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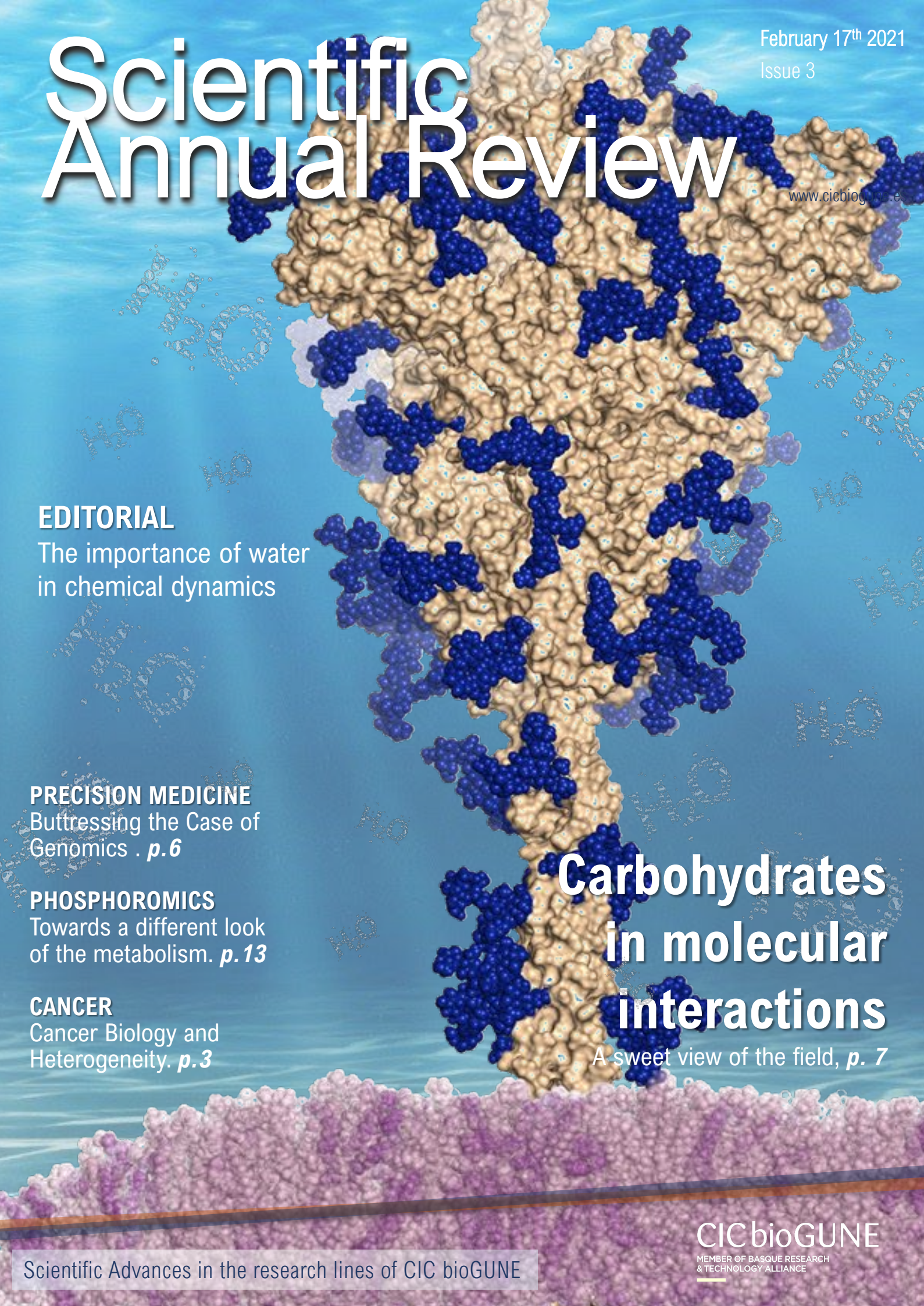

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Scientific Advances in the research lines of CIC bioGUNE

CIC bioGUNE
MEMBER OF BASQUE RESEARCH & TECHNOLOGY ALLIANCE

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The Center

CIC bioGUNE is a collaborative research center focused on Life Sciences, from Chemistry to Biomedicine, from basic to translational Science. Our cutting-edge scientific activity concentrates on discovering the molecular bases and mechanisms of disease to promote development of advanced therapies. Our activity explores four main biomedical research themes like Cancer, Metabolic Diseases, Rare Diseases, and Infectious Diseases organized in two research programmes "*Metabolism and Cell Signaling in Disease*" and "*Molecular Recognition and Host-Pathogen Interactions*".

With our collaborative philosophy we are deeply engaged in multidisciplinary research collaborations with local, national and international colleagues and technology experts. The center is impinged in a heterogeneous network of Academic and Clinical Entities, Research and Technology Centers, and is member of the Basque Research and Technology Alliance (BRTA).

The scientific activity is supported by cutting-edge infrastructures and technology platforms, including advanced equipment for nuclear magnetic resonance (NMR), now recognized as ICTS, electron microscopy, a facility for monoclonal antibody production, as well as different core technology platforms where genomes, proteomes and metabolomes can be analyzed.

General View

Direction

CIC bioGUNE activities are strongly related to our specific mission: to build up an EU-referent knowledge pole in biosciences, which should be able to favour the development of the emerging sectors in the bioscience and health fields, and the incorporation of the proper technologies to be able to enhance the competitiveness of the corresponding industrial (biotech, pharma, etc) sectors. Specifically, CIC bioGUNE acts with a strong commitment of collaboration and coordination with the rest of social and scientific agents in the Basque Country to optimize the existing capacities, and jointly conform an integrative scientific and technological offer of excellence. This offer should be able to boost the evolution of the economy by strongly increasing its intrinsically high added-value. Our research activities cover from the gene to animal models of cellular processes through the determination of biomolecular structure and assembly and the elucidation the key mechanisms and interaction patterns at the highest resolution. Our scientific objectives are transversal and target the complete characterization of the molecular basis of protein-based processes in human pathophysiology and immunological defence, cell proliferation, and development. The final aim is to translate our findings to the clinic, with special interest in precision medicine.

EDITORIAL

The importance of water in chemical dynamics

Gonzalo Jiménez, Osés *Computational Chemistry Lab*

Water is often called the “matrix of life”. These words reflect an ancient sentiment: Paracelsus in the 16th century said that “water was the matrix of the world and of all its creatures”. However, Paracelsus’s notion of a matrix –an active substance imbued with fecund, life-giving properties– was quite different from the classical view that, until very recently, molecular biologists have portrayed of water’s role in the chemistry of life. Although the unusual and important physicochemical properties of liquid water (its potency as a solvent, its ability to form hydrogen bonds, its atmospheric nature and abundance) have been acknowledged, biologists have regarded it only as the scenery on which life’s molecular components are arrayed and perform their “dance of life”. It was common practice, for example, to perform computer simulations of biomolecules in a vacuum. The prevailing (false) notion to justify this procedure was that water does little more than tempering the basic physicochemical interactions responsible for molecular biology. Computational and structural biologists agree today that this assumption was merely an excuse to ignore the very complex and computationally costly effects of water in biomolecular recognition and reactivity. Oddly enough, this neglect of water as an active component of the cell went hand in hand with the observation that environments without liquid water cannot sustain life. I fact, water is essential for life in many ways, and without it biomolecules might no longer truly be biomolecules. Water is important to the structure, stability, dynamics, and function of biological macromolecules. In protein folding, water mediates the collapse of the chain and the search for the native topology through a funneled energy landscape. Water actively participates in molecular recognition by mediating the interactions between binding partners and contributes to either enthalpic or entropic stabilization. Interestingly, it has been suggested that enthalpy/entropy compensation, which is an intrinsic feature of solvation, may be of evolutionary and functional advantage, providing a thermodynamic homeostasis that prevents harsh changes in free energy profiles of biological phenomena.

Accordingly, water must be included in recognition and structure prediction programs to capture specificity and correct energetics, features that are both crucial for in silico-based drug discovery & development. Water should not be treated as an inert environment, but rather as an integral and active component of biomolecular systems, where it has both dynamic and structural roles. Focusing on water sheds light on the physics and function of biological machinery and self-assembly and will advance our understanding of the natural design of biomolecules. Recent advances in computer simulations and the enhanced sensitivity of experimental tools promise major advances in understanding the dynamics of proteins, glycans and DNA/RNA, and water will surely play a major role in these forthcoming revolutionary discoveries.

Upgrading the Looking Glass of Cancer Biology

Arkaitz Carracedo, *Cancer Cell Signaling and Metabolism Laboratory*

The more we deconstruct cancer through the implementation of novel technologies and knowledge, the more this disease is shattered into tiny little pieces like an old glass. We learn about different cancers among patients, different cancers within a patient and changes in the same cancer as time passes by. From precision medicine in cancer (individualized, personalized) we feel the need to move into "ultra" precision (if we define Ultra as going beyond due limit). The profound individualization of the disease in space and time is aimed at generating catalogues of preferential alterations in tumors that are associated to pathological stages, therapy response or exposure to new microenvironments. In 2020, we reach the realization that these catalogues should account for both static (genomic) and dynamic (epigenetic) alterations; and that should focus on the cancer cells as well as in the non-cancerous cells that inhabit the tumor.

In 2020, the field of cell and cancer biology has experienced outstanding progress in many areas, some of which are highlighted in this section. Categorize, categorize, categorize. The vertiginous progress of single cell and sequencing technologies has generated the need of interrogating the heterogeneity within multi-cellular organisms, and by analogy within tumors. The capacity to assimilate and process large volumes of information has allowed the field to produce comprehensive cellular maps. In turn, a consortium[1] (has produced an atlas of human cells that will feed biomedical research in the coming years[2],[3],[4]. In cancer research, single cell technologies will aid in the refinement of computational tools that can nowadays predict the intratumor heterogeneity from bulk gene expression studies[5].

Miniaturizing Darwin. Natural selection shapes the identity and composition of tumors. Interestingly, we can exploit the laws of Darwinian evolution to ascertain the features and capacities of different cancer cell lines or cell clones. Thanks to cellular barcoding strategies, we can study clonal selection in vitro and in vivo at large scale, which has allowed the field to monitor cell competition and commensalism[6], to discover novel anticancer therapeutic strategies[7], and to ascertain natural selection and convergent evolution in high complexity pathological states such as metastasis[8].

Tumors feed creatively. Metabolism is associated to the uptake of nutrients, which requires that cells sense their energetic status and adapt their metabolic needs. In cancer, feeding can be taken in a broader sense, from sensing and uptake to the production and secretion of metabolic byproducts[9]. In 2020, we learned that nutrient handling is a cancer cell-intrinsic factor defining metastatic capacity[10]. In addition, the field reported that the increase in cancer frequency associated with age could be influenced by the accumulation of certain metabolites, such as methylmalonic acid[11]. Importantly, manipulating the production of reactive metabolic by-products by cancer cells can represent unprecedented anticancer strategies, as shown from methylglyoxal[12]. Same as nutrient handling, the sensing and adaptation to fluctuating oxygen in a tumor will influence its biology. The ecological habitats imposed by differential intratumoral availability of oxygen is at the core of cancer heterogeneity and evolution[13],[14], and can influence therapeutic efficacy[15].

Technology is pushing the boundaries of our understanding of cancer. In a moment where generation and processing of large sets of data is overwhelming, the trees should not prevent us from admiring the forest. Detailed and thorough mechanistic analysis of potential cancer drivers remains as a solid strategy to identify novel tumor stratification and treatment strategies. That said, our capacity to "imagine" cancer biology is starting to parallel with our technological capacity to analyze it. On the one hand, single cell technologies have adapted to the spatial dimension, and we can topologically position gene expression events in tumor slides. These technologies will become more versatile with regards to tissue of origin (frozen vs. paraffin embedded tissue[16], capacity to integrate diverse molecular signals[17] and they will increase in definition to reach single cell gene expression level. In turn, our view of factors influencing intratumor heterogeneity and cell-cell vs. cell-microenvironment interactions will become richer, and our capacity to infer biological factors influencing tumor progression, more powerful[18]. On the other hand, artificial intelligence is now revealing cancer features that we could hardly predict. The incorporation of deep learning strategies to cancer biology will be instrumental for next generation pathological analysis of tumors[19], but can open new avenues, from tissue processing-free histological characterization[20] to the identification of novel molecular alterations at the core of cancer biology[21].

Tackling Cancer Heterogeneity

Maria dM Vivanco, *Cancer Heterogeneity Laboratory*

The pandemic this year has affected our lives in many unexpected ways, however, it has not halted breast cancer progression. With this in mind, breast cancer research could not stop either and the fight has continued in many labs this year. As silent pandemic, breast cancer remains the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide (only in Europe, more than 138.000 women die in one year from breast cancer). Given the high incidence of the disease, development of resistance to therapy continues to be a serious clinical problem and, therefore, represents a challenge to health, wellbeing and demographic change. One of the most common subtypes of breast cancer is characterized by the expression and activity of estrogen receptor alpha (ER-positive tumors), which is treated with endocrine therapy. The recent identification and characterization of mutations in the ER gene in ER-positive metastatic breast cancer has demonstrated its role in conferring clinical resistance to endocrine therapy and has highlighted the roles of other hormones and steroid hormone receptors in resistance and development of metastasis[22],[23]. In addition to estrogen, ER can also be activated by other hormones, such as estrone, which dominates after menopause. A study in which we participated, has shown that the interaction between cancer cells and adipocytes stimulates estrone and pro-inflammatory responses, leading to more rapid cancer growth[24]. This work contributes to our understanding of why postmenopausal ER-positive breast cancer is increased in obese patients and highlights the increased risk of breast cancer progression and metastasis associated with the currently escalating epidemic of overweight and obesity. Furthermore, ER does not function alone, multiple co-regulators and associated factors have been implicated in controlling the hormone-driven transcriptional program[25].

Breast cancer cells also cannot function in isolation. In fact, breast cancer is a heterogeneous disease. There is a high degree of cellular complexity within tumors, which are populated by tumor epithelial cells, a small population of cells with stem cell properties (cancer stem cells, CSCs) and stromal cells, in addition to an ever-changing variety of immune cells. The presence of CSCs proves a challenge to therapy, as these cells are resistant to current cancer treatments and this can lead to CSC enrichment and recurrence. Interestingly, normal and cancer stem/progenitor cells share some markers, such as Sox2 and Sox9, as we previously showed[26] and, also another family member, Sox11, which we and our collaborators recently showed confers increased plasticity to breast tumors and elevated risk of developing brain metastasis[27]. As the tumor

progresses, neighboring cells in the tumor microenvironment, such as cancer-associated fibroblasts (CAFs), support proliferation, invasion, metastasis and therapy resistance through secretion of growth factors, cytokines, chemokines and matrix remodeling factors. CAFs have also become therapeutic targets. They are a heterogeneous population of cells in breast cancer whose composition also changes with progression, contributing to drug resistance[28]. Our recent collaborative work has shown that fibroblast-activating protein (FAP), one of the predominant stromal cell types, is involved in resistance to immunotherapy[29].

These recent findings further illustrate breast cancer heterogeneity at different levels, reinforcing the remaining scientific and clinical challenges to understand molecular mechanisms, identify new therapeutic tools and optimize treatments to eliminate cancer recurrence and metastasis and improve the quality of life of breast cancer patients.

2010-2020: the Decade of Cancer Immunotherapy

Asís Palazón, *Cancer Immunology and Immunotherapy Laboratory*

The aim of cancer immunotherapy is to unlock the potential of our immune system to attack malignant cells. 2020 marks a decade from the introduction of the first T cell targeted immunomodulators blocking the immune checkpoints CTLA-4 and PD1 or PDL1. In 2011, ipilimumab, the first antibody blocking an immune checkpoint (CTLA4) was authorized, followed by monoclonal antibodies targeting PD1 and PDL1. T-cell-targeted immunomodulators are now used as single agents or in combination with chemotherapies as first or second lines of treatment for about 50 cancer types. There are more than 3000 active clinical trials evaluating T cells modulators, representing about 2/3 off all oncology trials[30]. The main challenges of checkpoint receptor inhibitors are the discovery of suitable biomarkers to guide on patient selection, and the development of novel agents targeting other immunosuppressive pathways to achieve superior synergistic efficacy.

Cell therapy, especially chimeric antigen receptors (CAR-T), represents the other main approach to immunotherapy. The success of CAR-T therapies has been limited to certain types of haematological malignancies (B cell lymphoma and leukaemia). In 2020, the access of multiple myeloma patients that are refractory to conventional drugs to new CAR-T options (anti-BCMA) will soon become a reality[31]. The translation of this success into solid tumors is still a challenge. In this context, CAR-T cells must infiltrate the tumor, recognize their cognate antigen and perform their effector function in this hostile tumor microenvironment, to then differentiate and persist as memory T cells that confer long-term protection. Fortunately, recent advances in synthetic biology provide a wide set of tools to genetically modify CAR-T cells to overcome some of these obstacles[32].

Entering in 2021, more immune-oncology agents are being added to the current pipeline, and validated targets (PD-1 and CD19) enter the precision medicine arena (biomarker discovery). Moreover, other immune cell types beyond T cells are being explored for adoptive cell therapy, an example is Natural Killer (NK) cells. The Covid-19 pandemic has disrupted the global activity in oncology clinical trials, but it is expected that recovery will occur in early 2021.

In regards of early target discovery and first-in-class pre-clinical therapeutic approaches, the field of glycoscience is emerging as an ideal partner of immunotherapy: both malignant and immune cells suffer glycosylation processes in the tumor microenvironment offering opportunities for the development of novel monoclonal antibodies or cell therapies[33].

Chemical Strategies for Improved Saponin-based Adjuvants and Molecular Vaccines

Alberto Fernández-Tejada, *Chemical Immunobiology Laboratory*

Current subunit vaccine approaches based on homogeneous, structurally-defined antigens are less immunogenic than traditional whole-pathogen vaccines. As such, they require the use of an adjuvant, a substance that itself is not necessarily immunogenic, but increases the immunogenicity of the antigen, leading to more robust and durable immune responses. The issue, however, is that not many adjuvants show sufficient potency and acceptable toxicity for human use; in addition, their mechanisms of action are poorly understood[34]. Therefore, novel adjuvants and adjuvanting strategies are required to advance more effective subunit vaccines to the clinic, especially for those based on weakly immunogenic carbohydrate and glycopeptide antigen[35]. The development of new adjuvant–antigen conjugates, in which both components are covalently linked in the same molecule, is emerging as a promising approach for rational vaccine design, enabling a safer and more precise targeting of the immune system that can lead to more effective vaccine induced immunity. However, despite decades of investigation and clinical research, only mixed not entirely successful results have been obtained, and no fully-synthetic peptide- and/or carbohydrate-based vaccine is yet commercially available. In this sense, the primary research program in my group has a dual far-reaching mission based on developing and exploiting new chemical approaches to tackle the above clear gaps in the adjuvant/vaccine field.

Besides the latest successes of QS-21 as part of the recently approved malaria and shingles vaccines as well as the promising phase III clinical trial results of the M72/AS01 tuberculosis vaccine[36], QS-21 has the potential to serve as a suitable adjuvant in the development of a prospective Covid-19 vaccine based on previous experimental results[37]. Important review articles have been published this year in the field of synthetic carbohydrate[38],[39] and peptide-based[40] vaccines, highlighting the promise of such chemical approaches for vaccine development. Furthermore, this year has brought key review articles on the topic dealing with carbohydrate-based adjuvants and their mechanisms of action[41] as well as adjuvanted therapeutic vaccination strategies[42]. In particular our lab has published this year three original research articles on the development of synthetic QS-21 derived variants and new generation multivalent anticancer vaccines based on a Tn antigen (GalNAc-O-Ser) analogue[43],[44],[45]. Lastly, a landmark review article in the area has been published this year reporting a variety of chemical approaches for rational vaccine construction, including new antigen, adjuvant, and delivery systems with optimized properties to modulate and enhance antitumor immunity[46]. This review article is an outstanding compilation of excellent examples highlighting the power of chemistry to further the adjuvant/vaccine field.

Looking forward, significant achievements can be anticipated for 2021, including the development of subunit vaccine candidates against SAR-CoV-2 formulated with QS-21 as an adjuvant. From my perspective, ongoing and upcoming efforts in the Chemical Immunology Lab include the development of saponin chemical probes for investigations of their molecular mechanisms of action, as well as unimolecular self-adjuvanting vaccines incorporating saponin-derived in-built adjuvants in their structure, leading to new synthetic constructs and potentially improved vaccine approaches.

Multiscale Computational Modeling in Stem Cell Research

Antonio del Sol, *Computational Biology Laboratory*

Computational stem cell research aims at the development of computational models to characterize and predict the behavior of stem cells with the ultimate goal of designing novel therapeutic strategies. These therapeutic strategies include, for instance, cell transplantation where damaged or aged cells are replaced by healthy functioning cells and in vivo cellular conversion. However, a number of obstacles have to be overcome to design therapeutic interventions applicable in the clinics. For instance, although the identification of induced pluripotent stem cells has provided a framework for generating transplantable cells in vitro, a number of key bottlenecks, such as the low amount of successfully derived target cells, force researchers to spend large amounts of resources to collect enough target cells for clinical use. In this regard, the development of computational models simulating cellular behavior can provide testable predictions to overcome these issues.

Although the COVID-19 pandemic largely overshadowed research in the life sciences, exciting developments have been made this year. Especially the continuous improvement of single-cell technologies for the first time allowed the development of computational models at different scales of biological complexity, such as the intracellular and tissue level, that address key questions in stem cell research. In this regard, the gene regulatory network (GRN) based modeling of cellular conversions has been instrumental in the design of novel cell transplantation strategies. Great efforts have been made this year in the implementation of computational methods for inferring cell type and subtype specific GRNs[47],[48],[49]. These methods exploit the integration of transcriptomic and epigenetic data to identify critical regulators of cell identity, which can be employed to convert cells into a desired cell type. Moreover, multiscale modeling approaches have been developed that are able to determine niche signals underlying dysregulated stem cell phenotypes in disease and aging, thus, allowing the prediction of key signaling molecules to counteract the niche effect. These models rely on single-cell RNA-seq data to integrate signaling and GRNs for providing a general framework of stem cell niche interactions[50],[51]. Finally, cell-cell communication models have emerged that enable researchers to study the crosstalk of different cell types in the context of development, differentiation and disease[52],[53]. In particular, receptor-ligand mediated cell-cell interactions are inferred by combining prior knowledge with a statistical/machine learning framework. This allows the generation of reference cell-cell communication networks that can be exploited to detect pathological dysregulations and can guide the development of novel intervention strategies for restoring homeostasis.

With the increasing resolution of computational models, we envision a closer collaboration between experimental and computational researchers that will accelerate the development of therapeutic strategies and their translation to the clinics. This year's publications have already demonstrated that computational modeling can guide experimental efforts to address key questions in stem cell research and regenerative medicine. In the future, the generation and integration of different types of single-cell -omics data will allow the development of computational models that offer a more complete characterization of the biological system under study and an increased accuracy. These models will be instrumental in the generation of novel hypotheses for the design of regenerative medicine therapies.

Single Cells Analysis in Liver Disease

Malu Martínez-Chantar, *Liver Disease Laboratory*

Deregulated reprogramming of liver metabolism is a hallmark of liver disease, a series of multi-factorial conditions involving the progressive destruction and regeneration of the liver parenchyma leading to fibrosis, cirrhosis and liver cancer. Overall, along 2020 an improved knowledge of the regulatory mechanisms of liver metabolism during disease progression has provided novel druggable therapeutic targets which have definitely impacted in health and economic burden ascribed to liver disease. Recent improvements in drug design along with a greater understanding of the molecular mechanisms underlying pathologies have led during this year to important advances in the therapeutic treatment of patients. Precision medicine adapts to the genetic, molecular and phenotypic characteristics of the disease and, specifically targeting the causative components and intrinsic characteristics of the patient has blossomed in the recent years. In this respect, considerable progress has recently been made in the development of oligonucleotide-based therapies, in which the silencing of target genes is being incorporated in clinical studies[54]. Therefore, a real revolution is taking place in patient care in this sense[55].

The field of single cellular genomics has undergone a real technological transformation allowing the exploration of new cell types with unicellular resolution. In addition, it has revealed new information on tissue biology and mechanisms underlying diseases. The liver community has also embraced these new approaches, with a plethora of studies of single-cell RNA sequencing of the liver (scRNAseq). Single-cell RNA sequencing along with spatial mapping have demonstrated previously unknown molecular patterns. The use of scRNAseq has already provided a number of new insights into the functional attributes of hepatocytes across different areas of the human liver lobe and has facilitated a deeper understanding of how zoning is structured and regulated even during regenerative process[56].

Single cell analysis has also been a leading-edge technology that has defined better the tumor cell community and has enabled to identify new possible targets for immunotherapy or combination treatments[57]. Immune response is one of the main features to HCC mediated pathogenesis. Thus, this tumor is particularly appealed for immune-based therapies. However, the complex functional characteristics of the HCC tumor microenvironment highlight the presence of multiple non-redundant mechanisms of cancer immune-suppression, which synergise in defining a high barrier of resistance to immunotherapy. Rationale for a combinatory selection of immunotherapeutic intervention in a close future will pave the way for opening new opportunities to improve outcomes across the various stages of HCC[58].

Finally, it is necessary to emphasize in this 2020 one of the most important achievements in hepatology awarded for the Nobel Prize in Physiology or Medicine. Michael Houghton, Harvey Alter, and Charles Rice were awarded with the Nobel Prize for their decisive contribution to the fight against blood-borne hepatitis, a major global health problem that causes cirrhosis and liver cancer in people around the world. For the first time in history, the disease can now be cured, raising hopes of eradicating Hepatitis C virus from the world population[59],[60].

Let this race of scientific successes serve to reflect on the historically proven fact that a decisive commitment to scientific research is the best way to overcome public health problems. It has been so with hepatitis C, and it will be so with the COVID pandemic we are facing today. M Martinez Chantar.

Buttressing the Case of Genomics for Precision Medicine

Urko Martinez Marigorta, *Integrative Genomics Laboratory*

We analyze multiomic profiles of patient cohorts to illuminate our understanding of complex disease pathogenesis. Using inflammatory bowel disease (IBD) as a model, we focus on two aspects that are key for gearing genomic knowledge to achieve precision medicine, namely i) the need to understand the heterogeneity of mechanisms whereby each patient develops disease, and ii) the development of predictors of therapeutic response. These intermediate steps are key for the discovery and characterization of biomarkers useful in the clinic and that can serve to track prognosis and disease status in each patient.

Two developments crystalizing in 2020 demonstrate the exciting prospects for the purpose of implementing precision medicine approaches. The first one involves the incredibly disruptive availability of the full spectrum of human genetic diversity, and the lessons therein for prediction of disease. The release of the gnomAD "Genome Aggregation Database" serves as a case example of this potential. By compiling over 125,000 exome and over 15,000 whole genome sequences from people all over the globe, we have learned among others that about 3,200 genes are intolerant to inactivation[61]. In contrast, some 2,000 other genes – including many known drug targets – seem completely amenable for mutations. These results stress the inherent genetic complexity harbored by each person´s genome. The second exciting highlight of the year refers to actual examples of implementation of population-based genome sequencing for the screening and diagnosis of disease. This ranges from country-wide prenatal screening testing to improve the detection of genomic abnormalities[62], to active screening of genetic conditions such as hereditary breast cancer and familial hypercholesterolemia that are carried by over 1% of the population[63]. Given that these mutations lead to actionable clinical impacts, it is pressing to extend genomic profiling efforts across the population. Finally, it is worth mentioning the role that functional genomics has provided for understanding better the COVID-19 pandemic. In spite of an early study with strong contribution from Basque patients and researchers[64] studies of host genetics through GWAS have not provided much clues about the disease. Instead, multiomic profiling has demonstrated extremely useful to understand the pathogenic mechanisms that determine response and disease trajectory in each patient[65].

Besides a renewed focus on proof-of-concept successful implementation of precision medicine in diseases with obvious actionable interventions, I expect further deep diving into the functional and mechanistic aspects that underlie the genes associated with complex disease. Generalization of single cell studies will lead to innovative atlases of functional genomic profiles across cell types, tissues, developmental and disease stages. Coupled with accumulating evidence of prospective cohorts, this stream of new datasets will prove key for our purposes of developing new tools for longitudinal monitorization of patients and being a lab that allows for bridging genomic medicine with the clinic.

Long-term Innate Immune Responses and Lyme Cardiac Inflammation

Juan Anguita, *Inflammation and Macrophages Plasticity Laboratory*

The heart is the most energy demanding organ in the human body, with mitochondrial oxidative phosphorylation being responsible for nearly all of the ATP production in adult mammalian hearts. This high-capacity mitochondrial system is capable of oxidizing fuels such as fatty acids, which are the predominant energy substrate for the adult heart[66]. During infection, both a deficit in energy production and alterations in the source of energy substrates are associated with impaired cardiac function, in part by the myocardial infiltration of immune cells[67], accompanied by a strong activation of the cytokine system[68]. Therefore, abnormalities in mitochondrial function are known causes of cardiomyopathies, arrhythmias, abnormalities of the conduction system, ischemia-reperfusion injury, heart failure and ageing among others[69]. Besides the infiltration of immune cells, resident cardiac macrophages also play an essential role not only in homeostasis but in the inflammatory response to infectious agents[70]. Among the tissues affected during infection with the bacterium that causes Lyme borreliosis, Borrelia burgdorferi, the heart is one of the targets of inflammation. Lyme borreliosis is the most common arthropod-borne infection in the Northern hemisphere with around 300,000 cases every year in the US[71] and approximately 250,000 new cases yearly in Europe[72]. Symptoms associated with infection may be debilitating and long-lasting despite appropriate antibiotic treatment. The initial deposition of the bacteria in the skin is followed by the intradermal and hematogenous dissemination of the spirochete, which results in the invasion of different tissues and organs, including the joints and the nervous and cardiovascular systems. B. burgdorferi shows a marked tropism for cardiac tissue where it can persist for months to years even after antibiotic treatment[73] and in spite of the development of strong immune responses. Lyme carditis represents 0.3 – 4 % of Lyme borreliosis cases in Europe[74], and 4 – 10% in the United States[75]. Eighty to ninety percent of Lyme carditis patients suffer atrioventricular electrical block of varying degrees[76], while myocarditis, myocardial infarction, coronary aneurisms, pericarditis, pancarditis, dilated cardiomyopathy or endocarditis have been reported in a smaller percentage of infected individuals. AV block has been associated with the presence of spirochetes in the cardiac tissue and the ensuing inflammatory response[77]. Although apparent in a small percentage of Lyme cases, several fatalities associated with Lyme carditis have been reported since 1985[78],[79],[80].

The mechanisms used by the spirochete to persist in mammalian tissues and to induce persistent inflammatory responses are not completely known but may be related to co-evolutionary factors that affect the long-term responses of innate immune cells as well as immune escape mechanisms intrinsic to the bacterium. The existence of long-term consequences of the stimulation of macrophages with certain simple (e.g. beta glucans) or complex (e.g. BCG, the mycobacterial vaccine strain) stimuli has been termed innate immune memory[81],[82]. This concept originally evolved from observations in BCG-vaccinated individuals in which a level of protection against disparate pathogens was identified[83]. Innate immune memory has been defined in terms of the induction of soluble factors (i.e. proinflammatory cytokines)[84]. Responses identified as memory have been divided into innate immune training and tolerance; the difference being the nature of the secondary response (heightened versus reduced). The concept of long-term adaptations to the continuous exposure to infectious agents has expanded to include non-classical immune cells, such as fibroblasts and other cell type. In fact, a recent report has described innate immune memory-like characteristics in joint fibroblast-like synoviocytes of B. burgdorferi-infected mice while skin fibroblasts did not show signs of training[85]. The authors described a correlation between the ability of these cells to develop hallmarks of innate immune memory and the severity of the symptomatology.

It is expected that more correlative studies address the potential importance of long-term adaptative responses associated with innate immune memory during infection. Furthermore, and more critically, studies that address the mechanisms that control these responses in response to the spirochete, potentially identifying therapeutic intervention points

will likely continue in the following years. For this task, it is essential to understand the exact relationship between inflammation and the functional defects observed during persistent infections, such as Lyme borreliosis, particularly related to cardiac inflammation and the role of innate immune memory in this context.

A Sweet View on Glycans in Molecular Interaction

Jesús Jiménez-Barbero, *Chemical Glycobiology Laboratory*

Carbohydrates (saccharides, sugars, glycans) are everywhere. They play key roles in diverse biological events and have distinct physical, chemical, structural, and biological properties that make them useful for a wide range of applications. For many years, it has been known that they can be used as energy source and as building materials. In the past decades, it has been also demonstrated that they act as recognition points for many essential processes in life and disease. In fact, glycans interact with many classes of proteins, comprising enzymes, antibodies, and lectins. The specific recognition of glycans constitutes a major pathway in which the complex information contained in the structures of glycans may be deciphered to promote a given particular bioactivity.

In this 2020, we have been seriously beaten by the COVID-19 pandemic. This coronavirus is currently the last example of a large family of viruses that threaten our lives. As described in other cases, SARS-CoV-2 attaches to cell-surface receptors as the initial step in the infection process. Indeed, many viruses that infect mammals have evolved to recognize glycan receptors, glycoproteins or glycolipids, on the cell surface. As a matter of fact, Indeed, more than half of all virus families that attack to mammals bind glycan receptors, including families with either membrane envelopes or protein capsids, and RNA- or DNA-encoded genomes. In particular, the spike protein of the zoonotic MERS strain recognizes a (DPP4) as a primary receptor, while sialic-acid containing glycans act as co-receptors, as also described for coronaviruses. Alternatively, coronaviruses infecting the respiratory tracts in the avian world display spike proteins that recognize similar sialic acid epitopes, while their counterparts in the gastrointestinal system specifically interact with non-sialylated complex N-glycans that contain multiple LacNAc disaccharide repeats[86].

Regarding SARS-CoV-2 recognition, it has been proposed[87] that the virus attachment and infection event involves the initial attachment of the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein to cellular heparan sulfate, thus enhancing the binding to the angiotensin-converting enzyme 2 (ACE2). It has been suggested that heparan sulfate or heparin promoted the particular conformation of the RBD (the so-called open form) that effectively binds to ACE2. In fact, Clausen et al. have shown that different types of heparin and heparan sulfate effectively block the binding of the spike protein thus abolishing the infection by pseudo-typed virus and the authentic SARS-CoV-2 virus. In the same context, although the direct involvement of sialic acid residues as initial requirement for infection by SARS-COV-2 remains an open question, Gibson and coworkers[88] have demonstrated that a sialic-acid-based lateral flow system can detect the SARS-COV-2 spike glycoprotein in a virus-like particle in less than 30 minutes, with a detection limit of 5 µg/mL, using a nanoparticle decorated with sialic acid residues.

From a different perspective, we have investigated[89] the interaction of the glycans attached at the RBD with different human lectins expressed in the organs affected by the infection. The interacting glycan epitopes specific for each lectin (including galectins, C-type lectins, and siglecs) have been elucidated as proof-of-concept to study the possible modulation of the infection by the presence of different lectins and antibodies, by interfering with the interaction with ACE2. Indeed, the existence of novel molecular pathways using host lectins and signaling processes that might contribute to the infection and to the subsequent immune exacerbation has been postulated[90]. Moreover, it has been also suggested that some C-type lectins promote virus transfer to permissive ACE2+ cells. Interestingly, a small molecule, a glycomimetic compound, strongly inhibits this event[91].

Therefore, it is evident that the glycoscience field is experiencing a boost from different perspectives: from the fundamentals to the applications, taking advantage on the spectacular developments in chemical synthesis, structural methodologies, and cell and molecular biology protocols. There is no doubt that new applications in biology and biomedicine will emerge in the coming year, with implications in the discovery of new glycan-related targets and receptors to combat a variety of pathologies, from bacterial and viral infections to immune diseases and cancer.

Ubiquitin-like Modifications in Health and Disease

Rosa Barrio, *Ubiquitin-likes and Development Laboratory*

We are interested on the regulation of developmental processes and diseases by post-translational modifications by the Ubiquitin-like (UbL) SUMO of specific transcription factors. Among those, the Spalt-like (SALL) family are necessary for numerous biological processes. Mutations in SALL1 are associated to Townes-Brocks Syndrome (TBS), a rare disease causing kidney defects, deafness and polydactyly. TBS patients might develop kidney failure, requiring dialysis or transplant. TBS interferes with the function of cilia, cellular antennas that play crucial roles in cell signalling, which opened new opportunities of intervention and advanced in the understanding of the molecular mechanism of the disease. UbLs, like SUMO, are attached to target proteins altering their function, thus regulating nuclear integrity, proliferation and transcriptional regulation, contributing to diseases like cancer or neurodegeneration. We develop new technology to identify modified targets in a subcellular manner, as well as factors that only interact with the modified substrates. Importantly, targeted degradation is getting more and more relevance in the development of biomedical strategies.

Great advances related to SALL1 and kidney formation have been published during 2020, specially involving the role of SALL1 in the generation of in vitro models of kidneys from human pluripotent stem cells (hPSCs)[92]. The final aim would be the regeneration of human kidneys in animal models to contribute to transplantation therapies.

Evidences of the role of SALL1 during development were also published, specifically on microglia formation in the peripheral nervous system[93],[94],[95]. Other SALL members have also been implicated in diseases like cancer[96],[97],[98]. Importantly, relevant for the field of targeted degradation, SALL proteins have become important targets that open the avenue for new intervention strategies[99],[100],[101]. SUMO and other UbLs has also been related to variety processes and disorders during 2020: Parkinson[102]; cancer[103], [104]; pluripotency during development[105]; infection[106],[107],[108]; DNA repair[109],[110] among other processes.

The advances in the mechanisms of cilia dysregulation in TBS individuals opened new possibilities of intervention by treating the cells with specific drugs and/or genome editing. Studies in model systems will be necessary to develop these treatments. The role of members of this family of transcription factors in other diseases like cancer will also benefit. Targeted degradation strategies will be increasingly important in the biomedical field, including rare diseases[111],[112]. SUMO and other UbLs are involved in a plethora of diseases, many times involving the cross-talk among different modifications. New technologies

are needed to approach those studies, making proteomics a crucial technology. Biotin-based techniques will be extremely useful in those developments[113,114].

Understanding Prions to Improve Diagnostic and Treatments for Encephalopathies

Joaquín Castilla, *Prion Research Laboratory*

The group of fatal neurodegenerative disorders known as Transmissible Spongiform Encephalopathies (TSE) are caused by prions and affect several mammals including humans. The central event leading to pathogenesis is the misfolding of the cellular prion protein (PrPC), expressed abundantly in the nervous system, into a pathogenic isoform (PrPSc) that presents the ability to induce its conformation to the cellular counterpart and triggers an unknown neurotoxicity pathway leading to neuronal death. The scarce knowledge on molecular mechanisms underlying prion pathogenesis, limit the advances on complex phenomena such as the existence of prion strains, the role of cofactors defining their unique characteristics or understanding interspecies transmission. Moreover, this lack of knowledge also hinders early diagnosis and the development of new therapies.

During 2020 some progress on deciphering the three-dimensional structure of prions was made, an essential milestone for understanding prions, with new models derived from cryo-EM studies with synthetic fibrils[115] and molecular dynamics simulations with the most credible models[116]. In this regard, the highly infectious recombinant prions we are able to obtain now in unprecedented amounts, are being also applied for structural studies using both ssNMR and cryo-EM. In terms of understanding prion propagation and neurotoxicity mechanisms, uncoupling of infectivity and toxicity of purified prion fibrils has been suggested[117] and infectivity of small molecular weight protein fragments, characteristic of atypical prion diseases, has been shown[118]. Along this line, new insight on the role of polyanionic cofactors and posttranslational modifications on determining strain features in vivo[119,120] has been published, pointing towards the relevance of variations in PrPC glycosylation for the interaction with cofactors and therefore, determination of strain features. Although an in vitro study suggests that the 3D structure of each prion strain determines glycosylation and cofactor preferences, a view shared by our group and that we are trying to demonstrate through our synthetic prion preparations done in the presence of distinct cofactors. Closely related to strain phenomenon, relevant findings on interspecies prion transmission, have also been published, revealing strain adaptation during transmission and the risk of acquiring the disease through finger incision[121]. Studies on species resistant to prion transmission, which are of utmost interest for the development therapies, have drawn considerable attention in 2020, mainly studying dog[122]; and porcine PrP[123]. Many other promising therapeutic strategies have been also made public[124,125];, being specially interesting those dealing with the search of anti-prion auto-antibodies[126] and PrPC expression lowering strategies[127]. The area of TSE diagnosis is also rapidly evolving thanks to genome-wide association studies looking for new risk factors[128] and the search of novel biomarkers in CSF and blood[129,130]; including miRNA[131] and transcriptomic signatures[132]. Finally, three areas that were especially relevant during this year are worth mentioning. The worrisome spread of Chronic Wasting Disease in cervids across north America and Europe promotes multiple studies on PrP polymorphisms that could influence transmission and strain properties[133,134], as well as on rapid detection methods[135]. Decontamination of prion-infected surfaces and instruments has received special attention also with the development of new decontaminants[136] and new methods to assess their efficacy[137,138]. The search of appropriate models to study such complex disorders was also relevant during 2020, since two new cellular models were presented[139,140], which will add much to the few cell models available for these diseases.

In 2021, advances are expected mainly in the three more rapidly evolving areas: early diagnosis through a combination of novel biomarkers in blood and CSF and in vitro prion propagation techniques, that allow detection of minute amounts in tissues or body fluids. The latter, which are being applied also to other more prevalent neurodegenerative diseases with promising results, will likely advance further next year facilitating considerably the differential diagnosis. Regarding therapy, positive preliminary results from PrPC expression lowering strategies are presumed via publication of efficacy experiments in animal models. Last, concerning resolution of the 3D structure of prions, based on the slow but steady advances, new information is expected that will further support or discard some of the models that have been proposed. Undoubtedly, strain phenomenon and interspecies transmission will be again under the spotlight as the most complex phenomena in prion diseases that require understanding, although advances in these areas, which are highly dependent on the 3D structure of prions, are supposed to be slower.

Viral three-Dimensional Structure

Nicola G. A. Abrescia, *Structure and Cell Biology of Viruses Laboratory*

Never as before the past 12 months have shown to the entire world the importance of studying viruses. Viruses are pathogens to humans and animals others are allied of humans in controlling bacteria proliferation. Others are manipulated and used as delivery systems of drugs or repairing genes into humans (gene therapy). Our recent projects involve mainly the study of eukaryotic animal and human viruses (enveloped and not), including SARS-CoV-2, at the stage of assembly, virus entry and antibody recognition. The ultimate goal is to provide a solid conceptual/knowledge-based framework for exploring new avenues for therapeutic interventions or biotechnological applications. Research on viral pathogenesis at basic or translational level is a global health challenge.

The field of Structural Virology has seen pivotal advances on several fronts in tackling human and animal health threats such as Influenza (infecting humans)[141], African Swine Fever (infecting pigs)[142,143,144] and lately SARS-CoV-2 viruses. The last 12 months, however, have demonstrated, once more, the power of cryo-EM and X-ray in the virology field. Both techniques have allowed to quickly visualize in 3D key viral SARS-CoV2 proteins and pivotally understanding the process of viral entry, antibody recognition and viral proteins druggability. Biomedicine (and society) would have lost even more time in setting-up prophylactic measures without this basic but essential information. Viruses are also miniaturized machines that perform complex biological tasks and comprehending how members of the Virosphere are related beyond the similarity in their primary sequence (which is easily lost when analyzing distant viruses) remains a powerful tool to exploit/discover new virus applications and understand how the 3D structure can steer evolution (keep in mind the origin of the new SARS-CoV-2). Further, discovering new viruses or engineering new ones for biotech applications as in the case of adeno-associated virus (AAV)[145] can be considered an outstanding scientific advance.

Advances for the coming year will mainly concern:

- HR cryo-EM: it will develop even further. Smaller molecules (~50 kDa) and faster data collection with K3 and Falcon IV direct detection cameras (a current reality), Selectris X imaging filters is rendering EM, the technique of choice to determine not only the 3D structure of a wide size range of macromolecules (including the attached carbohydrate) but also the conformational dynamic of such proteins. Pharma companies

are heavily involved in drug screening programmes using cryo-EM and cryo-EM will be used in the clinical area. Further, to democratize the EM, more accessible microscopes have been lunched on the market[146]

- FIB/cryo-ET, soft X-ray tomography and high-resolution correlative microscopy: their combination allows a direct correlation of cellular morphological changes with virus entry pathways and consequent antiviral treatment.
- The above structural techniques will contribute to provide a 'Google Map' of distinct (pathological) human cells and thus helping to navigate with predictive capabilities in the landscape of human diseases.

Three-dimensional Cryo-Electron Microscopy of Flexible Filamentous Plant Viruses

Mikel Valle, *Cryo-EM of biologicals macromolecules Laboratory*

We use cryo-electron microscopy (cryoEM) to explore the structure and to understand the functioning of several biological complexes such as ribosomes and filament plant viruses. In the field of flexible filamentous plant viruses we did contribute with the structure of three viruses from different families and we have also explored the structure of Viral-like Particles (VLPs) from one representative.

In this regard, a new structure for this type of viruses has been reported this year, and it is the highest resolution (2.2 Å) cryoEM structure[147]. Regarding ribosomal structures there are numerous reports, since ribosome and translation are extensively explored by cryoEM. This way, from the total set of cryoEM structures deposited in the EM databank[148] (around 11% (1414 out of 12855) are entries related to ribosomal structures. To mention a some: 1) time-resolved cryoEM structures display the structural changes that govern the incorporation and selection of cognates aminoacyl-tRNAs into the ribosome, and shows how closing/opening of the head from the small ribosomal subunit plays a central role during proofreading[149]; and 2) the most complete eukaryotic initiation complex described so far, and it could become a seminal work to understand the complex regulation of recruitment of mRNAs for initiation of translation[150].

Advances in cryoEM field have dramatically changed the technique in the last 5 years. During 2020 the highest resolution structure for a cryoEM study has been published (at an astonishing resolution of 1.22 Å)[151]. The cryoEM map not only unveils all the large molecular weighted atoms in the structure of apoferritine, but also reveals electron clouds for hydrogen atoms when difference maps between the cryoEM density and the atomic model are calculated. This is really a breakthrough.

Finally, the arrival of a direct detection camera for our in-house electron microscope platform, and the incoming high-resolution 300 kV Titan Krios (at the Biophysics Unit) is greatly improving our capabilities to deliver high resolution cryoEM data. We use cryo-electron microscopy (cryoEM) to explore the structure and to understand the functioning of several biological complexes such as ribosomes and filament plant viruses. In the field of flexible filamentous plant viruses we did contribute with the structure of two viruses from different families. In the last year we have also explored the structure of Viral-like Particles (VLPs) from one representative. In this regard, a recent publication has presented the structure of VLPs from another species, Potato Virus Y (PVY)[152].The VLPs from PVY are different from the previously described ones (including our own structure). It is a filament of stacked rings that allow for high resolution in cryoEM. This sample is very well suited for structural characterization of the interaction of phytosanitary compounds with antiviral properties, a line of research that we are also carrying out. With this in mind, we have already produced these same VLPs from PVY for the next step in our research.

On the other hand, we are lagging in the advance of cryoEM, where our center is acquiring a direct detection camera that will considerably improve the performance (attainable resolution) of our microscope.

Molecular and Functional Characterization of Extracellular Vesicles

Juan M. Falcón-Perez, *Exosomes Laboratory*

All cells of the body secrete extracellular vesicles (EVs) to communicate intercellularly with proximal and distal cells. They carry complex signals and cargo such as enzymes, lipids, nucleic acids and sugars, to deliver into recipient cells and they travel through our body fluids. EVs, depending on their intracellular origin, can be classified as microvesicles, exosomes and apoptotic bodies. Currently, they are being studied as pathological agents in different neurological and metabolic diseases, as a biomarker in liquid biopsies and as a vehicle for targeted drug delivery. The field is also trying to develop isolation and phenotyping methodologies to separate EVs, while keeping vesicle integrity, and characterize their sub-populations.

During this year, more than 2000 articles in the field of extracellular vesicles have been reported covering many aspects of the biology of these vesicles from the isolation and phenotyping to their implications in the development of different diseases. This year has been monopolized by the COVID-19 pandemic and, of course, the EV field could not progress staying away from it. Exosomes may play a role in spreading coronavirus infection[153], since EVs secreted from infected lung epithelial cells carry viral RNA that has been after found in cardiomyocytes[154]. On the other hand, exosomes can be used for clinical applications as a COVID-19 treatment and as vehicles for delivering drugs. The International Society for Extracellular Vesicles (ISEV) made a statement on the use of EVs as therapeutic agents against COVID-19[155], which highlighted the potential use of EVs, but warned about the importance of following ethical and regulatory guidelines. EVs secreted from mesenchymal cells seem to be a potential therapeutic tool for COVID-19 pneumonia[156],[157]. Currently, there is an active clinical trial that has proven the safety and efficacy of exosomes derived from allogeneic bone marrow mesenchymal cells[158]. There is still a need to find an isolation technique that becomes the standard-of-use in the EV field. Tangential flow filtration (TFF) presents as a good candidate for the isolation and concentration of EVs. Since 2015, there has been an increase in the use of this technique[159] thus gaining an interest in the field of exosomes. TFF is capable of processing high volumes of samples and concentrating them down to low volumes[160]. In addition, TFF has a great potential to isolate exosomes for clinical use because it can be used under GMP conditions and under sterile conditions[161]. Nature Biotechnology has published an article pointing out this year's economic impact of big pharmaceutical companies developing EVs for drug delivery[162]. Pharma using EVs as nucleic acid carriers seem to add up to a value of more than $2 billion. Exosomes are gaining a lot of interest in becoming carriers of nucleic acids, such as RNAi and siRNAs, because they are capable of delivering longer sequences than other carriers do. This year, ISEV has published a meeting report of the workshop on EV-based Clinical Theranostics, carried out the past November 2018[163]. In this report, there is summarized the state of the art of the EVs isolation methods, EV omics and biomarkers, applications in clinical diagnosis and EV-based therapies. Some of the conclusions were the need of a high throughput validation process for EV biomarkers and the high potential of the EVs to act as drug-delivery systems.

The perspective in the EV field for the next year continues to be the finding of a consensual, well-performing isolation technique. Also, a better understanding of the EVs role in the development and spreading of human diseases and an improvement on the targeting and distribution of the EVs as drug delivery systems.

Endosomal Trafficking. The Retromer complex

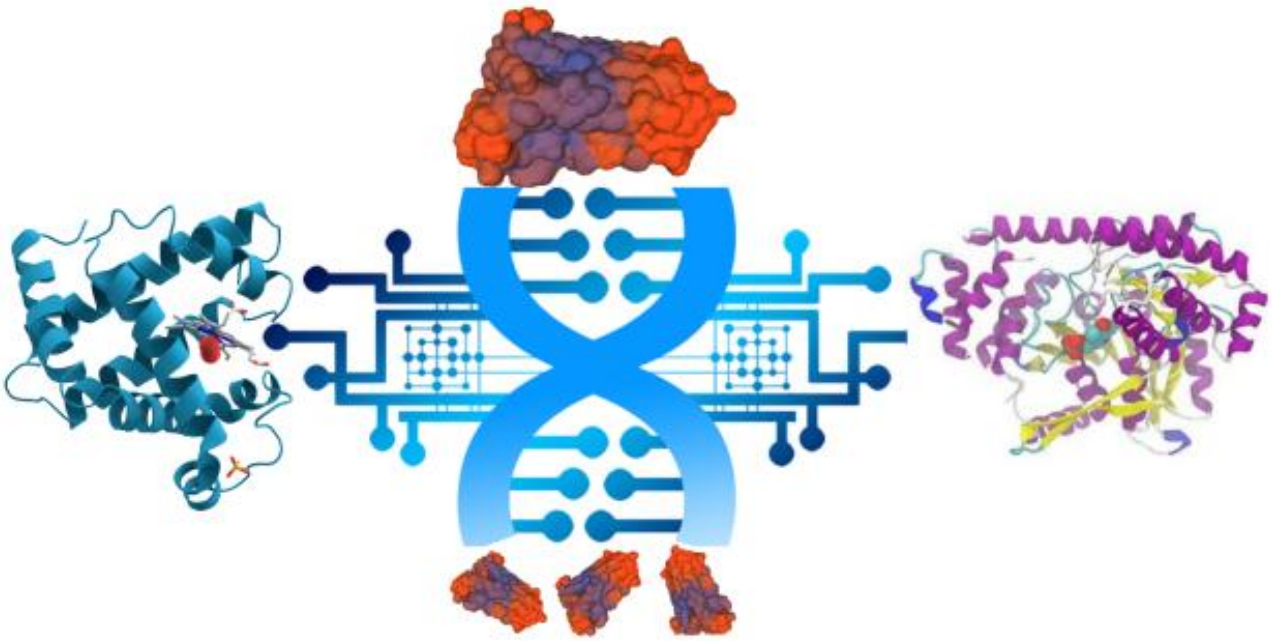
Aitor Hierro, *Membrane Trafficking Laboratory*

Living cells are constantly moving material as part of their inherent activity and environmental interactions. The endosomal network is a mayor hub in protein trafficking. It receives a large number of transmembrane proteins, receptors and ligands, termed cargo, from the secretory and endocytic pathways which then are routed to the lysosome for degradation or recycled for re-use. Protein recycling has a direct impact on metabolic balance and cellular homeostasis, and is altered with aging. Of the cargo molecules delivered to endosomes, about two-thirds are rescued from degradation and recycled for reuse. Each recycling pathway utilizes distinct sets of molecular machineries to transport channels and receptors involved in a wide range of physiological processes such as nutrient intake, cell signaling, polarized transport, cell differentiation, immune response and nerve transmission. These endosomal assemblies have a large number of components and interactions regulating the formation of specific exit routes. Nonetheless, our understanding of the mechanisms that regulate the assembly of these recycling machineries on endosomes, the concentration of cargo in pre-budding domains, and the coupling of cargo with the biogenesis of transport carriers remains very limited. Spatiotemporal control of these events not only is essential for general proteostasis and neuroprotection, but also is subverted by numerous pathogens. Our goal is to elucidate the molecular mechanisms for recognition, packaging and sorting of integral membrane proteins into specific membrane-coated structures.

More than 78 papers have been published during 2020 in the retromer field related to different disease, highlighting the role of retromer in different context like SARS-Cov-2, transport proteins, Alzheimer, and Papilloma Virus. Daniloski *et al.*[164] studied the genome-scale CRISPR loss-of-function screen in human alveolar basal epithelial carcinoma cells, identifing essential host genes in SARS-CoV-2 viral pathogenesis. Overall, the top-ranked genes clustered within several protein complexes including vacuolar ATPases, Retromer, Commander, ARP2/3, and PI3K highlighting the importance of endosomal trafficking in viral infection. In a previous study relevant to Alzheimer's disease it was identified R55 as a chemical chaperone that stabilized retromer and decreased the pathogenic processing of APP in cells. In the current work, the authors identified a new chaperon similar to R55 that binds and stabilizes retromer, and exhibits neuroprotective effects in iPSCs derived motor-neurons from patients suffering from Amyotrophic Lateral Sclerosis[165]. Another work describes that Human Papillomavirus (HPV) trafficking through the retrograde pathway requires TBC1D5, a retromer-associated, Rab7-specific GTPase-activating protein. Intriguingly, while classical retromer cargos require only GTP-bound Rab7 for trafficking, HPV relies on cycling between GTP- and GDP-bound Rab7[166].

For the intracellular trafficking role of Retromers, using an NMR/X-ray approach, describes the structure of the complex between retromer subunit VPS29 and the accessory proteins VARP (Ankyrin repeat domain-containing protein 27). The structure identifies a unique four-cysteine microdomain stabilized by a single Zn++ ion that is present only in VARP homologues[167]. *Ye et al.*[168] describes This work describes that VPS29 is required for retromer recruitment via Rab7 and TBC1D5. Remarkably, the absence of Vps29 in Drosophila does not affect the expression or stability of other retromer components but impairs lysosomal proteolysis and autophagy, leading to a progressive nervous system dysfunction. *Yong et al.*[169] uncovers a a bipartite sorting motif for cargo recognition through the association with the phox homology domains of SNX5 and SNX6. The bipartite motif is present in over seventy putative SNX-BAR ligands that are sorted to the plasma membrane or the trans-Golgi network. Interestingly, some of the ligands could cooperate with the SNX-BARs, SNX27 and retromer to form a “supercomplex” that mediates endosome–to–plasma membrane transport.

The process of tubular endosomal budding and trafficking is responsible for the subcellular localization of hundreds of cargo proteins such as signaling receptors, nutrient transporters, ion channels and adhesion molecules. Not surprisingly, numerous mutations affecting cargo recognition and recycling have been associated to a variety of diseases, most of which affect the nervous system. In the future, detailed description of how disease mutations compromise tubule-based endosomal sorting is certainly required. This knowledge is of pivotal importance for future therapeutic intervention as it might provide new targets for the development of scaffolding drugs that either promote or inhibit protein-protein interactions to interfere with specific trafficking pathway(s) without affecting others.



TECHNOLOGIES

Mass Spectrometry based Proteomics Challenges

Felix Elortza, *Proteomics Platform*

Proteomics has reached to levels hard to foresee few decades ago. The most notable achievements have come from the optimization of chromatographic systems coupled on-line to tandem mass spectrometers (nLC MS/MS), and software development. Nowadays identification and quantification of thousands of proteins can be considered routine. Nevertheless, some aspects make proteomics analyses challenging:

-Sensitivity: sometimes less is more. In clinical analysis the less sample needed for any clinical test, the less invasive will be for the donor/patient.

-Reproducibility: not only standard operating procedures (SOPs) all the way along the sample acquisition and biobanking must be carefully controlled, the performed analytical procedures need to be robust and reproducible.

-Throughput: due to human heterogeneity, large cohort studies are necessary to carry out in order to get robust enough data and thus, useful biomedical information. Moreover, precision medicine projects which are based in big data analysis are fed by very large studies where thousands of analyses are integrated.

-High throughput post-translational modification analysis: eventually every human protein is processed during its life spam. Protein post-translational modifications are of paramount importance since they will affect to their location, modulate activity and interactions with other proteins and molecules etc. etc. Post-translational modifications (PTMs) analysis in a universal way is very challenging since they are very different from the physic-chemical point of view. Eventually different type of modifications shall need specific sample preparation and mass spectrometry acquisition methods.

-Data analysis: latest generation of proteomics platforms can generate incredible amount of data. Digging into these oceans of

data to get useful information is an enormous task only achievable with dedicated algorithms and software.
During lasts years several outstanding advances have been reported in proteomics field. Hereby some of them:

-Single cell-proteomics has been lately in the spotlight for high sensitivity proteomics rush. In a recently published paper by Cheung [170] they take care of some methodological aspects to take into consideration and they introduce a software tool called Single-Cell Proteomics Companion that recommends instrument and data analysis parameters for improved data quality.

-Nano scale chromatography has been used in proteomics to help increasing sensitivity. The drawback of this has been the limited robustness of the entire chromatography and the reduced sample per day analysis capability. The irruption of a novel nano scale LC system, the EVOSEP ONE, with the improvements coming from pre-formed gradients and offset gradients for peptides refocusing has been a big step forward analytically. The minimized overhead times and the optimized analytical column and gradient length allows to notable speed up of data acquisition. Moreover, the minimization of cross-contamination even with complex mixtures such as digested crude plasma samples enables the necessary robustness to tackle high sample numbers requiring projects[171].

-Protein phosphorylation events are important since among others they rule many relevant signal transduction events by activation/inactivation of many effectors. Phosphoproteomics has been in the forefront of the PTM analysis since beginning of this century. The constant improvements have generated robotized sample preparation workstations, miniaturized sample preparation pipelines and optimized data acquisition methods. Altogether these optimizations have enabled to quantify thousands of phosphoproteins and phosphopeptides in the same study[172].

-Ion mobility spectrometry (TIMS) coupled to a quadrupole time-of-flight (QTOF) analyzers provide information containing four-dimensional (4D) data space spanned by m/z, retention time, ion mobility and signal intensity. MaxQuant Software, one of the gold standards for proteomics and freely available, has launched a module that permits to put in value all this analytical data allowing to extract more useful proteomics information[173],[174].

At CIC bioGUNE´s proteomics platform we want to foster the application of some of the newest developments in the proteomics arena. Among others we want to pursue the analysis clinically relevant yet very scarce starting material samples: mini biopsies (even FFPE) or even human oocytes. Besides, we want to scale up our sample processing pipeline to be able to analyze high number of samples to better face precision medicine projects and keep on optimizing methods for post-translational modification analysis.

By applying these new technological and methodological proteomics advancements, we envision that the coming years will be full of new findings not only in different areas of molecular and cell biology but also in clinical research.

Landmarks in structural biology

Isaac Santos and **Adriana Rojas**, *Electron Microscopy Platform*

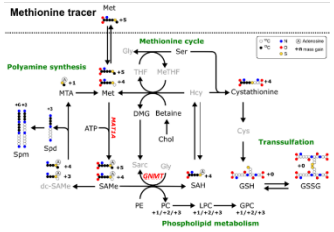
Cryo-EM has emerged as a powerful method to elucidate structures at near-atomic resolution. This year, two independent research groups have performed the remarkable feat of resolving individual atoms of a protein called apoferritin. Radu Aricescu and Sjors Scheres' group at the MRC and Holger Stark´s group at the Max Planck obtained a 1.22 Å and 1.25 Å resolution reconstruction, respectively[175],[176]. Each group used different approaches to achieve atomic maps in which hydrogen-bond networks were clearly visible. They took advantage of newly developed devices such as the electron source, energy filter, and camera in the first case. In the second case, they got a similar resolution implementing changes in the existing hardware, applying a monochromator to the electron beam, reducing the optical aberrations. The substantial improvement in the cryo-EM density map quality is highly relevant for using cryo-EM in structure-based drug design. However, one bottleneck is that image collection takes hours to days to generate enough data for extremely high-resolution structures. In this sense, X-ray crystallography is still an advantageous technique, particularly for screening thousands of compounds bound to a protein in a short time. Other developments in software and hardware have improved the determination of macromolecar structures by Cryo-EM. Among them, the better image denoising allows higher Signal-to-Noise Ratio (SNR), improving the quality of the collected data[177]. Another enhancement has been the design of new grids[178], the known as HexaAufoil grids, can eliminate the buckling of the amorphous ice during irradiation reducing electron beam-induced particle movement to less than one-angstrom distance.

Lastly, the "democratisation" of the Microscopes will come with the new imaging device, called Tundra Cryo Transmission Electron Microscope which will be available with unparalleled ease-of-use at an affordable price, extending the technique to almost any laboratory interested. On the other hand, this year MicroED has been consolidated as a powerful tool in structural biology. This technique uses electron diffraction of nano-crystals to generate structures of a different kind of samples, including soluble and membrane proteins. Tamir Gonen group has led the development of this technique, improving sample preparation and data collection[179]. An outstanding achievement has been determining the structure of a membrane protein embedded in a lipid matrix[180]. Also, a great breakthrough in structural biology was the recent advance of Deepmind company, that using artificial intelligence (AI) has developed a structure prediction software, called AlphaFold, that from only the amino-acid sequence, has been able to predict protein structures with an accuracy close to that obtained from traditional experimental approaches[181].

In the next years, the outstanding developments will come from observing proteins in their natural context, inside the cells, combining the capabilities of two typically separate microscopy platforms: light (or fluorescent) microscopy (LM) and electron microscopy (EM), what is currently known as Correlative Light and Electron Microscopy (CLEM). The challenge in the future will be to integrate LM, cryoFIB and EM into a single platform with automated workflows.

Metabolomics based Toxicity

Juan Manuel Falcón, *Metabolomics Platform*



The metabolomics specially based on high resolution liquid chromatography-coupled to mass spectrometry (hrLCMS) has advanced dramatically in recent years. Our platform has implemented many methodologies to deliver reliable bioanalytical studies in different areas and for a diverse set of objectives.

Regarding endogenous metabolisms we focus on the quantitative analysis of metabolites related to the methionine and Krebs cycles by targeting metabolites like citrate, succinate, glutathione, polyamines and phospholipids. Conventionally these

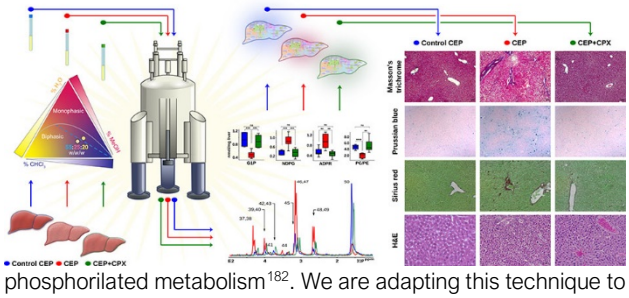
analyses are carried out by analysing total fractions of metabolites from biological samples. With these methods it is difficult to establish dynamic behaviour because of high background signals. These background signals obscure subtle changes in metabolite levels. In order to get more in details about how the metabolites are incorporated in the pathways, fluxomics has been implemented recently by using non-radioactively labelled precursors to track the metabolic changes through the pathways in a targeted, time dependent manner. These hrLCMS-based fluxomics methods constitute an advance over conventional metabolomics because allow to study the dynamics of the cellular pathways in an unparalleled sensitive and specific manner. In this context, during this year versatile, cell-based assays (hepatocytes, prostate cell lines, macrophages etc.) to follow the metabolism of labelled glucose, glutamine, methionine and choline has been implemented including an automated data-pipeline to efficiently analyse and report the relevant information.

Other important area where the application of metabolomics is advancing significantly is in drug metabolism. One of the main reasons drug candidates do not reach the market or are withdrawn after roll-out is due to adverse effects. Many of these adverse effects are triggered by unfavourable metabolic properties. Filtering out problematic compounds at an early stage will significantly reduce development costs. To overcome this problem preclinical drug development is employing metabolomics along with medium-throughput assays to assess metabolism and toxicity of new chemical entities (NCEs) and drugs in an early stage. These assays are strongly based on FDA guidelines for preclinical drug discovery and are aimed at medicinal-chemistry groups. The assays cover 1) Cytochrome P450 (CYP) metabolism, 2) reactive metabolite screening and 3) pharmacokinetic/pharmacodynamic (PKPD) support. Remarkably, in the drug metabolisms characterization it is crucial to know how a drug candidate behaves in vivo with respect to adsorption, distribution, metabolism and excretion (ADME). These parameters are determined via mathematical PKPD models. As an input these models require accurate levels of the candidate drug and its metabolites.

Phosphoromics: Towards a different look of the metabolism

José M Mato and Óscar Millet, *Precision Medicine and Metabolism Laboratory*

One of the complications of metabolism is its inherent complexity, which precludes a proper determination and dampens its interpretation. Thus, it is important to find innovative yet simpler ways to provide a holistic view of the metabolism in health, disease and as a response to treatment. In this context, we reckon that phosphorylated metabolites occupy a prominent position in all anabolic and catabolic pathways. In our laboratory, we have developed a 31P-NMR-based method to study the "phosphorome" in tissue samples through the simultaneous identification and quantification of multiple hydrophilic and hydrophobic phosphorylated metabolites. The methodology included the standarization and optimization of the protocol to yield a robust and quantitative measurement of the



phosphorilated metabolism[182]. We are adapting this technique to define the metabolic landscape in several organs (including liver, brain, heart, and pancreas) of mouse models and of human tissue samples as well. For instance, we have characterized the liver from a mouse models of the rare disease disorder congenital erythropoietic porphyria (CEP), as well as several murine models of nonalcoholic steatohepatitis, one genetic, methionine adenosyltransferase 1A knockout mice, and three other models based on the diet, mice fed a high-fat and/or choline deficient diet. In many cases, we observed alterations in the concentrations of phosphorylated metabolites that are readouts of the balance between glycolysis, gluconeogenesis, the pentose phosphate pathway, the tricarboxylic acid cycle and oxidative phosphorylation, and of phospholipid metabolism and apoptosis. Moreover, these changes correlate with the main histological features: steatosis, apoptosis, iron deposits and fibrosis. Strikingly, treatment with the repurposed drug ciclopirox improves the phosphoromic profile of CEP mice, an effect that was mirrored by the normalization of liver histology.

Al together, these findings indicate that NMR-based phosphoromics may be used to unravel metabolic phenotypes of liver injury and to identify the mechanism of drug action.

Nuclear Magnetic Resonance Methodological Advances

Tammo Diercks, *NMR Platform*

NMR metabolomics at our NMR facility is now supported by two 600 MHz IVDr spectrometers. While the strict protocols leave marginal room for NMR methodological advances, one profiling study on live cancer cells recorded 2D 1H,13C HSQC[183] to remedy the low 1H dispersion. Tracer based studies employed glucose with 2H labelling to quantify Warburg metabolism in cancer cells via produced HDO and lactate isotopomers[184], and 13C labelling to monitor pancreatic β-cell response to fuel pressure with sensitivity enhancement by DNP. In cell NMR mostly relies on overexpression of 15N labelled proteins to selectively record their 2D 15N HSQC spectrum, which was shown to correlate with the host cells' growth phase[185]. The living cells can be stabilised by methylcellulose hydrogel as a matrix mimetic.

Glycan NMR also suffers from low 1H signal dispersion that can be similarly alleviated by direct 13C detection with unexpectedly low losses, which could be further reduced by selective glycan 13C labelling [186]. Resolution in 1H observation can be enhanced by homodecoupling, as by BIRD decoupling in all dimensions, mirror symmetric perfect echo, and improved CHIRP pulses.

NMR relaxation accesses molecular dynamics on different timescales. For fast local motions, the dynamic 15N[1H] NOE experiment and theory of relaxation in field cycling relaxometry were revisited, and the deleterious effects of 13C-13C dipolar coupling on 13C relaxation were analysed[187]. Functionally more relevant slow motions can be sampled by 13CH3 relaxation dispersion experiments, where pure TROSY coherence preparation and compensated 1H refocussing pulses were presented for artefact suppression. CEST (chemical exchange saturation transfer) ideally complements relaxation dispersion studies by identifying and quantifying the exchange with invisible minor states. New CEST experiments were presented for 15N[21], glycine 13CAHA2 groups, and high power applications to extend the covered time range[188].

Further NMR methodological advances include a labelling scheme along with filtered NOESY experiment to distinguish intermolecular methyl contacts in protein aggregates, an experiment to measure protein backbone dihedral angles cross-correlated relaxation, MQC artefact suppression in 13C,13C TOCSY, combinatorial 13C(13C,H)n editing of constant-time 13C-HSQC for protein. It was also shown how intermolecular CH protein-ligand interactions can be identified and quantified by the

induced 1H signal shift, how protons in fast exchange with H_2O can be detected with much enhanced sensitivity, and how double F1-F2 frequency selection alleviates bias from spectral overlap[189].

Non-Uniform Sampling (NUS) of indirect spectral dimensions allows enormous gains in resolution-vs-measurement time, where improved schemes to measure exact NOE intensities and minimise artefacts with maximal robustness were proposed. Machine learning can further improve and speed up NUS data reconstruction[190] while we presented NOSCO-NUS for NMR titration series.

The suitability of 2- vs. 3-fluorotyrosine incorporation in proteins was analysed to enabled 19F relaxation dispersion studies on a very large protein complex[191], and was made available to RNA studies by introducing [5-19F,13C]-uridine. Separated 19F and paramagnetic tags on a membrane protein allowed PRE studies of unprecedented resolution. 19F NMR also facilitates fragment based drug discovery (FBDD), for which a dedicated fluorinated compound library with maximal chemical diversity and an optimised broadband 19F experiment were presented. Binding ligands can then be affinity ranked by the new CSAR method[192] and binding modes could be predicted from measured 19F chemical shifts[193].

In 2021, we expect that the expansion of ultra-high 1.2 GHz magnets and AVANCE NEO spectrometer electronics with multiple receivers will greatly drive NMR methods development. The former requires new schemes for broadband excitation, decoupling, and Hartmann-Hahn mixing, probably designed with Optimal Control algorithms, while the latter enables parallelised NMR experiments to exploit more polarisation especially for small molecules.

Going Beyond Basic Genomics Analysis

Ana M Aransay, *Genome Analysis Platform*

The deeper research in Genomics is increasing the necessity of developing new tools and/or adapt existing ones to answer very specific questions. In this sense, during 2020, the Genome Analysis Platform at CIC bioGUNE has worked hard in order to fine-tune protocols that allow the study of challenge RNA samples, such as the ones obtained from RiboTag and RIP (RNA-ImmunoPrecipitation) experiments, and the generation of 16S-rDNA amplicons out of DNA extracted from exosomes and other tissues different from stool, which means with very low bacterial content, in order to characterize their metagenome. RiboTag is a Ribosomal Tagging strategy to analyze cell-type-specific mRNA expression in-vivo from cells that are difficult to isolate by conventional methods, such as neurons[194]. RIPseq technology aids characterizing the RNAs that are captured or interacting with a protein of interest, and thus, understanding unexpected gene expression regulation of genes contained in the detected RNAs. The platform, besides standard services, has run some prolific collaborations that have yielded significant scientific reports in genomic and functional advances in cancer, digestive and cardiovascular disorders and central nervous system malignancies[195,196,197,198]

Due to the increasing interest in single-cell genomic approaches, CIC bioGUNE has acquired a 10x Chromium System from 10x Genomics company, which allows to obtain catalogues of expressed mRNAs (sc-mRNAseq) and open chromatin regions (sc-ATACseq) for each individual studied cell, as well as to classify the studied cells based on their protein contents. Hence, the Genome Analysis Platform at CIC bioGUNE has started setting-up the basic strategies for single-cell RNAseq and, in 2021, we will focus on running parallel protocols that allow sc-mRNAseq and sc-ATACseq for each individual studied cell. Our next obvious step will be to complement these data with the classification of the studied single-cells based on their protein content, too.

Furthermore, it is clear the need to describe the gene expression not only at single-cell level but through 2D-tissues. Accordingly, we will set-up protocols for spatial (multidimensional) transcriptomics, and for this goal, we bet on the Visium Spatial Gene Expression Solution developed by 10x Genomics. Such spatial-transcriptomics strategies will facilitate uncovering how the complex and rare cell populations interact in specific tissues or organs, and so, revealing which the aberrant functional regulations and cell communications that cause diseases are.

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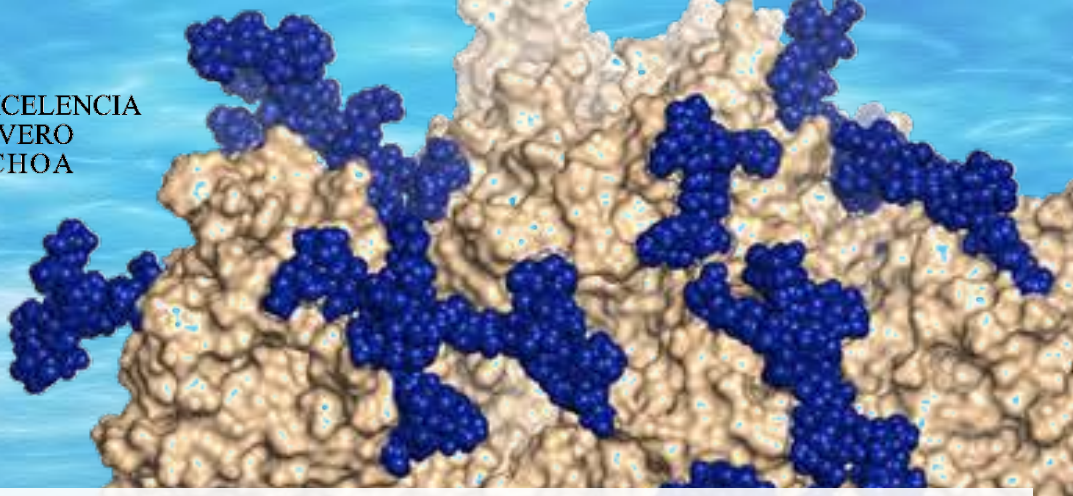

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