus*, to the treatment with different antibiotics (vancomycin, teicoplanin, and dalbavancin). We monitored the survival rate of the larvae, analysed different cellular and humoral markers of the immune system, and evaluated the bacterial load in the hemocoel, performing experiments at 37 °C to reproduce human physiological conditions.

While AMP expression and bacterial load resulted to be inadequate to our purpose, we demonstrated that larval survival rate, hemocyte viability, and activity of prophenoloxidase system respond to the infection as reliable and reproducible markers. Considering the limited costs of using *Bombyx mori* as infection model, the quickness of analysis, and the easiness of use, the three selected markers might represent potential tools for accelerating the discovery and development of novel antimicrobial drugs.

20-OH-ecdysone and extracellular ATP are not pro-autophagic factors for the lepidopteran fat body cell line, IPLB-LdFB

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Autophagy is a conserved self-protective cell process that may also help the induction of type I programmed cell death (PCD-I, apoptosis) and, specifically during insect metamorphosis, it also represents the foundation of type II programmed cell death (PCD-II). The double role played by autophagy in insects has been mainly studied in *Drosophila melanogaster* and *Bombyx mori*, thus we investigated PCD-II initiation in an alternative *in vitro* insect model, IPLB-LdFB, a cell line from the lepidopteran pest *Lymantria dispar*. IPLB-LdFB derive from larval fat body, an organ encompassing metabolic as well as immune-related functions that is deeply rearranged during metamorphosis. After a 2 h-incubation with the ATP synthase inhibitor oligomycin A (OA, 10 µM), IPLB-LdFB cells release in the conditioned medium (CM) uncharacterized pro-autophagic factors (PAFs) able to induce PCD-II in naïve IPLB-LdFB. In this study, we looked for the identity of PAFs and the signaling pathways they activate. Size-exclusion centrifugation of CM evidenced that PAFs are retrieved into the < 3 kDa fraction. Proteomic studies on CM did not provide < 3 kDa PAF candidates, thus we investigated the effects of 20-OH-ecdysone and extracellular ATP as potential non-protein candidates. Surprisingly, 20-OH-ecdysone (0,1 ÷ 10 µg/mL) was ineffective on IPLB-LdFB cells and did not promote neither PCD-II nor other forms of cell death, after 72 h-incubation. Conversely, morphological, flow cytometry and TUNEL assays showed that extracellular ATP (0,1 ÷ 5 mM) induced PCD-I in IPLB-LdFB cells after a 6 h-exposure. Accordingly, luminometric assays showed CM does not contain significant levels of ATP, excluding also this molecule from the potential PAFs released under OA treatment. In order to elucidate the signaling pathways elicited by the still unidentified PAFs, we assessed whether single protein kinase (PK) inhibitors could block the CM effects. Cell viability, morphological and flow cytometry analyses showed that all the inhibitors tested (namely H-89 for PKA, calphostin C for PKC and wortmannin for PI3-kinase) acted on cell size and cytoplasm organization, but they did not induce cell death and did not prevent CM lethal effects after 24 h-experiments. This suggests that the PAFs in the CM may promote PCD-II through multiple signaling pathways. Our studies confirm the complexity of the PCD-associated processes also in an *in vitro* system, and suggest that, in spite of the high conservation of the basic mechanisms from the unicellular organisms to the metazoans, autophagy can be carried out under various stimuli and following multiple and diversified signaling pathways.

Discovery and characterisation of novel CHHs involved in the immune response of the red swamp crayfish *Procambarus clarkii*

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Crustacean hyperglycaemic hormone (CHH) has many functions to regulate carbohydrate metabolism, ecdysis and reproduction including ion transport in crustaceans. Furthermore, rapid mobilization of neuropeptides, including CHHs, from the sinus gland in the eyestalks represent the primary response to stress.

Here we report the identification of two potential transcripts pertaining to the CHH family in *Procambarus clarkii*, thanks to a whole-transcriptome sequencing approach.

These two members, named CHHip (CHH Immune-related *Procambarus*) and CHHop (CHH homologous *Procambarus*) share the typical features of the CHH (the presence of a CHH-precursor-related peptides between the signal peptide and the peptide, and 6 conserved cysteines involved in the three typical disulfide bonds of the CHH-superfamily) and they have been found up-regulated following the knock-down of the main CHH isoforms through RNA interference.

To shed light on their possible role in the immune response of *P. clarkii* two experiments have been performed to evaluate CHHip and CHHop relative expression in the eyestalk in response to a lipopolysaccharide (LPS)- and a *Staphylococcus aureus*-challenges (1 µg/animal (body weight 40 g) of LPS and *S. aureus* 3 x 108 CFU/100 µl per animal, respectively).

Following the LPS injection, only CHHip increased significantly (7-fold at 2 h post injection–hpi– and 14-fold at 4 hpi, $p \leq 0.01$). *S. aureus* injection triggered CHHip at 6 and 12 hpi ($p \leq 0.05$), but not at 24 hpi. Conversely, CHHop resulted up-regulated exclusively at 24 hpi of *S. aureus* ($p \leq 0.05$).

These preliminary findings suggest an early immunological response to bacterial infection driven by CHHip followed by an up-regulation of CHHop, with the nearly contemporary down-regulation of CHHip, at 24 hpi. These results seem to support the existence of a combined action of CHHip and CHHop in response to LPS and Gram - bacteria in a time-dependent manner and increase the spectrum of action of this family of hormones up to their direct involvement in the immune response.

Hemocyte depletion as a tool for studying immune cell dynamics and contribution to fundamental biological processes in the freshwater snail *Pomacea canaliculata*

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Pomacea canaliculata is an invasive freshwater gastropod native to South America which spread all over Asia, with severe consequences on ecosystems and public health. This animal has been recently retrieved also in EU and USA and it is listed among the "100 of the world's worst invasive alien species". Because of the uncommon adaptive features of this snail, its immune system (IS) has been investigated through cytological analyses, flow cytometry, immunohistochemistry, proteomic and molecular techniques. Major attention has been paid to the characterization of the cellular component of *P. canaliculata* IS, especially circulating hemocytes. Although the morphology and ultrastructure of these cells have been described, their development, maturation and the distribution of hematopoietic sites still remain unclear. Similarly, the roles played by circulating hemocytes in fundamental activities, like tissue repair, wound healing and organ regeneration are unknown. On these bases, we decided to study circulating hemocytes during hemolymph repopulation, after the application of either repeated hemolymph withdrawals or injection of a pro-apoptotic drug specifically targeting phagocytic cells in mammals and insects, *i.e.*, clodronate liposomes. Samples of withdrawn hemolymph were analysed with optical microscopy and via the ImageStream®X-II imaging flow cytometer, while hemolymph from clodronate-treated snails underwent cytocentrifugation and was stained with combined histochemical and fluorescent staining. The observation of hemolymph after multiple withdrawals showed limited variations in its composition and a complete deletion of circulating hemocytes has never been observed, suggesting the presence of significant hemocyte reservoirs in our model. Our first data from clodronate-treated animals, instead, indicate that the drug could efficiently and temporarily remove circulating hemocytes in a short-time incubation, without affecting snail survival. On the whole, multiple hemolymph withdrawals and injections of clodronate liposomes followed by morphologic and/or cytofluorimetric analyses, represent diverse and valid approaches for in-depth and novel studies on hemocyte balancing, turn over and maturation in molluscs. Moreover, collected information and tools may be extended to investigate the contribution of the IS in other processes relevant for the snail survival, *e.g.*, wound healing and organ regeneration.

microRNAs: possible new players in bivalve antiviral immunity

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Deep sequencing is contributing to enlarge the view of small non-coding RNAs (sncRNAs), a group of RNA molecules not producing proteins while serving other roles. Among the variety of sncRNAs (short interfering RNAs, extracellular RNAs, small nuclear ribonucleoproteins, small nucleolar RNAs and piwi-interacting RNAs), microRNAs (miRNAs) have been widely studied because of their role in disease and as biomarkers. Mature miRNAs are short sequences (20-23 nt), which originated from stem-loop precursor RNAs (pre-miRNAs). miRNAs mostly act as post-transcriptional repressors by targeting 3' untranslated regions (3'-UTRs) of messenger RNAs, with paramount functions across various cellular and developmental processes such as immunity, cell behavior and host-microorganism interactions. Evolutionary studies showed that miRNA families have been continuously added along the evolution of bilaterians, and that miRNAs are conserved under strict negative selection. Because of these features, including also a conserved biogenesis mechanism and a small probability of convergent evolution, miRNAs have been proposed as powerful markers for phylogenetics.

We have previously demonstrated the conservation of the miRNA complement genes in bivalves. However, the knowledge regarding bivalve miRNAs are fragmentary with sncRNA sequences available only for a few species (*Pinctada martensii, P. fucata, Crassostrea gigas, C. hongkongensis, Chlamys farreri, Tegillarca granosa* and *Ruditapes philippinarum*). Aiming to identify conserved and novel bivalve miRNA families and to verify their contribution during viral infection, we produced sncRNA sequencing data of two species, the blood clam (*Scapharca broughtonii*) and the Pacific oyster (*C. gigas*). Using the corresponding genomes, we analyzed 167 and 151 bona-fide miRNAs for clam

and oyster, respectively. By measuring their expression levels during *Ostreid herpesvirus* (OsHV-1) infection in both species, we reported specie-specific expression patterns involving different miRNAs. Several of the most modulated miRNAs have been reported as involved in antiviral immunity also in shrimp, underpinning possible functional conservation. In conclusion, we provided a first comparative view of bivalve "miRNAome", tracing commonalities and differences within invertebrates.

Evolution and molecular diversity of mytilin-like defense peptides in marine mussels

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The cysteine –stabilized alpha/beta motif (CS-αβ) is a widespread structural scaffold found in several low-molecular weight cationic peptides with defense function. While many CS-αβ antimicrobial peptides have been described as major players in bivalve immune response, several aspects concerning their evolution are still unclear and a clear-cut definition of the relationship among defensins, mytilins, myticins and other structurally similar peptides is still lacking.

Here, we redefine the distribution of mytilin-like CS-αβ peptides thanks to a bioinformatic screening of the sequence resources available for Mytilida. We highlight that, in spite of limited primary sequence similarity, these AMPs retain a nearly identical three-dimensional folding, stabilized by eight highly conserved cysteine residues. We discuss that the variable position of the C1-C5 disulfide bond has a significant effect on the structural flexibility of the mytilins identified in *Perna* spp., as well as in a few novel *Mytilus* spp. mytilins.

We further describe the organization of the *Mytilus galloprovincialis* mytilin gene cluster, reporting the presence of two pseudogenes in addition to the four canonical genes previously identified, and discuss the presence of additional dispensable mytilin genes subject to presence/absence variation.

Session 3. Chairmen: Adriana Vallesi, University of Camerino,Camerino, Italy and Gianfranco Santovito, University of Padua, Padua, Italy

Earthworm cellular immunity: an old story re-loaded from the nanomaterial's point of view

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Broad commercial and biomedical applications of silver nanoparticles (AgNPs) may result in unintentional release of the AgNPs and their byproducts into the environment posing potential impacts on various living beings. It is largely unexplored how immune system reacts against AgNPs, albeit those particles can be effectively recognized and engulfed by innate immune cells. In this regard, special attention should be paid to nano–immuno interactions. Earthworm macrophage-like cells (coelomocytes) offer potentially sensitive and accessible resources of monitoring the complex interactions of NPs with biological systems.

Previously, we studied the evolutionary conserved molecular and stress mechanisms in earthworm coelomocytes cross-referencing to human monocytic cells exposed to AgNPs. In our recent study, we have further focused around the species differences within the earthworm taxa, revealing fine differences of various immune and stress-related parameters from two closely-related earthworm species *Eisenia andrei* and *E. fetida* following *in vitro* exposure to AgNPs and AuNPs. Finally, we touch on the emerging aspects of nanoparticle-protein interactions in which earthworm-specific proteins may play an unexpected role in pattern recognition.

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Immunomodulation of selected NPs in *Mytilus* hemocytes: a morphofunctional study

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The nanoparticle (NP) industry is continuously increasing in production and creativity; producing NP with diverse size or surface coating that change their properties and will likely also change their interactions with the immune system of organisms.

In a reducing strategy for the amount of animal used in science, to test such amount of NPs, alternative protocols are needed and *in vitro* experiments have shown to be promising for future testing strategy. In this line, the hemocytes of *Mytilus galloprovincialis* were utilized in *in vitro* short time (30 min) exposure to screen the immunomodulatory effects of several NPs of different size, material and surface modifications (metal based zero valent, nano-oxides, amino functionalized nanopolystyrenes). Moreover, to evaluate the role and interactions of NPs with proteins from the biological media, experiments were performed using NP suspensions in either artificial seawater (ASW) or hemolymph serum (HS). Upon exposure, several functional immune

parameters (lysosomal membrane stability, phagocytosis, lysozyme and ROS release) were evaluated. In addition, the effects of different NPs of hemocyte morphology were investigated by scanning electronic microscopy (SEM).

The results on determination of immune parameters showed activation of immune defences specific to the NPs type and underline the importance of exposure media in the experimental design. The images obtained at the SEM contributed to understand the original hemocyte morphology in different media and their potential morphological changes upon NPs exposure, showing peculiar cell activations.

These data will contribute to build information on the role of size and functionalization of NPs for *Mytilus* hemocyte for both functional and morphological responses.

Effects of different nanoparticles types on benthic foraminifera *Ammonia parkinsoniana*

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Benthic foraminifera, with their developed processes for cellular internalization, can take up nanoscale and microscale particles for example during seawater endocytosis or in sediments via reticulopodia, which are used for gathering food such as bacteria and algae. The potential adverse effects of nanoparticles (NPs) including nanoplastics, in marine environments have recently attracted considerable attention, though remain relevant knowledge gaps, particularly on their effects on the benthos. NPs are expected to make their way into the aquatic environment where sedimentation of particles will likely occur, putting benthic organisms at particular risk.

In this work, the effects of exposure (1 mg/L, 24 h) to different types of NPs (TiO_2, 25 nm; polystyrene 42 nm and SiO_2, 92 nm) were investigated in the benthic foraminiferal species *Ammonia parkinsoniana* using a combination of techniques (TEM; SEM; ESEM; EDS and CMLS). The results show the internalization of NPs in the cytoplasm of *A. parkinsoniana*, as well as the associated intracellular lipid accumulation, free radical production and the alteration of mitochondria. In particular, nTiO2 induced the highest ROS production. Moreover, preliminary gene expression analysis revealed that nTiO2 potentially affects the following molecular pathways: endocytosis, exocytosis, lysosome, ROS and biosynthesis of unsaturated fatty acid. This latter in particular may have a pivotal role in the detoxification process.

These are the first data on the effects of different NPs in a benthic unicellular eukaryote. The results suggest that NPs can have deleterious effects on benthic organisms, which are commonly neglected in the research despite representing an important link in the trophic web. The ability to internalize NPs coupled with NP persistence may also promote their transfer and biomagnification the foodchain.

In light of the increasing occurrence of NPs in marine environments, the evaluation of their cytotoxicity and the internalization capability of bottom-dwelling organisms is an essential step to predict future exposure scenarios. These benthic marine organisms may represent a new model by which to assess future NP impact scenarios in the marine benthos.

Session 4. Chairmen: Giuseppe Scapigliati, University of Viterbo, Viterbo, Italy and Giovanna Parisi, University of Palermo, Palermo, Italy

Identification of novel lumbricins in *Eisenia andrei* earthworms – a missing link of annelids' antimicrobial peptides

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Antimicrobial peptides (AMPs) are the structurally conserved ancient weapons of innate immunity from plants to human. To date only one particular AMP, the lumbricin and its relatives were identified in certain earthworms, but not in the *Eisena andrei*.

Hereby we report the identification of the mRNA for lumbricin and -serendipitously- a novel lumbricin-related peptide in *E. andrei* earthworms. The characterized mRNA sequences for *E. andrei* lumbricin and lumbricin-related peptide are composed of 477 and 575 nucleotides. The proline-rich *E. andrei* lumbricin and the related peptide contain 63 and 59 amino acids, and the anticipated molecular weights are 7413.35 and 7066.84 Da, respectively. The novel *E. andrei* lumbricin and

lumbricin-related peptide revealed the closest relationship with lumbricins from the leech, *Hirudo medicinalis* and *Lumbricus rubellus* earthworms by phylogenetic analysis. Most prominent mRNA expression occured in the foregut (pharynx, gizzard), while other organs had fair (body wall, midgut, ovary, metanephridium, seminal vesicles, ventral nerve cord) or low (coelomocytes) levels. By means of coelomocyte sorting, only the amoebocyte subpopulation has evidenced the mRNA expression for both peptides. During embryogenesis, a gradually increasing expression pattern was observed in the various embryonic stages that were coincident with the mRNA expression pattern during tissue restoration processes. Following 48 h of *in vivo Staphylococcus aureus* bacteria challenge both mRNAs were significantly elevated in coelomocytes, while *Escherichia coli* bacteria or *zymosan* stimulation had no distinguishable effects.

Since *E. andrei* earthworms are widely applied in various toxicological assays, these novel peptides can be monitored as potential molecular targets that may be modulated by environmental contaminants.

Acknowledgements

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Characterisation of the complement system of a colonial protochordate: study of the expression of C3, CR1, C3AR and their role in nonself recognition

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The complement system is one of the most ancient immune effector mechanism of bilaterian metazoans. Three complement-activation pathways are known in vertebrates: the classical, the alternative and the lectin pathways.

In the compound ascidian *Botryllus schlosseri*, a reliable model organism for the study of immunobiology, we demonstrated the presence of the lectin and the alternative pathways. All the complement components identified so far, are expressed by morula cells, the most abundant circulating hemocytes.

In mammals, once the complement system is activated, C3 is cleaved to C3a and C3b, the former exerting a chemokine–like activity, the latter acting as opsonin and, ultimately, activating the lytic pathway. In the present work, we continued our analysis of the role of C3 in *Botryllus* immunity by studying the modulation of BsC3 transcription during the colonial blastogenetic cycle and the effect of bsc3 knockdown on immune responses.

In addition, we looked for putative complement receptors. In mammals, the best-known receptor for C3a is C3aR, whereas CR1 is the receptor, on the phagocyte surface, able to recognize and bind C3b. Here, we describe, in *B. schlosseri*, a gene showing similarity with vertebrate C3aR and three genes with similarity to CR1 (two soluble forms and one transmembrane). We also studied their transcription in the course of the colonial blastogenetic cycle. Results indicate that complement receptor mRNAs are located in different immunocytes, suggesting the presence of a cross-talk between phagocytes and morula cells. Only morula cells, and no other immunocytes type, were labelled by the antisense probe for BsC3aR and the soluble CR1s, whereas phagocytes and young, undifferentiated cells known as hemoblasts were the cells stained by the probe for the membrane-linked BsCR1. Both the *bsc3ar* and *bscr1* genes are constitutively transcribed; however, a modulation of transcription occurs during the colonial blastogenetic cycle as the amount of BsC3aR mRNA abruptly decreased at take-over, whereas no differences were observed when early-cycle and mid-cycle were compared. This is probably related to the renewing of circulating cells at TO, when 20 - 30 % of hemocytes undergo cell death by apoptosis and are replaced by new, differentiating cells entering the circulation in the same period.

Evidence for the chimeric origin of a pheromone-coding gene in *Euplotes raikovi*

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Ciliates, unique among eukaryotes, evolved two types of nucleus which are distinct in both structure and function. A diploid transcriptionally silent germline micronucleus (MIC) with an orthodox chromosomic structure coexists in the same cytoplasm with a polyploid transcriptionally active somatic macronucleus (MAC) showing a unique sub-chromosomic structure. This structure is acquired in coincidence with every sexual event, when the ex-conjugant (or ex-autogamic) cell initiates a new life cycle developing a new MAC from a mitotic product of the synkaryon. In spirotrichous ciliates such as *Euplotes*, the chromosomes of this product undergo impressive phenomena of polytenization, fragmentation in thousands of DNA fragments known as 'Macronuclear Destined Sequences' (MDSs), and DNA elimination. Under the guide of noncoding RNA templates synthesized by the old MAC before being destroyed, these MDSs are assembled into sub-chromosomic ('gene-size') DNA molecules which, amplified to thousands of copies, compose the new MAC. The way to a correct MDS assembly may be crossed by errors, with the consequent generation of functional chimeric genes which can stably be integrated and expressed in the MAC genome.

One of these chimeric genes came to light by studying the genetic basis of the pheromone-mediated self/not-self recognition mechanism in *E. raikovi*. The genome of type I cells secreting pheromone E*r*-1 was found to contain two structurally distinct MAC E*r*-1 coding genes, both expressed via a mechanism of intron splicing responsible for the synthesis of the E*r*-1 soluble

form and a membrane-bound E*r*-1 isoform functioning as autocrine pheromone receptor. The sequence of one gene resulted unmistakably homologous throughout its length with the sequences of other members of the *E. raikovi* pheromone gene family. In contrast, the sequence of the second gene resulted unique at level of a 359-bp segment of the 5' region destined to specify the cytoplasmic domain of the E*r*-1 membrane-bound isoform. By showing that this segment arises from a wrong assembly of a MDS destined to a 2417-bp gene with no relationship with the signaling pheromone system, we provide additional evidence that the generation of functionally active chimeric genes from not-programmed phenomena of somatic MDS recombination is an effective and MIC-independent source of gene variants in the ciliate MAC genome.

The Mediterranean anthozoan *Anemonia viridis* (Forsskål, 1775) for the study of inflammation and regeneration

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Regenerative capability in anthozoans is an important adaptive strategy for their survival to environmental disturbance of natural and anthropogenic origin such as predation or anchoring, that can cause injuries or removal of entire parts of the animal body, and it can be also considered indirectly a further tool of innate immune system. In the context of "self"-"non self" recognition, is significant the interaction with the endosymbiont of the genus *Symbiodinium* and the recognition of pathogens and foreign agents capable of invading the injured tissues.

From these premises and the growing stressors that can cause injuries, it is significant to understand how species respond to physical damage and how they manage to recover and regenerate compromised tissues.

Our research team, studied in the Mediterranean anthozoan *Anemonia viridis* (Forsskål, 1775), the natural seasonal variability of its morphology and enzymatic biomarkers involved in inflammatory process, the immune system response following injection of molecules varied in type and dimension. In particular, after the infection of two pathogenic bacteria *Escherichia coli* and *Vibrio alginolyticus* a particular and strong reaction was observed. These previous knowledge allowed us to examine the activity of enzymes such as proteases (SDS-PAGE on gelatin and fibrinogen substrate), peroxidase and alkaline phosphatase as biomarkers traditionally involved in wound healing event and in the rearrangement of extracellular matrix.

The regenerative process, in this Mediterranean species of anthozoan, was analyzed by subjecting groups of animals to differential tentacle cuts (n = 10, 20, 30) and observing and estimating the regenerative potential after 7, 14 and 21 days from cutting. A morphological and histological observation of the tentacular regrowth event was conducted in addition to the evaluation of the expression of proliferating cell nuclear antigen (PCNA) using the immunoblotting technique.

The future goal is to increase the knowledge of the processes that trigger the immune response in inflammation and regeneration, for the considerable interest in basic sciences and for the transferability of results in biotechnological field.

Morpho-functional characterization of the microenvironment in muscle cell development of *Hirudo verbana*

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Muscle regeneration is a process of great interest to the scientific community because is involved in a big number of disorders that are still without a cure. To study the mechanisms behind this process, the most used animal models have always been rodents, for their closeness to humans. Nonetheless, the rising ethical awareness has encouraged a reduction in the use of vertebrates in the research, but at the same time the complex puzzle in which the different factors interact for muscle regeneration has not been clarified, so the contribution of the *in vivo* experiments is still fundamental.

The leech is an inexpensive and easily manageable animal model and represents an invaluable and necessary alternative for these studies. In fact, in a simple body organization it is able to elicit complex processes (wound-healing, angiogenesis, immune response, and muscle regeneration) characterized by the same phases described for vertebrates.

For all these reasons, taking also in consideration that regeneration recapitulates some of the processes employed during development and that no satellite cells were identified in leech, we aimed to study the structure and composition of the microenvironment where myogenic precursors mature during the body wall growth of the juvenile leech.

In this regard, we performed morphological analyses of the adult and of the juvenile of *Hirudo verbana,* focusing on the extracellular matrix (ECM) organization and on the cell's level of differentiation. Moreover, we also reconstructed one of the main pathways by which mechanical stimuli are integrated and transduced into transcriptional activity: the *Hippo signaling pathway*.

Our data suggest that ECM has a pivotal role in controlling myocytes' proliferation, migration and differentiation thanks to the control exerted by Yap1. Moreover, many cellular types, which concur in this development as stem-like cells of different lineages, were identified in these processes. These preliminary data confirmed that juvenile leech can be a good model for understanding how muscle fibres can growth and differentiate in relation to the

stiffness of ECM and to its conformations, to other mechanical, and chemical stimuli and to the interactions with different cell types.

In silico characterization of the leech *Helobdella robusta*'s matrisome

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Leeches (Phylum: Annelida) have been extensively studied to elucidate sophisticated evolutionary-conserved biological processes that are similar to those of mammals. Recently, the growing interest on extracellular matrix (ECM), owing to its multifaceted roles in physiological and pathological processes, has led the scientific community towards the definition of the matrisome as the list of all the structural ECM components, of those proteins affiliated to them, and of all secreted factors that the ECM is usually impregnated with. This annotated list is an invaluable tool to get a better insight on the role of a substantial component of every process and could help in the design and analysis of data coming from both classical and "-omics" experiments.

Despite the human matrisome as well as those of several animal models have been already annotated, no specific information regarding Annelids and leeches in particular is available. In this work, using a combination of in silico evolutionary conservation, gene onthology, and structural analyses we conduct a first annotation of the *Helobdella robusta*'s matrisome.

Session 5. Chairmen: Magda de Eguileor, University of Insubria, Varese, Italy and Loriano Ballarin, University of Padua, Padua, Italy

T2 ribonuclease-mediated tumor suppression: an evolutionary conserved process involving a cross-talk between cancer cells and the immune system

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The link between cancer development and immune system dysregulation has long been established, and both the innate and adaptive immune systems are widely acknowledged to establish a complex crosstalk *in vivo* with cancer cells, which might culminate into a pro- or anti-tumorigenic response. In this context, cells belonging to the monocyte/macrophage lineage represent a key cellular component of the innate immune system involved in the control of the tumorigenic process, since advanced human tumors are frequently infiltrated with Tumor-Associated Macrophages (TAMs) which actively contribute to tumor growth and dissemination. However, the long established functional plasticity displayed by macrophages can provide these cells with a marked anti-tumor properties as well. Thus, experimental approaches aimed at promoting a macrophage shift *in vivo* from pro-tumor to anti-tumor phenotype represent a deeply investigated research field to develop immune system-mediated oncosuppresive therapies. The human *RNASET2* oncosuppressor gene has recently emerged as a potential tool for macrophage-mediated tumor suppression. T2 Rnases represent very ancient, pleiotropic and evolutionary conserved enzymes involved in a wide range of biological processes, among which host defense is frequently observed. A key role for human *RNASET2* in the regulation of macrophage activity in both *in vitro* and *in vivo* experimental models has been recently reported. Of note, the ability of *RNASET2* to tune the macrophage phenotype has been recently observed in both vertebrate and invertebrate experimental model, thus pointing at a very ancent role for T2 Rnases in the modulation of the innate immune system. Moreover, recent reports suggest a functional role for *RNASET2* in a broader range of immune cell types, pointing at T2 Rnases as a putative regulators of several functional features within the immune system.

The antibacterial role of *Hirudo verbana* RNASET2: evaluation *in vivo* and *in vitro*

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Recent data obtained in the medicinal leech *Hirudo verbana* revealed that the enzyme RNASET2, belonging to T2 ribonucleases family, took part in the regulation and modulation of the innate immune system, by the direct recruitment of immunocompetent cells during bacterial infection. Indeed, the injection of LPS (lipopolysaccharides) and LTA (lipoteichoic acid), principal components of the bacterial cell wall of Gram-negative and Gram-positive respectively, into the leech body wall, triggered an increased expression of the endogenous leech enzyme *Hv*RNASET2 in both macrophages and granulocytes. These results suggested a possible antibacterial role for this enzyme, as observed for the RNase 3 and the RNase 7 in vertebrates. In order to better clarify this aspect, here we conducted *in vitro* experiments by treating *Escherichia coli* and *Staphylococcus aureus* bacterial strains with the recombinant protein *Hv*RNASET2. The effect on microorganisms was analyzed by light, transmission (TEM) and scanning (SEM) electron microscopy, after 3 and 24 h from

the incubation. The images showed an evident agglutination in both cases and immunogold assays indicated a direct interaction between *Hv*RNASET2 and the bacterial cell walls. Moreover, experiments performed both *in vivo* and by using the Matrigel biomatrix (MG), confirmed the presence of *S. aureus* bacteria aggregates in the leech body wall, supporting the idea that, during leech innate immune response, *Hv*RNASET2 triggers bacterial clumps formation to promote a more rapid phagocytosis by macrophages and to elicit a rapid and effective eradication of the infecting microorganisms from inoculated area recruiting phagocytic cells.

Hyaluronan, a new neuroimmune modulator of the microbiota-immune-gut-axis

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The gut saprophytic commensal flora has a fundamental role in the modulation of several local functions including regulation of host immune system and defense against pathogenic microorganisms. Alterations in the symbiotic relationship between the microbiota and the enteric microenvironment underlays development of complex gut disorders such as chronic inflammatory disease (IBD). In recent years, we have focused on hyluronan (HA), an unbranched glycosaminoglycan (GAG) component of the extracellular matrix, as a new molecular player involved in neuroadaptive changes of enteric neuronal circuitries in the inflamed gut. The GAG is a key molecule mediating the host immune response to commensal and pathogenic bacteria by binding to toll-like receptors, TLR2 and 4. Since both TLRs have been localized to enteric neurons and may regulate intestinal inflammation by controlling enteric nervous system (ENS) structural and functional integrity, HA represents a potential molecular tool involved in development of myenteric neural plasticity by tuning adaptive signals at the intersection between the microbiota-innate immunity axis and ENS. This hypothesis is innovative and opens new scenarios in the study of the molecular mechanism involved in the onset and severity of bowel diseases as well as for the development of new therapeutic agents for the treatment of diseases with clinical and social impact, all with underlying derangements of the microbiota-immune-gut axis, such as IBD.

Preliminary data on senescence in hemocytes of the colonial ascidian *Botryllus schlosseri*

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Senescence is a cellular response to damage that limits the proliferation of aged or effete cells and plays physiological roles as it is required for tissue homeostasis.

Colonies of the protochordate *Botryllus schlosseri*, undergo cyclical generation changes or takeovers (TOs) during which adult zooids are replaced by their buds reaching adulthood. The period of time between two TOs is referred to as blastogenetic cycle. During the TO, cells of adult zooid tissues die by apoptosis and are cleared by circulating phagocytes that, in turn, undergo phagocytosis-induced apoptosis and are cleared by new phagocytes in a recurrent, apparently endless, process. In the present work, we demonstrate that phagocytes, after the engulfment of effete phagocytes enter a senescence status and home, in the following mid-cycle, in the ventral islands, on both sides of the endostyle, where undifferentiated (stem) cells are also found. Senescent cells remain in the homing site at both sides of the endostyle and contribute to form the dark masses representing the remains of the previous zooid generations.

Identification of a LPS induced chemo attractive peptide from *Ciona robusta*

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Previously published work demonstrated that in *Ciona robusta,* the LPS-induced inflammatory response leads to the overexpression of a truncated form of an immune-related mRNA by means of a CR-APA whose biological activity has not be investigated so far. By 3D in silico modelling we observed a certain degree of homology to a protein domain of the human cancer-related signaling adaptor protein CT10 Regulator of Kinase (CRK), a vertebrate gene that has been demonstrated to induce cytoskeletal reorganization during cell migration. Starting from this observation, we postulated its possible involvement in cell migration mechanisms upon LPS challenge. We decided to study the biological function of the derived peptide, named CrCP (Ciona robusta Chemoattractive Peptide) using a primary epidermal human cell line (HuDe). To evaluate the potential cytotoxic effect of CrCP peptide on HuDe cells, MTS assays were performed using different concentration of CrCP peptide (from 200 nM to 3.2 μM) for 24 h. Data show that CrCP peptide do not impair the cell viability of HuDe cells. Then, the CrCP peptide (400 nM) was used in in vitro assays to study its involvement in cell motility events. By means of a wound healing and Boyden chamber we demonstrated that the CrCP peptide can induced HuDe motility (p = 0.0277). Furthermore, by Real Time PCR, we investigated whether the CrCP modulated the expression of genes involved in cell motility pathways. HuDe cells treated for 16 h with the peptide showed a statistical significant increase in matrix metalloproteinase-7 (MMP-7) (p = 0.0431) mRNA expression and a significant reduction in E-cadherin (CDH1) levels (p = 0.0277), compared to control cells. Finally, Western Blot analysis demonstrated that the treatment with 400 nM CrCP induced activation of NF-κB signaling pathway. In

conclusion we demonstrate that the LPS-induced peptide CrCP is capable of inducing cell migration of a human primary dermal cell line via a NF-κB-dependent mechanism, leading to the modulation of the expression of relevant genes involved in re-epithelialization restoring tissue architectures probably trough the loss of cell-cell contact allowing a migratory phenotype.

TLR4 present and future perspective

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The mammalian TLRs act as key molecular patterns pathogenic associated (PAMP) sensors, such as bacterial LPS, lipopeptides and flagellins, which are present in microbial cells but not in host cells. It was therefore considered that the TLRs played a central role in the discrimination between "self" and "non-self". However, since the discovery of their microbial ligands, many studies have shown that even molecules derived from the host can act as TLR4 agonists. These endogenous TLR4 ligands tend to fall into the categories of released intracellular proteins, ECM components, oxidatively modified lipids and other soluble mediators. This review aims to summarize the evidence supporting the intrinsic capacity of TLR4 stimulation in some of these proposed endogenous ligands and showing an activity different from the TLR4 receptor agonism.