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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 key biomedical research topics 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).

Our 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 capability for monoclonal antibody production, integrated in our animal facility, as well as diverse 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 scientific pole in biosciences that, with the incorporation of the proper state-of-the-art technologies will develop emerging areas in the life science and health fields to enhance the competitiveness of the corresponding industrial (biotech, pharma, etc) sectors in the Basque Country. Specifically, CIC bioGUNE is strongly committed in the collaboration and coordination with the other scientific and technology entities integrated in BRTA and with other social, academic and health agents in the Basque Country to optimize our existing capacities, and jointly conform an integrative scientific and technological offer of excellence. This offer should be able to boost the evolution of our 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 of the key mechanisms and interaction patterns related to health and disease at the highest resolution. Our scientific objectives, specially focused on precision medicine, are transversal and target the complete characterization of the molecular basis of key processes involved in human pathophysiology, immune defence, cell proliferation and development with the final aim of translating our findings to the clinic.

The power of synthetic proteins towards CRISPR-based treatments

Ylenia Jabalera and Raul Perez-Jimenez, *Synthetic Biology Laboratory*

During 2023 we have witnessed once again that CRISPR-based technologies are the present and future for the treatment of thousands of diseases with a genetic component. CRISPR-Cas enzymes guided by RNA, can cleave specific regions of the genome that can subsequently be modified following different approaches such as homology direct repair (HDR), base or prime editing (BE and PE, respectively) or transposon integration. Nevertheless, CRISPR system are not perfect, and further developments are still needed for full, safe, and efficient implementation. Common problems and limitations relate to off-target mutations, dependency of a Protein Adjacent Motif (PAM) recognition for targeting, lack of efficient delivery systems and potential immune response to current CRISPR systems derived from bacteria. Considering this, much research has been conducted towards alleviating these problems and several potential solutions are emerging. In this sense, during this year researchers have presented results both at laboratory and clinical level, that shed light on the new routes that are set to provide the safety and efficiency needed for the implementation of CRISPR tools in clinical applications.

One such development is the utilization of base editors (BE) in clinical applications. This technology relies on the construction of a molecular complex encompassing a nickase version of CRISPR-Cas9 fused to a nucleoside deaminase enzyme, allowing for the replacement of a single base without the need of doble-stranded break. Thus, this methodology offers the advantage of avoiding off-target effects. This year, the first successful clinical implementation of BE was accomplished by treating a 13-year-old girl with acute lymphoblastic leukaemia. This achievement was possible by applying BE to generate off-the-shelf chimeric antigen receptor (CAR) T cells for cancer treatment[1]. Other clinical trials will be reported soon as these precision tools has also entered clinical trials in US in 2023[2,3]. Although BE provides a tool with immense potential, its main limitation resides in the size. A CRISPR-Cas nuclease fused to a deaminase has a considerable size (over 1500 a.a), and it has been recognized that this may represent a potential problem for current delivery systems. In this sense, researchers have recently investigated the potential of smaller CRISPR systems such as Cas12f, and more importantly engineered versions of such systems[4,5,6,7]. These systems are about half the size of regular Cas9 and represent a good alternative to facilitate delivery in in vivo applications. Another challenge for RNA-guided CRISPR-Cas systems involves modifications that entail large-scale perturbations, such as inserting gene-sized payloads. To overcome this limitation, scientists have been exploring the use of CRISPR-associated transposases (CASTs)[8,9]. This system enables programmable DNA integration by merging DNA-binding Cas effector proteins with DNA-inserting enzymes known as transposases. The initial findings are promising, showing effectiveness in integrating DNA cargo sizes of up to 15 kilobases[10].All these developments have provided a new generation of CRISPR tools; nevertheless, some of the limitations persist, and perhaps the most relevant one is the need for a PAM recognition sequence. The PAM sequence is a mechanism that bacteria use as a double-check point to prevent cleavage of their own genetic material and secure cleavage of external genetic elements. PAM sequences are highly specific (e.g., NGG for Streptococcus pyogenes), and each Cas nuclease recognizes a unique sequence before cleaving the target region a few bases away from the PAM sequence. This mechanism represents a limitation for CRISPR application, and this is especially significant in BE where high precision is needed. BE is based on replacing one base at a time, the need for PAM recognition makes it very difficult to alter and change any desired base. Therefore, the application of BE is limited to those bases where the appropriate PAM sequence is present. To alleviate this limitation, over the past years, many scientists have worked in the development of engineered PAMless Cas nucleases. Only molecular evolution approaches have been able to create such nuclease[11].

With all these developments in mind as well as the development of new tools for discovering novel systems (such as the fast locality-sensitive hashing-base clustering, FLSHclust[12]), the future seems to offer an opportunity for synthetic biology and protein design as viable alternative to design new engineered enzymes with improved properties, and designing PAMless Cas nucleases is a route that can extent the applicability of the most precise CRISPR tools. Will it be 2024 the year that sees a new fully efficient Cas nuclease for super-precise gene editing?

Natural and Artificial Intelligence Against Breast Cancer

Maria dM Vivanco and Robert Kypta, *Cancer Heterogeneity Laboratory*

Artificial intelligence (AI) has been developing and improving consistently over the last few decades. However, this year AI has trespassed the wonders of the science-fiction books and the limits of the specialized research laboratories to reach the front-page news with the amazing development of ChatGPT and many other AI tools. The extraordinarily fast expansion of these tools has also highlighted their potential risks, which has led to a political deal on comprehensive rules for trustworthy AI in the European Parliament, published on December 9th 2023. This agreement aims to “ensure AI in Europe is safe, respects fundamental rights and democracy, while business can thrive and expand”, as reported in the European Parliament News web page[13]. Beyond business opportunities, scientists have been trying to use AI to try to find solutions for complicated biological and medical problems, such as breast cancer, for many years. Considerable advances in basic and translational breast cancer research have significantly improved the quality of life of breast cancer patients, while increasing their survival, and yet, this disease continues to plague our modern society. According to the latest GLOBOCAN published data, female breast cancer is the most commonly diagnosed cancer with almost 2.3 million of new cases only in 2020[14]. How can AI help? No doubt at many levels. One important problem in breast cancer is early detection and diagnosis, as it has been previously shown that delays in diagnosis leads to poorer survival. The possibility of automating this task, through digital pathology approaches and combination of various imaging modalities has been attracting a surge in research activities thanks to the advent of deep learning, availability of improved hardware and accessibility of large enough dataset required for training AI algorithms. Different imaging modalities have been exploited by researchers to automate the task of breast cancer detection including mammograms, ultrasound, magnetic resonance imaging, histopathological images or any combination of them. Some of the work published this year reflects these efforts by using a massive number of cases. For example, analysis of over one hundred thousand consecutive mammograms from a population-based screening programme has shown the potential to reduce false-positive screens and increase cancer detection rates[15]. Furthermore, in the context of a prospective clinical trial, incorporation of readings by AI from over 55,000 women, also confirmed this potential in a real-world setting[16]. These studies illustrate just one example of the promise of AI to supplement clinical diagnosis, alleviating the growing pressures on our health care systems and improving our understanding of breast physiopathology.

As 2023 nears its end, we marvel at the recent developments in artificial intelligence, creative applications of mathematical modelling to identify risk of resistance to therapy[17] and many other advances in breast cancer research[18], which are often driven by new technologies and experimental approaches. Evidently, this should happen in parallel to promoting interactions between basic and translational scientists, clinicians, drug developers and patient advocates with the final aim of improving breast cancer management.

Generative AI Revolutionizes *de novo* Protein Design

Gonzalo Jiménez-Osés, *Computational Chemistry Laboratory*

Just after a couple of years into the now widely recognized as post-AlphaFold era[19] the field of Protein Sciences, particularly Structural Biology and Protein Design are experiencing a whole paradigm shift due to the emergence of extremely accurate and computationally efficient algorithms able to predict the structural outcome of sequence generation and alteration, allowing researchers to accomplish tasks that were unthinkable only a few years ago. A new open-source deep neural network developed by David Baker's group at the Institute for Protein Design, University of Washington has taken a huge step in solving the problem of sequence design. This open-source tool called ProteinMPNN[20], is now able to idealize either natural or designed protein folds by instantaneously generating multiple mutations able to increase the expression yield, solubility and stability of proteins, even in the most difficult cases such as membrane proteins[21]. Our group has utilized ProteinMPNN in conjunction with AlphaFold for protein structure prediction, and Baker's Rosetta as an energy estimator building upon our previously developed method[22], to increase the thermodynamic stability of human frataxin – a protein causing the rare genetic disease Friedrich's ataxia – by nearly 20 ºC and even rescue severely destabilizing pathological mutations[23].

This year has also witnessed the roaring of a plethora of different deep-learning based tools based on so-called diffusion methods, to quickly design new-to-Nature protein structures, with astonishing results. Diffusion models are a class of generative AI models that generate high-resolution images of varying quality. They work by gradually adding Gaussian noise to the original data in the forward diffusion process and then learning to remove the noise in the reverse diffusion process. Instead of images, videos or sound, programs such as Baker's RFdiffusion[24], ProteinGenerator[25], RoseTTAFoldNA[26], Generate Biomedicines' Chroma[27] and Microsoft's FrameFlow[28] use structural and genetic information encoded in a powerful neural network to create unconditional or function-guided proteins completely from scratch. These methods have been proven capable of designing new proteins to form symmetric oligormers, catalyze unnatural chemical reactions[29], predict protein–nucleic acid complexes with high accuracy[30], act as biosensors[31] and bind to therapeutic targets with picomolar affinity[32].

While researchers struggle to keep pace with all these new developments, DeepMind and Isomorphic Labs have announced the capabilities of the new AlphaFold model, which claims that "can now generate predictions for nearly all molecules in the Protein Data Bank (PDB)"[33] his upgrade promises to significantly improve prediction accuracy in multiple key biomolecule classes, including ligands (small molecules), proteins, nucleic acids (DNA and RNA), and those containing post-translational modifications (PTMs) such as glycosylation, methylation, palmitoylation, etc. These different structure types and complexes are essential for understanding the biological mechanisms within the cell. The model's long-awaited expanded capabilities and performance will surely help accelerate biomedical breakthroughs and realize the next era of "digital biology", giving new insights into the functioning of disease pathways, genomics, biorenewable materials, plant immunity, potential therapeutic targets, mechanisms for drug design, and new platforms for enabling protein engineering and synthetic biology.

In this occasion, however the Baker lab has taken the lead on this challenge and released RoseTTAFold All-Atom first[34]: a deep network capable of modeling full biological assemblies containing proteins, nucleic acids, small molecules, metals, and covalent modifications given the sequences of the polymers and the atomic bonded geometry of the small molecules and covalent modifications.

More than ever, the swords are drawn in the protein design battlefield…

Computer-Guided Strategies for Tissue Rejuvenation

Antonio del Sol and Sascha Jung, *Computational Biology Laboratory*

Aging is a multifactorial process that is characterized by a progressive physical and functional decline of tissues over time. To date, age-related diseases, such as metabolic syndrome or neurodegeneration, account for more than 50% of the healthcare burden among adults in the world. Without strategies to prevent age-related tissue dysfunction, the main intervention remains the specific treatment of symptoms after their onset. Thus, the discovery of interventions that can rejuvenate tissues is a major challenge impacting society at large. Currently, the most promising approach remains to be 'partial reprogramming' in which the transient activation of pluripotency genes leads to the reversion of age-related cell phenotypes. However, although promising results have been obtained in animal studies, the translation to humans is overshadowed by serious safety concerns. Computational approaches can help to discover urgently needed therapeutic interventions by generating predictions to guide experimental and clinical research. Indeed, these computer-guided approaches include the modeling of age-related dysregulations at multiple levels of biological organization as well as machine learning methods to identify patterns in biological and clinical data.

Lately, the aging field has witnessed an upsurge in computational strategies to predict candidate therapeutic targets for tissue rejuvenation. In this regard, a computational cell-cell communication model was developed to characterize the tissue- and sex-specific age-related dysregulations in ligand-receptor interactions[35]. As a result, several candidate molecules have been pinpointed whose modulation may rescue specific tissues functions, such as growth, angiogenesis or extracellular matrix organization. Apart from such modeling approaches, the use of machine learning techniques to quantify biological age led to the discovery of a few novel rejuvenation factors. For instance, one study employed physical measures, biochemical assays, genomic data, and cognitive functions from more than 50,000 healthy individuals and identified CST3 as a candidate therapeutic target whose expression explains the difference between chronological and biological age in 10 tissues[36]. Moreover, a gene expression-based multi-tissue clock has been developed that predicts biological age and can inform about age-dysregulated processes[37]. Using this framework, a seminal study demonstrated how gene-expression based clocks can prioritize therapeutic targets in large-scale genetic screens. The study discovered the splicing factor SRSF1 as a novel rejuvenation factor in human fibroblasts that reduces senescence and rescues several dysregulated processes[38]. Moreover, upregulation of SRSF1 rescued the wound healing capacity of human cells as well as of the skin in animal models. Importantly, SRSF1 does not activate pluripotency genes and is therefore expected to be a safer alternative to 'partial reprogramming' while showing comparable rejuvenation effects.

The remarkable developments in computational approaches that discovered new candidate therapeutic targets to guide experimental aging research raises the hope of designing rejuvenation protocols that are efficacious and safe in humans. In this regard, biological aging clocks are to play a key role in this endeavor by providing an interpretable readout for drug- and genetic-screening approaches without the need for large-scale experimentation. In the coming years, we believe to see tailor-made aging clocks for various tissues and an expansion in large-scale screening studies, which is expected to accelerate the discovery of healthspan extending interventions significantly.

Glycosciences in Action

Ana Ardá, June Ereño-Orbea, Ana Gimeno, Luca Unione, and Jesús Jiménez-Barbero, *Chemical Glycobiology Laboratory*

In 2023, Glycosciences have witnessed remarkable advancements that spans from basic science to promising applications in biomedicine. This knowledge has also been fueled by ground-breaking studies that redefine our capabilities for glycan design and synthesis. A pioneering approach developed in a collaboration between Delbianco's with our lab[39] has introduced a new paradigm, demonstrating the unique ability of designed glycans to fold into a stable secondary structure -a glycan hairpin, a topology not found in nature. Moreover, standing out in the first category, Anggara et al have revealed for the first time how covalently attached sugars are arranged in glycoproteins and glycolipids[40], capturing single molecule images by scanning tunneling microscopy (STM) at cryogenic temperatures and unveiling the intricacies of glycan-decorated molecules. These advances in the direct decoding of the "sugar code" provide indispensable insights that hold immense promise for crafting effective cancer treatments and propelling this field into new frontiers. In tandem with this, chemoenzymatic glycan synthetic methodologies have been implemented to mimic complex biosystems, such as the cell, contributing to impact on more realistic molecular recognition studies. As a key example, Derda et al. have constructed genetically-encoded liquid glycan arrays (LiGA) featuring complex type N-glycans using a phage platform[41]. At the biomedical level, as the relationship between glycosylation and disease becomes increasingly robust, scientists continue trying to unveil the specific elements that regulate cell glycosylation. For instance, the exploration of glycans in cancer takes us on a comprehensive journey from understanding immune evasion mechanisms to potential therapeutic break-throughs. Hutter et al. have delved into the Siglec–sialic acid axis, uncovering a pivotal "don't eat me" signal and elucidating a critical mechanism in glioblastoma. Their groundbreaking study[42] illuminates the inhibitory impact of Siglec-9 on immune cells, proposing a compelling therapeutic target. In parallel, our collaborative research[43] has unveiled the glycosylation-dependent interaction between the Siglec-15 glycoimmune checkpoint receptor and T cells. These findings open new avenues for advanced therapies, potentially targeting Siglec-15 or its glycosylated receptors (such as integrin CD11b/CD18).

Regarding infections, most viruses attach to the surface of host cells by binding to specific human glycans. This year, a transoceanic collaboration among world leader scientists in USA, UK, and Japan has elegantly assessed the impact of specific glycan receptors on virus binding, infection, and growth in the context of human influenza[44], identifying those that are recognized by the virus to infect human cells. They engineered different cell lines by overexpressing specific enzymes known to produce the glycans and then evaluated their susceptibility to infection by different influenza virus strains. These contributions underscore the dynamic evolution of the field, setting the stage for further discovery, and represent the guiding light to show that only collaborative efforts among chemistry, biology, and biomedicine will provide reliable solutions to the challenge of decoding the biological role of glycans.

Rethinking Cancer: Slow, Furious, Hungry and Computerized

Arkaitz Carracedo, *Cancer Cell Signaling and Metabolism Laboratory*

The 2023 consolidates a recurrent perception that has emerged in this century since the sequencing of the first human genome: data above everything else. In the past, every single result would be thoroughly scrutinized, analyzed, and interpreted. In the advent of high-throughput technologies, we have instead embraced a new way to do research: we generate terabytes of data from which we only exploit a small fraction. We even generate data that is dominated by the absence of information, matrices governed by zeroes such as single-cell transcriptomics, and apply potent inference strategies to generate new data and assumptions. This research strategy is tremendously powerful but also overwhelmingly inefficient. Although we have at hand multiple computational tools to integrate data and move from altered mRNAs to perturbed pathways, we leave much of the biological data in the pipeline. Despite this observation, the indiscriminate generation of massive amounts of data in primary studies opens the door to secondary research projects in the scientific community, which are inspired or dominated by the exploitation of publicly available data. In turn, we have the opportunity to build a hypothesis based on original data that remains to be fully exploited.

The capacity to interrogate epigenetic and transcriptional reprogramming with single cell resolution has enabled the identification of novel molecular processes that underlie cancer progression. On the one hand, Terkhanova et al.[45] constructed a dataset with single cell RNA Seq and chromatin accessibility data from more than 200 specimens. With over 1 million cell sequenced, the authors identified molecular regulators of cancer transitions that were conserved in different tumor types specific to a given cancer. A major challenge in cancer biology is the assessment and relevance of intratumor heterogeneity. To study this important aspect, Gavish et al.[46] compiled single cell RNAseq data from 1163 samples representing 24 different tumor types to identify 24 metaprograms that led to 11 hallmarks of transcriptional intratumoral heterogeneity. The translation of this effort to an accessible website ensures that this compilation exercise will inspire multiple future studies.

Lastly, the combined power of sample collection and analysis is well-illustrated by the TRACERx strategy, which has made seminal findings since its creation. The clinical and molecular data contained in TRACERx has promoted the development of research studies covering cancer evolution, subclonal selection, ctDNA-based disease monitoring and the influence of biometric characteristics of patients (body composition) in the development of cachexia[47],[48],[49],[50],[51],[52]. Our perception of cancer is largely illustrated by a cell proliferating without control. Whereas proliferating cancer cells are responsible for generating a tumoral mass, there are many instances where the cells that prevent the disease from being eradicated are those that remain quiescent. This state can be observed in circulating, dormant or drug-tolerant cancer cells. Therefore, understanding the specific molecular requirements of these stages is critical to refine current therapeutic strategies. Two interesting strategies are worth reporting in 2023. On the one hand, the analysis of slow-cycling cells upon drug treatment allowed two independent groups to identify relevant processes in drug tolerance, namely PINK1-mediated mitophagy[53] and DPPA3-HIF1a axis[54] On the other hand, the combination of cellular barcoding with single-cell RNA Seq technologies has

allowed the identification of cellular states and trajectories that are associated with reduced sensitivity to anticancer agents[55,56] and the characterization of increased heterogeneity in homogeneous cell cultures[57]. The metabolism of the tumor microenvironment which comprises dynamic interactions between cancer cells and surrounding stromal components plays a fundamental role in cancer. During next year we will experiment increase knowledge of cellular mechanism of cancer through nutrient competition, hypoxia-induced adaptations, and crosstalk with stromal cells such as adipocytes or macrophages contribute to a complex metabolic landscape.

Expanding Horizons towards Genomic Medicine: Multi-Omics, AI, and Broadening GWAS Focus

Urko Martinez Marigorta, *Integrative Genomics Laboratory*

At the Integrative Genomics lab, we analyze large-scale multi-omic profiles from patients to improve our understanding of disease. We aim to gear knowledge about how patients develop diseases to come up with new predictors of disease prognosis and response to treatment. Gearing genomic data towards development of precision medicine in the clinic is the overarching context of our research. We will discuss here three important developments in genomic medicine in 2023.

The first one involves the consolidation of multi-omic approaches for precision medicine. Integrating layers such as genomics, transcriptomics, and metabolomics enables insights into diseases, but can also revolutionize precision health initiatives. For instance, the Acute Care Genomics program swiftly diagnosed critically ill children with suspected genetic conditions, showcasing a 54% diagnostic yield through integration of whole-genome sequencing with functional assays[58]. In another example, a study based on the UK Biobank leveraged genomic and metabolomic data to improve a risk prediction model for cardiovascular disease, with significant gains in predictive power[59]. These developments underscore that multi-omics will slowly gain a pivotal role in our ambitions towards genomic medicine. The second development involves the promise of artificial intelligence (AI) as a key tool to accelerate genomics for precision medicine. AI can analyze vast genomic datasets, aiding in the interpretation of complex genetic information. For instance, AI-driven platforms like Sei excel in predicting the effects of unexplored genetic elements that were identified through GWAS, namely genome-wide association studies[60]. Moreover, AI can play a crucial role in applying genomics for drug discovery and development. By incorporating genomic insights, platforms like Open Targets use omics data through AI models that expedite drug repurposing efforts[61]. The final development involves the application of GWAS to identify genetic regions linked to disease prognosis and drug response. For instance, the IMSGC consortium discovered significant genetic modifiers that shape long-term outcomes in multiple sclerosis[62]. In turn, Zhang et al. capitalized on data from several clinical trials to carry out a GWAS for response to anti-IL17 therapy in inflammatory conditions[63]. These pioneering efforts mark a shift beyond the exclusive focus on GWAS for diagnosis. Leveraging genetic data to predict disease trajectories and drug response represents a more promising strategy for genomics to impact clinical practice and patient lives.

For the future, I anticipate that integration of multi-omics with other sources of information, such as the exposome, through advanced AI-driven analyses will likely revolutionize the prospects for genomic medicine. As we move beyond purely genetic data and simple focus on diagnosis, the convergence of methodologies towards characterizing the full architecture of disease will propel us closer to tangible clinical impact.

Genomics for Precision Medicine in Functional Gastrointestinal Disorders

Leire Torices, Mauro D'Amato and Cristina Esteban, *Gastrointestinal Genetics Laboratory*

A subset of “organic" irritable bowel syndrome (IBS) patients is defined as "carbohydrate malabsorbers", typified as carriers of defective variants in the sucrase-isomaltase gene (SI). SI encodes an intestinal disaccharidase responsible for breaking down sucrose and starch. Similar to cases of congenital sucrase-isomaltase deficiency (CSID), our investigations indicate that defective SI variants producing enzymes with reduced disaccharidase activity may contribute to an increased risk of IBS[64]. This work predominantly focuses on the impact of SI genotypes in the response to diet in IBS patients, suggesting a high efficacy of a diet that specifically targets SI substrates, sucrose and starch-restricted diet (SSRD), compared to low-FODMAP diet[65]. This pioneering approach presents a promising avenue for future trials, emphasizing the relevance of personalized dietary interventions in IBS, with a positive impact on symptoms and enhanced quality of life. Our latest research initiative expands beyond the SI gene looking into carbohydrate-digesting enzymes (Carbohydrate-Active enZYmes, hCAZymes). Through genomic sequencing of DOMINO trial participants[66], we identified defective variants in 25 pivotal hCAZyme genes for carbohydrate digestion and absorption, including AMY2B, LCT, MGAM, MGAM2, SI, and TREH. Our findings reveal that defective hCAZyme genotypes significantly influence the response to a low-FODMAP diet. The therapeutic advantage was most pronounced in patients with multiple dysfunctional hCAZyme genes, reinforcing the potential of a gene-dosage effect in improved clinical response (manuscript submitted for publication). Based on pioneering work, a novel and unconventional approach has shaped another main research line, focused on the exploration of bowel (dys)function through genetic characterization of endophenotypes. Endophenotypes, quantifiable traits with demonstrable genetic inheritance and clinical relevance, serve as focal points in our endeavor to dissect the molecular underpinnings of bowel function. This research demonstrates our dedication to push the boundaries of traditional methodologies, providing a promising path for a better understanding of GI disorders. The insights gained exploring the genetics of GI endophenotypes will contribute to advance scientific knowledge for the use of these methodological approaches for complex conditions and potentially offer novel opportunities for therapeutic interventions in the realm of bowel (dys)function. Our groundbreaking research unravels pivotal genetic factors in both IBS and dysmotility syndromes, marking a transformative leap towards precision medicine. These findings not only deepen our understanding but also introduce actionable targets, reshaping the clinical approach to functional GI disorders[67].

Virus Structures in the Cellular Context.

Juan Diego Rivero, Ikera Arriaga and Nicola Abrescia, *Structure and Cell Biology of Viruses Laboratory*

Traditionally, researchers have examined viruses within infected cells using fluorescently labelled viruses and light microscopy (with a resolution worse than 200 nm) or employed cell-sectioning techniques for subsequent imaging with an electron microscope. The latter approach involves various cell and sample preparation methods that have been developed and optimized to maintain conditions close to the native state of the

virus and cell. However, they often require using harsh fixation or embedding procedures that might end up introducing artifacts within the cell. These efforts have been enhanced by advances in acquiring routine tomographic data. Cellular tomography provides depth information at a higher resolution than the serial addition of ultrathin 2D sections.

One significant challenge in interpreting the structure of virus-infected cells arises from the complexity of macromolecular crowding within the cell. To create an atlas detailing the various stages of viral infection, including the entry, uncoating, replication, assembly, and exit of viruses from the cell, researchers investigate structural changes at different timeline points during infection. Tomograms are collected for each timepoint, and various computational techniques, from segmentation to template matching, are utilized to identify recurring cellular or virus structures. Sub-tomogram averaging of equivalent structures improves the signal-to-noise ratio, allowing the refined structure to be reintegrated into the cellular landscape, as demonstrated in previous structural work on SARS-CoV2[68,69].

Our group has been incorporating in situ structural cellular techniques that have allowed to image viruses infecting cells in quasi-native conditions. We have been using cryo-structural illumination microscopy (cryo-SIM) at Diamond Light Source facility (UK) to generate 3D images of volumetric samples of viruses hijacking and overpowering the host cell with superior resolution than any other confocal microscopy. The scope of cryo-SIM at cryogenic temperatures is its combination with correlative ultrastructural imaging. This has been achieved by collecting from the same illuminated region tilt series using soft X-rays tomography. Then the reconstructed 3D tomograms are correlated with the cryo-SIM volume. Further, we have implemented cellular cryo-Focus Ion Beam (FIB) milling and cryo-electron tomography (cryo-ET). The operation of a focused ion beam system is to ablate or mill a sample/cell/tissue using a strongly focused ion beam, usually gallium or plasma FIB xenon which basically removes sample's material until a thin layer of the cell if left (~200-300 nm). The final cell fragment called lamella is thin enough to be targeted by cryo-ET allowing the visualization in detail of the cell content, from organelle and cell compartments' organization, to macromolecules, and zooming in up to protein structures and virus particles in their native context.

The integration of the above techniques enables us to produce a multiscale resolution 3D imaging of quasi in-vivo infected cells and structurally analyse at different time points the cell physiopathology upon virus infection (and in presence of antiviral drugs) and the morphogenesis of viral particles.

Extracellular Vesicles for Interorgan Communications and Therapeutic Vehicle

Juan M. Falcón-Perez, *Exosomes Laboratory*

Extracellular Vesicles (EVs) are cell-secreted entities that mediate intercellular communication that can provide valuable information about organs, tissues, and cells via liquid biopsy, and are an ideal diagnostic window into the human body. This hypothesis is backed by the fact that there are two FDA-approved EV-based diagnostic tests: Bio-Techne's ExoDx Prostate IntelliScore EPI-CE IVD Test for prostate cancer and Guardant's 360 CDx test for non-small cell lung cancer[70]. With almost 30.000 scientific publications EVs in the last 20 years and 7800 in 2023[71], and more than 100M€ privately invested to develop EV based diagnostics and therapeutics products, the EV market forecast is expected to reach $2.28 billions by 2030. Globally, there are 204 clinical trials on exosome studies, of which 114 trials are evaluating exosome-based therapeutics and 74 are testing exosome-based diagnostic tests (source: ClinicalTrial.gov). These numbers reflect the great expectations that academic and pharma industry researchers have in EVs, however there are many challenges that need to be solved before EV research impact significantly in the society.

There were works during 2023 showing the role of EVs in intercellular and interorgan communications and the use of EVs as biomarkers for the diagnosis of diseases. They provide evidence that Adipocyte-derived extracellular vesicles (AdEVs) from diet-induced obese (DIO) mice serve as a signal that can amplify insulin secretion through the transfer of a functional insulinotropic protein cargo into pancreatic beta-cells. Therefore, AdEVs reflect the metabolic state of the tissue. EVs participate in cellular communication between different cell types in the brain and might be involved in the dissemination of pathological changes from the brain to other tissues. Several reviews this year such as Kong et al., 2023[72] and Guo et al., 2023[73] show the enormous potential of EVs as biomarkers for brain disorders. This is a great challenge for clinical psychiatry as current diagnosis is almost dependent on report symptoms and physicians' experience. EVs can pass the blood-brain barrier and brain-derived EVs have been detected in rodent and human peripheral blood. However, one limitation in this area of research is the identification of tissue specific EVs by liquid biopsy. It is necessary to develop more specific brain derived EV markers or brain region specific EVs, and even specific for certain cell types such as neuron- or glia-EVs. Identify the functions of EVs with high histological or pathophysiological specificity will become a research hotspot of mental illness. Eitan E, et al described how blood biomarkers can improve drug development for Alzheimer's disease (AD) and its treatment[74]. Neuron derived extracellular vesicles (NDEVs) in plasma offer a minimally invasive platform for developing novel biomarkers that may be used to monitor the diverse pathogenic processes involved in AD. The fabrication of more physiological models is another area of interest for the EV community. Thus, Jian H et al.[75] as reported an in vitro construction of liver organoids with biomimetic lobule structure by a multicellular 3D bioprinting strategy. HepaRG cells are used on this strategy to optimize a bioink system using materials with opposite charges. Two distinct bioinks are used: (a) Sodium Alginate-Based Bioink (Bioink 1): This is chosen for its ability to provide structural support to the resulting liver organoids. (b) Dipeptide-Based Bioink (Bioink 2): Dipeptides offer flexibility and allow for precise design of the liver organoid structures. The primary objective was to create liver organoids that closely mimic the characteristics of natural liver tissue.

Another interesting article in this area reported by Nguyen et. Al.[76] reported a human kidney and liver organoid-based multi-organ-on-a-chip model to study the therapeutic effects and biodistribution of mesenchymal stromal cell-derived extracellular vesicles. This article discusses the therapeutic potential of sEVs derived from mesenchymal stromal cells (MSCs), particularly in the context of kidney injury. These organoids maintained their physiological functions, and holds the promise for accelerating the development of MSC-sEV-based therapies for kidney injury and potentially other diseases.

The Rise of Antibody-Drug Conjugates

Asís Palazón, *Cancer Immunology & Immunotherapy Laboratory*

The field of oncology witnessed a significant milestone with the rise of Antibody-Drug Conjugates (ADCs). ADCs uniquely blend the specificity of monoclonal antibodies with the potency of cytotoxic drugs, aiming to target tumor cells while sparing healthy tissue.

The concept of ADCs was initially envisioned over a century ago, evolving into a promising therapeutic strategy in oncology. The first FDA-approved ADC in 2000 marked the

beginning of a new era in targeted cancer treatment. The rapid advancement in ADC technology has catalyzed the development of numerous ADCs, addressing various tumor types, especially those resistant to conventional treatments. Paul Ehrlich's early 20th-century concept of a 'magic bullet' laid the foundation for ADCs. The hybridoma technology, crucial for monoclonal antibody generation, significantly contributed to realizing Ehrlich's vision. The FDA's approval of gemtuzumab ozogamicin in 2000 marked a new phase in ADC development, integrating improved understanding of ADC mechanisms with technological advances. An ADC comprises three fundamental elements: an antibody targeting a tumor-associated antigen, a cytotoxic payload, and a linker. The design of each component significantly influence the ADC's efficacy and safety. Modern ADCs predominantly use humanized or fully human antibodies, reducing immunogenic side effects. The linker technology ensures payload release at the tumor site, enhancing therapeutic efficacy while minimizing systemic toxicity. ADCs exert their antitumor action primarily by delivering cytotoxic payloads directly to cancer cells. The binding of the antibody to its target antigen triggers internalization into the tumor cell, followed by linker breakdown and payload release. This targeted approach allows for a more efficient and less toxic treatment compared to traditional chemotherapy.

Despite their targeted nature, ADCs are associated with unique toxicities. Common adverse events include pneumonitis, ocular, and skin toxicities. Understanding these side effects is crucial for optimizing treatment[77]. Recent ADC developments have focused on expanding their therapeutic scope, incorporating immune-stimulating agents and other novel payloads. The ongoing exploration of combination therapies and engineered toxin bodies exemplifies the dynamic nature of ADC research, aiming to further enhance their effectiveness while minimizing side effects[78]. As with any cancer therapy, resistance remains a challenge in ADC treatment. Mechanisms of resistance include alterations in target antigen expression, mutations affecting payload sensitivity, and changes in ADC internalization. Understanding these mechanisms is critical for developing strategies to overcome resistance.

HER2-targeted Antibody-Drug Conjugates (ADCs) represent a significant advancement in the treatment of HER2-positive cancers, particularly breast cancer. These ADCs include trastuzumab deruxtecan (T-DXd), designed to deliver cytotoxic agents directly to cancer cells overexpressing the HER2 protein. These ADCs have shown remarkable results in improving patient outcomes, including those with metastatic or treatment-resistant forms of cancer[79]. As research continues to evolve, ADCs are poised to play an increasingly vital role in oncology, potentially changing the landscape of cancer therapy.

The Role of Microbiota in Colorectal Cancer

Naiara Gutiez, Héctor Rodríguez and Juan Anguita, *Inflammation and Macrophages Plasticity Laboratory*

A healthy microbiota is critical to maintain homeostasis, while its imbalance (dysbiosis) can promote the development of several intestinal disorders, such as colorectal cancer (CRC). CRC is the fourth most diagnosed cancer and the second leading cause of cancer-related deaths. The latest advances in the field have shown how intestinal microbes influence all stages of the disease, from tumor development to post-treatment outcomes.

Several microorganisms are related to a poor prognosis in CRC. One of the most prevalent is Fusobacterium nucleatum, linked to inflammation, tumor cell proliferation, chemoresistance and metastasis. F. nucleatum modulates host epitranscriptomic modifications to increase CRC aggressivity and enhance cancer cell migration and its invasive capacity through cytoskeleton reorganization[80]. In contrast, commensal bacteria might prevent CRC development including members of the Lachnospiraceae family that maintaining the immune surveillance function of CD8+ T cells[81].

There is interest in the stratification of patients based on their gut microbial communities and distinguishing the causes of early-appearance CRC from those diagnosed at an advanced age. A recent classification showed that enrichment with pathogens such as F. nucleatum, or those belonging to the Pseudomonadota phylum have poorer outcomes than patients retaining commensals from the Bacillota and Bacteroidota phyla[82]. The microbiome and metabolome profiles of early- and late-onset CRC have also shown differential signatures and underlined the relevance of the diet in the former case[83]. Due to the slow progression from premalignant lesions to carcinoma, the search of chemopreventive agents is a priority. Statin use correlates with a reduced incidence of CRC. It induces the modulation of host immunity by increasing the levels of the tryptophan metabolite, indole-3-lactic acid, by Lactobacillus reuteri[84]. Once CRC has developed, treatment often includes the surgical resection of the colon or rectum and the reconnection of the remaining bowel ends (anastomosis). Poor healing-induced anastomotic leaks increases cancer recurrence, with a causal role by preoperative inflammatory gut microbiota linked with certain bacterial species[85]. Gut microbiota also affects the efficacy of cancer treatments. Metabolomics and microbiome data analysis from a CRC cohort revealed an increased abundance of F. nucleatum and its derived metabolite, succinic acid, in patients that did not respond to anti-PD1 immunotherapy, which limits CD8+ T cell infiltration into the tumor microenvironment[86]. Targeting F. nucleatum also provides microbial neoantigens that induce the infiltration of CD8+ T cells, effectively achieving an immunologically 'hot' tumor[87]. On the other hand, the delivery of capecitabine, the first-line chemotherapeutic agent for CRC, inside nanoparticles composed of stearic acid conjugated with xylan, a polysaccharide prebiotic, delays its blood clearance and increases its intratumoral accumulation, promoting anti-tumor immune responses and the growth of probiotics[88].

Microbiota-based approaches provide a framework for improving challenges posed by CRC. Microbes and their metabolites have potential as non-invasive biomarkers for early detection, patient stratification, and treatment efficacy prediction. They are also powerful therapeutic targets for intervention, alone or combined with classical treatments, such as surgery, chemotherapy and immunotherapy. Therefore, expanding the knowledge about microbiota and integrating it with classical medicine is key for the progress of personalized medicine.

Great Hope and Expectation for the Imminent Start of Clinical Trial for Prion Diseases.

Joaquín Castilla and Hasier Eraña, *Prion Research Laboratory*

During 2023, research on prion diseases or transmissible spongiform encephalopathies (TSE), a group of rare and invariably fatal neurodegenerative diseases, has been marked by important advances in therapy, diagnostics, and a more profound understanding of some of the molecular mechanisms underlying these disorders. Prion diseases affect humans and other mammals and are caused by an unconventional pathogen solely constituted by an aberrantly folded protein, called prion or PrPSc (Prion Protein Scrapie isoform). This misfolded protein originates from a poorly known structural change of an endogenous protein

especially abundant in neurons, named PrPC (Prion Protein Cellular isoform).

One of the most important advances in 2023 was the announcement of a new clinical trial led by Ionis for an anti-sense oligonucleotide (ASO)-based therapy, aimed to reduce PrPC levels in brains of patients at early stages of disease[89]. Nonetheless, despite the imminent clinical trial, other therapeutic strategies have also been investigated during 2023[90,91,92,93,94]. However, given the difficult diagnosis of these disorders, and the benefits that early treatment would suppose for the therapeutic efficacy of any drug, developing highly specific and sensitive diagnostic methods is instrumental. For this reason, research on prion disease diagnostics is another highly active field, focused in 2023 on finding biomarkers in easily accessible biological fluids and tissues[95,96,97]. Along this line, the search of new prodromal biomarkers that may allow predicting disease onset in at-risk individuals has also seen some advances this year[98]. Finally, in terms of diagnostics, retrospective evaluation of tests currently in use in the clinical practice has been quite active this year[99,100,101]. Our understanding of the molecular mechanisms underlying these disorders has also advanced, mainly due to the definitive demonstration that structural differences define prion strains and determine their inter-species transmission[102], and the new evidences defending the existence of multiple PrPSc conformers within a single prion strain[103].

Overall, it has been a very exciting year in prion research, mainly due to the imminent and highly promising clinical trial and because of the advances in diagnostic methods, which in a near future will definitively allow the early diagnosis from easily accessible body fluids. For the following years, the results of the clinical trial will be in the spotlight while novel therapeutic approaches will keep developing given the likely necessity of combination therapies aiming to multiple targets to cure definitively these devastating diseases. With regards of the molecular basis of prion diseases, for the near future we expect new high-resolution structures of distinct prion strains that will contribute to understand the different structural motifs that determine the differential biological features of prion strains. Therefore, it seems that all the efforts done to comprehend these diseases for the past 50 years are finally crystalizing into highly promising therapies and diagnostic methods. This transformative development brings about radically new and positive prospects for all those affected by these hitherto incurable diseases and very exciting times for researchers in the field.

Transformative Landscape of Incretin-Based Therapies and Precision Medicine in Liver Disease

Malu Martinez-Chantar, *Liver Diseases Laboratory*

The landscape of liver disorders has undergone a transformative shift with the emergence of metabolic dysfunction-associated fatty liver disease (MAFLD, acknowledging the intricate interplay between metabolic dysfunction and fatty liver disease. This updated terminology not only captures the diverse manifestations of the condition but also emphasizes the critical role of addressing metabolic factors in its management. Ongoing research and clinical efforts in this field play a fundamental role in advancing our understanding and improving outcomes for patients.

Since the introduction of exenatide in 2005, marking the onset of the glucagon-like peptide 1 (GLP-1) agonist era, there has been a notable surge in these molecules. By 2023, their prominence extends beyond merely controlling blood sugar levels in type 2 diabetes, encompassing the management of associated conditions such as obesity, cardiovascular disease, diabetic kidney disease, and notably, metabolic dysfunction-associated steatohepatitis (MASH)[104,105];

The development and utilization of GLP-1 agonists have become a valuable tool for managing metabolic syndrome, addressing various facets of the condition. From inducing weight loss to improving insulin sensitivity and reducing cardiovascular risk factors, these medications hold promise for enhancing clinical outcomes in patients with metabolic syndrome[106,107];

Incretin-based therapies mark a revolutionary shift in approaching liver-related conditions, going beyond the conventional focus on non-alcoholic fatty liver disease (NAFLD). Distinguished by their intrinsic metabolic and anti-inflammatory properties, these therapies exhibit remarkable potential across a spectrum of liver-related conditions. Research and preclinical evidence unveil a promising role for incretin-based therapies in mitigating liver fibrosis, presenting a transformative approach by addressing underlying metabolic dysregulations and inflammation. The potential to decelerate or even reverse fibrotic progression opens a novel therapeutic avenue, offering optimism for patients grappling with this challenging condition[108]. In the realm of cirrhosis management, incretin-based therapies are also gaining prominence beyond their metabolic effects. These therapies may play a pivotal role in promoting liver regeneration and function, potentially alleviating complications associated with cirrhosis. Ongoing research delves into nuanced mechanisms, enhancing our understanding and clinical applications. Incretin-based therapies extend beyond non-malignant liver conditions, actively under investigation for their role in preventing the development or progression of hepatocellular carcinoma (HCC). Operating as preventive measures by addressing metabolic factors contributing to liver cancer risk, these therapies offer a multifaceted approach to liver health.

Precision medicine approaches add sophistication, aiming to identify patient subgroups deriving specific benefits from incretin-based therapies based on unique liver condition characteristics. This tailored approach holds the potential to maximize therapeutic impact while minimizing adverse effects, ushering in a new era of personalized medicine. To substantiate these findings, longitudinal studies are underway to gather robust, real-world data on the sustained effects of incretin-based therapies across diverse liver-related conditions. These studies contribute valuable insights into therapeutic durability and the potential for reshaping the natural history of liver diseases, fostering evidence-based and patient-centric care. Additionally, long-term data on weight loss preservation will be crucial determinants of the therapeutic applicability of these agents. Ongoing efforts to address these liabilities will shape the future prospects of incretin combinations.

In conclusion, the broadening scope of incretin-based therapies goes beyond addressing just NAFLD, extending to cover fibrosis, cirrhosis, and hepatocellular carcinoma. The dynamic research landscape augurs well for reshaping the treatment approach across various liver disorders, fostering optimism for better outcomes and heightened patient well-being[109]. Pioneering strategies are delving into combination therapies that integrate incretin-based treatments with existing approaches for liver-related conditions. By leveraging synergistic effects, these innovative approaches are poised to establish more comprehensive and effective treatment strategies. Science Journal has recognized GLP-1 agonists, developed for obesity treatment, as the scientific breakthrough of the year.

Ubiquitin-like Modifications in Health and Disease

Rosa Barrio, *Ubiquitin-likes and Development Laboratory*

Our primary focus is the regulation of developmental processes and diseases through post-translational modifications

by the Ubiquitin-like (UbL) family. UbL proteins can attach to target proteins, altering their function and regulating cellular processes such as proliferation and transcriptional regulation. The study of UbL modifications is challenging due to the limited quantities of a given modified protein and the transient nature of the modification. We developed biotin-based strategies for recognizing UbL-modified proteins (BioUbL[110]), identifying interactors of modified proteins (SUMO-ID [111],[112]), and identifying specific targets of E3 ligases (BioE3[113]). These approaches are particularly relevant in the context of targeted protein degradation (TPD), a novel strategy that hijacks the ubiquitin-proteasome system (UPS) to degrade disease-causing proteins. We are particularly interested in rare diseases, especially those caused by dysfunction of the primary cilia, and in the role of the UbL family in these processes. Specifically, we investigate the implication of protein homeostasis in Townes-Brocks Syndrome (TBS), a rare condition characterized by kidney defects, deafness, and polydactyly, resulting from mutations in the transcription factor Spalt-like 1 (SALL1).

Important advances in TPD have been achieved, increasing the number of PROTACs in Phase I/II clinical trials, most of them for oncological treatments. Interestingly, other diseases different than cancer are being approached, i.e. a Phase I trial of a IRAK4 degrader for the treatment of inflammatory skin disease[114], showing a promising reduction in inflammatory biomarkers. Of particular relevance is the development of BacPROTAC[115]. Directed to the degradation of bacterial proteins, it showed more than 100-fold more potent killing efficiency over M. tuberculosis, than antibiotic. This strategy might be used as well for other pathogens and be crucial in the fight against antibiotic resistance. The growing interest on TPD raised the need to increase the E3 ligases repertoire. A web portal has been developed, E3Atlas[116], to analyze the suitability of a given E3 to be considered in TPD.

Biotin-based technologies are continually evolving, particularly in the context of the UbL field: TransitID[117], identifies subcellular trafficking of proteins across compartments; CsFiND[118], combines Split-TurboID / Split-GFP to identify proteins at the interface of organelles; LOV-Turbo[119] uses light to enable spatiotemporal control of labeling; BioTAC identifies targets of small chemicals; and BioE3[120], identifies targets of E3 ligases; SATTs[121], focuses on targets of SUMO-specific ligases.

There have been advances in the understanding of the role of SALL proteins during cardiac development[122] and their interaction with SMADs proteins to stablish microglia identity[123]. New cases of TBS have been reported[124,125,126] one case with mutations in the DACT1 gene, associated to TBS2[127].

Anticipated growth in the field of TPD underscores the need for an expanded E3 ligase repertoire. Given the current limitations in the types and quantity of E3 ligases employed in TPD, broadening the E3 toolbox is crucial for enhancing the precision of TPD applications. The exploration of new strategies, particularly those based on biotin proximity proteomics, is highly promising due to their exceptional specificity and sensitivity. The regulation of protein homeostasis by UbL components in the context of centrosome regulation and ciliary formation and function is also a growing field with biomedical interest, particularly in rare diseases.

Three-Dimensional Cryo-Electron Microscopy of Dynamic Macromolecular Complexes

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

In the field of Structural Biology, the use of AI based predictive methods has dramatically changed the landscape. The Alphafold software and the associated database already provide predictions for the structure of all known proteins. The current efforts are centered in modeling of surface interactions between proteins or docking of small molecules as potential drugs. It seems that docking based on predicted structures for proteins is not working well, probably due to the bad quality of the amino acid conformers. That is, AI methods are very accurate at the level of the protein backbone but fail to describe the position and orientation of side chains. We have already worked with Alphafold predicted structures in proteins from non-model organisms, such as hummingbirds[128], which allowed us to link structural data with metabolic functions.

In cryoEM there are some trends that include the use of time-resolved techniques, or the preparation of the sample directly from the natural source. In the latter, it has been shown that classification techniques can separate macromolecules present in the sample, allowing the structural characterization of several samples in one shot. One of these works describes ten cryoEM structures for liver enzymes obtained from a raw human liver microsome lysate[129]. This approach has the potential to develop a diagnostic tool at molecular level in human diseases.

Chemical Strategies to Boost the Development of Molecular Adjuvants and Vaccines

Alberto Fernández-Tejada, *Chemical Immunology Laboratory*

The clinical success of anticancer and antiviral vaccines often requires the use of an immunological adjuvant, a substance that helps stimulate and direct the body's immune response to the vaccine, making it work better. However, few adjuvants are sufficiently potent and non-toxic for clinical use; moreover, it is not really known how they work. Current vaccine approaches based on weak carbohydrate and glycopeptide antigens are not being particularly effective to induce the human immune system to mount an effective fight against cancer. Despite intensive research and several clinical trials, no such carbohydrate-based antitumor vaccine has yet been approved for public use. In this context, the research topic in the Chemical Immunology group has a double, far-reaching goal based on applying chemistry to address the above clear gaps in the adjuvant/vaccine field. Saponins are plant-based natural products (standing out those derived from the bark of the South American tree Quillaja Saponaria (QS)) that are able to increase the immunogenicity of the coadministered antigen, boosting the resulting immune response. QS-21 is one of the most potent adjuvants and is approved as a component of the AS01 adjuvant system (in combination with monophosphoryl lipid A (MPLA) in a liposomal formulation) in shingles (herpes zoster) and malaria vaccines. Moreover, QS-21 has also shown promise as part of the M72/AS01 tuberculosis vaccine Phase III clinical trials. Additional formulations incorporating saponin adjuvants have gained a great deal of interest in the context of HIV and SARS-CoV-2 viruses[130], including the saponin-based Matrix-M nanoparticulate adjuvant used in the approved Novavax COVID-19 vaccine[131,132].

Notably, this year has seen a key milestone in saponin research with the publication of a ground-breaking study in Science elucidating the biosynthetic pathway of saponin adjuvants from Quillaja Saponaria[133]. This work provided key insights into how these molecules are biosynthesized and diversified, and by reconstituting this pathway in a tobacco expression system enabled production of advanced QS saponin structures such as the vaccine adjuvant QS-7. In a related study, several genes and enzymes for saponin biosynthesis (e.g. triterpene oxidation and glycosylation) were identified in Saponaria vaccaria, an annual herb containing saponins

structurally similar to QS-21[134]. Overall, these fundamental studies open the door to access and engineer high-value saponin molecules (such as vaccine adjuvants) in heterologous expression systems through synthetic biology and metabolic engineering approaches. In 2023, an interesting review article has been published comparing the similarities and differences of chemical structures and physical characteristics of two liposomal vaccine adjuvants based on MPLA and QS-21 (AS01 and the Army Liposomal Formulation ALFQ), where the authors comment on some of the potential mechanisms of safety and adjuvant activity[135]. While most of the approved vaccines are simply co-formulated with adjuvants, state-of-the-art synthetic constructs are being developed in which the antigen and the adjuvant components are covalently linked in the same molecule. This concept enables simultaneous uptake by the same antigen presenting cell, ultimately leading to more targeted and enhanced immune responses. In 2023, important studies have been published on this front, namely using tumor-associated (TA) MUC1 glycopeptide antigens in anticancer vaccine designs. In an elegant example, Gabba et al. have developed a tricomponent candidate incorporating a high-affinity N-acetylgalactosamine (GalNAc) glycocluster targeting the macrophage galactose-type lectin (MGL) on antigen presenting cells[136]. More recently, Corzana and coworkers have reported the rational design of synthetic MUC1-glycopeptides modified with an unnatural hydroxynorvaline amino acid, which effectively mimicked the threonine-derived antigen both in terms of conformational dynamics in solution and enhanced antibody binding affinity[137]. From the Chemical Immunology Lab, we have also contributed to this area with a study that was published in Chemical Science early this year. We developed synthetic self-adjuvanting anticancer vaccine candidates based on our minimal saponin adjuvant and TA-MUC1 glycopeptide antigens, which were chemically conjugated together with a T helper peptide[138]. Unlike the dicomponent molecules, these tricomponent constructs self-assembled into stably organized aggregates not disrupted upon dilution (assessed by NMR experiments) and elicited significant levels of TA-MUC1 specific and functional IgG antibodies, correlating higher structural stability and increased immunogenicity. Finally, in 2023 we have also published a chapter on carbohydrate-based antiviral vaccines[139] as well as a mini-review article discussing chemical biology tools to investigate the effects and roles that glycosylation of proteins with α-O-N-acetylglucosamine (α-O-GlcNAc) has in immune cells and immunity[140].

In the foreseeable future, significant achievements can be anticipated for 2024 from our side, including the development of novel saponin-based vaccine constructs and chemical probes for molecular level mechanistic investigations. It is expected that critical new insights into the molecular mechanisms of QS-21 and saponin-derived adjuvants can be gained, although it is unlikely that a single, universal mechanism of action can be applicable to the range of natural and synthetic saponin adjuvants being investigated in the field.

Endosomal Trafficking. The Retromer Complex

Aitor Hierro, *Membrane Trafficking Laboratory*

Membrane trafficking is a fundamental cellular process essential for the proper functioning of cells. It involves the movement of proteins, lipids, and other molecules within and between different cellular compartments, such as the endoplasmic reticulum, Golgi apparatus, endosomes, and lysosomes. This dynamic process enables cells to regulate diverse functions including signal transduction, nutrient uptake, waste disposal, and organelle maintenance. The retromer complex plays a pivotal role in endosomal membrane trafficking. It ensures proper protein and lipid movement within the endosomal system, aiding in the recycling of molecules from endosomes to various cellular compartments, including the Golgi apparatus and the plasma membrane. Dysfunctional retromer activity has been linked to neurodegenerative diseases, bone disorders, and viral and bacterial infections, highlighting its broader impact on human health. The goal of the membrane trafficking lab lead by Aitor Hierro is to elucidate the molecular mechanisms for recognition, packaging and sorting of integral membrane proteins into distinct coated tubulo-vesicles. For this purpose, the lab integrates X-ray crystallographic studies of retromer and associated proteins with relevant cargos, together with cryo-electron microscopy analysis of reconstituted tubulo-vesicles. The number of scientific papers published in the field of retromer during 2023, according to PubMed, is 95. Among all the published papers and high-performing research in this field, two standout contributions are particularly noteworthy:

Cellular determinants of targeted membrane protein degradation using lysosome-targeting chimeras (LYTACs). These LYTACs utilize receptors, such as the cation-independent mannose 6-phosphate receptor (CI-M6PR), to direct extracellular proteins to lysosomes. The team employed a genome-wide CRISPR knockout approach and found that disrupting retromer genes improved target degradation by reducing LYTAC recycling to the plasma membrane. Additionally, they identified Neddylated cullin-3 as a facilitator of LYTAC-complex lysosomal maturation and a predictive marker for LYTAC efficacy. The study's findings provide insights into the design of next-generation LYTACs and shed light on aspects of cell surface receptor occupancy and trafficking[141]. The Commander complex, which is linked to Ritscher-Schinzel syndrome, is crucial for the endosomal recycling of various transmembrane cargos. Comprising the Retriever and CCC complexes, the Commander complex includes unique subunits such as COMMD proteins and coiled-coil domain-containing (CCDC) proteins. Through a combination of X-ray crystallography, electron cryomicroscopy, and in silico predictions, the researchers assembled a complete structural model of Commander. The study provides insights into disease-causing mutations and highlights the molecular features essential for the function of this evolutionarily conserved trafficking machinery[142].

Our lab fosters an integrative approach through international collaborations with experts in areas of cell biology, neurobiology and infectious diseases for the effective assessment of novel mechanisms and models. Our long-term goal is the identification of distinct recognition mechanisms to provide new tools for organelle-specific delivery of biologically active molecules.

TECHNOLOGIES

Unveiling Genomic Enigmas

Ana M Aransay, *Genome Analysis Platform*

In the year of the 70th anniversary of DNA double helix by Doctors Franklin, Crick, Watson and Wilkins[143,144,145] genomic advances through advanced High-Throughput Sequencing (HTS) methods have boosted the understanding of cellular biology. Single-cell RNA Sequencing (scRNAseq) dissects gene expression profiles at single-cell level, enabling the identification of cell types and unveiling intricate regulatory networks and, single-cell Assay for Transposase-Accessible Chromatin using Sequencing (scATACseq) maps open chromatin regions, unravelling the dynamic structure and regulatory elements of the genome. During 2023, the Genome Analysis Platform's projects have been dedicated to characterization of transcriptomes (polyA-RNAs as well as long-noncodingRNAs and smallRNAs), methylomes, ATACseq and RNA-protein interactions both, in bulk tissues and at single-cell level. In addition, some of those have yielded these publications thanks to their fruitful collaborations[146,147,148,149,150,151].

In the landscape of advanced genomics, we have started to work in the following disciplines:

- Single-cell Methylome Sequencing (scMethylseq): provides insights into DNA methylation patterns, offering a comprehensive view of epigenetic regulation and aiding in the study of diseases.

- Long-read sequencing technologies: produce an unprecedented view of genomic structures by enabling the sequencing of entire DNA molecules without fragmentation. This approach is pivotal for resolving complex genomic regions, detecting structural variations, and comprehensively characterizing alternative splicing events. We are adapting this strategy to actual scRNAseq as well as to metagenomics studies.

- Genomic classification of Extracellular Vesicles (EVs), including exosomes and microvesicles, that represent a burgeoning field. For this aim, adjusting protocols for EVs isolation and sequencing of nucleic acids (DNA, microRNAs, mRNAs, and other RNA species) encapsulated within them is

required. This cargo reflects the molecular status of their cellular origin and, since EVs serve as mediators of intercellular communication, it holds potential diagnostic and therapeutic implications. This work runs under the Twinning project EVCA[152], in collaboration with the metabolomics and proteomics platforms.

- Account of CRISPR off-target effects is an essential component, particularly when utilizing CRISPR-Cas9 for genome editing. CRISPR off-target characterization involves the identification and assessment of unintended genomic modifications resulting from the CRISPR-Cas9 system. One method commonly employed for CRISPR off-target description is HTS. These protocols include whole-genome sequencing, targeted deep sequencing, and amplicon sequencing. By comparing the genomic DNA of edited and unedited cells through accurate bioinformatics analyses, researchers can pinpoint potential off-target sites and assess the frequency and nature of unintended modifications.

Furthermore, CIC bioGUNE is generating an Integrative Biology group by joining together the staff of all the technological platforms to make possible the integration of the outputs by the mentioned techniques, involving robust bioinformatics tools and statistical methodologies and ensuring the holistic interpretation of multi-omics data. Considerations for sample quality, data integration challenges, and rigorous statistical analyses underpin the scientific soundness of these genomic tactics, fostering reliable and reproducible results that withstand scientific scrutiny.

Novel Technological Achievements in Mass Spectrometry-Based Proteomics

Felix Elortza, *Proteomics Platform*

Molecular basics of life are complex. Even the understanding of the molecular details of a human single cell is still a challenge. Governed by the expression of approximately 20.000 genes our proteome is dynamic and enormously complex. The temporal regulation of gene expression dictates that not all genes are concurrently active. It is hypothesized that a somatic human cell expresses between 10 to 12.000 gene products within a specific cellular context. The comprehensive cataloguing and quantification of these proteins have posed a formidable challenge in the field of proteomics.

The proteomics community is excitedly observing remarkable advancements made possible by novel mass spectrometers, allowing closer examination towards accomplishing comprehensive proteome assessments. This year it has been launched a mass spectrometer that combines a mass-resolving quadrupole, the Orbitrap, and the novel Asymmetric Track Lossless (Astral) analyser. This instrument enables very fast acquisition of high-resolution accurate mass (HRAM) MS/MS[153]. Although the instrument is yet in hands of very few laboratories, the combination of narrow-window data independent acquisition and >200 Hz MS/MS scanning speed, allowed to Olsen´s group to report up to 10.000 proteins identified in just in half hour acquisition time[154]. Besides, the latest version of timsTOF instruments called timsTOF Ultra, which combines trapped ion mobility spectrometry (TIMS) with quadrupole time-of-flight (QTOF), allows to acquire at 300 Hz speed and shows impressive sensitivity enabling single cell proteomics studies with unprecedented depth. Metchler´s group reported in last American Society for Mass Spectrometry Conference (ASMS, Houston, TX) that by using the timsTOF Ultra are able to measure roughly 6,000 protein groups with a median coefficient of variation of 10 percent in a 250-picogram standard (approximately equivalent to the amount of protein in a single cell). Slavov´s[155] and Olsen´s[156] groups have reported 3.700 and 5.000 proteins identified respectively starting from somatic single human cells.

The depth of analysis achieved for regular samples such as in-vitro cell lines and/or tissue samples is impressive and will for sure help getting deeper knowledge about many still obscure cellular mechanisms. Besides, among research and clinical applications of single-cell proteomics, we can mention that this type of analyses will contribute to our understanding of how protein levels change as cells differentiate by analysing lineage-specific transcription factors and their abundance over time, ultimately leading to the maintenance or emergence of a lineage trajectory[157]. Moreover, the single cell proteomics will be framed within single cell-multiomics studies. Characterizing cell states and activities by simultaneously integrating various single-modality omics methods that profile the transcriptome, genome, epigenome, epitranscriptome, proteome, metabolome and other(emerging) omics will revolutionize molecular cell biology research[158]. Focusing on spatial proteomics, the study presented by Mann´s group is an example of how spatial single-cell mass spectrometry can define zonation of the hepatocyte proteome[159]. Although preliminary, it clearly points that soon will be possible to face 3D single cell proteomic studies that shall help shedding light on, for instance, relevant tumour-tissue microheterogeneity aspects.

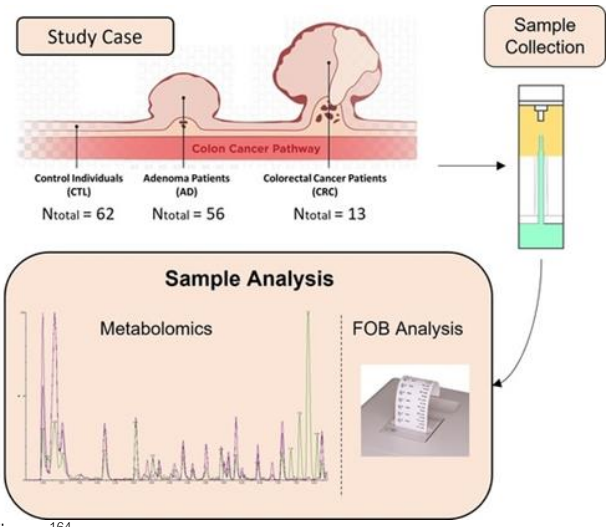
All these new technological achievements in mass spectrometry-based proteomics lead us to an incredibly exciting future, where we anticipate a positive impact on the production of cell-specific atlases, tumor immunology, and translational research.

Cell and Plant Metabolomics

Oihane Alboniga, Diana Cabrera, Sebastiaan van Liempd and Juan M. Falcon-Perez, *Metabolomic Platform*

We supported numerous research projects in their metabolite analysis by high resolution liquid chromatography coupled with high resolution mass spectrometry (hrLCMS) to target the metabolites of interest. During this year we have demonstrated for the first time the use of metabolomics as a complementary high-content technology to analyze the Faecal Occult Blood test remanent of screenings population[160], what will allow improving the detection of novel early candidate biomarkers for colorectal cancer and making more efficient the already established population screenings. Remarkably, we have performed a pre-clinical study showing the interplay between the microbiota and the host metabolism, specifically a specific probiotic was able to reduce the blood glutamate levels in mouse[161]. In addition, the implementation of untargeted metabolomics and lipidomics protocols for polar and apolar metabolites in positive and negative mode has provided the platform with a powerful tool to cover a broad range of small molecules in different biological systems to unravel biomarkers and mechanisms of action of physiological and pathological processes. Our core facility is also focused on analysis of metabolic members of the methionine cycle, polyamide synthesis, transsulfuration pathway and the tricarboxylic acid (TCA, Krebs) cycle. We have further implemented the steroid hormones into the possibilities of the platform[162]. We have expertise to approach Plant metabolomics by analyzing how ammonium nutrition interacts with iron homeostasis in Brachypodium distachyon as experimental model for improving resources sustainability of the society[163] in the framework of a project funded Spanish Ministry. We have also further implemented stable-labeled flux analysis methods to our portfolio. The essence of these methods is the tracking of stable labelled atoms from a precursor metabolite throughout a metabolic pathway. In our platform we use 13C5-methionine and 13CD3-methionine to probe the methionine cycle while we determine fluxes trough the TCA cycle with 13C6-glucose and 13C5-glutamine. However, if a labeled precursor is available, we can track most pathways with our hrLCMS setup. The great

advantage of these stable labelled experiments is the absence of background signals of endogenous metabolites. We are currently setting-up assays focused on pentose phosphate pathway and also on drug metabolism. Especially cytochrome P450 inhibition, reactive intermediate trapping and liver toxicity have our interest. These assays are part of the pre-clinical drug discovery pipeline and thus of great importance to the pharmaceutical industry.



Figure[164]

Nuclear Magnetic Resonance Methodological Advances

Tammo Diercks, *NMR Platform*

Over the past years, the CiC bioGUNE NMR facility has seen a continuous increase in usage and applications, paralleled by a massive investment in new hardware which, in 2023, has culminated in the installation of a 80 MHz benchtop spectrometer (to translate NMR metabolomics to clinical applica¬tion), modernisation of our most versatile 600 MHz research spectrometer (allowing to implement newest NMR methodology), and arrival of an ultra-high field 1 GHz spectrometer (with extreme resolution and sensitivity to open the doors for novel cutting-edge studies). Thus, the NMR facility now houses seven spectrometers (80, 400, 600 (3x), 800, 1000 MHz) to support our main research lines (molecular recognition, dynamics, and structure; metabolomics; compound analysis) and enable the widest range of further upcoming applications (e.g., in-cell NMR studies).

The spread of ultra-high field magnets (800 – 1200 MHz) and machine learning algorithms (artificial intelligence, AI) with their enormous potential to improve data acquisition and analysis, respectively, will now drive the further development of NMR. Yet, such large magnetic fields require novel power optimised schemes for broadband excitation, decoupling, spin locking etc. Thus, new broad-band-selective 90º and 180º pulses[165], composite CHIRP pulses[166], and pulse optimisation algorithms[167] have emerged. Other pulses were developed to suppress artefacts like coil ringing[168] and coil background[169]. By now, AI has also been used to design novel pulse shapes, e.g., for broadband excitation[170] or 1H excitation without H2O saturation[171]. The broad, upcoming contributions of AI in NMR have been reviewed recently[172] and include the deconvolution of spectra[173] or exponential decay curves[174], structure based spectra prediction[175], identification of metabolites[176] and molecular substructures[177], prediction of chemical shifts for 15N[178], protein signal assignment by ARTINA[179] or AssignSLP for sparse labelling[180], virtual homodecoupling during NUS spectra reconstruction[181], and optimised NMR shimming[182]. Most likely, AI will have the strongest impact in metabolomics, as presented in a recent review[183] and study on age prediction[184].

Isotopic labelling is fundamental for enabling advanced NMR studies, where recent progress includes novel routes for the selective insertion of 2H[185] or 13C[186] into aromatic amino acids, enantioselective 2H and 15N introduction[187], late-stage 14/15N isotopic exchange in primary amines[188], multi-site specific glutamine labelling[189], and segmental protein labelling and multiple ligation[190]. Specifically for cell-free protein expression, site-specific alanine introduction[191] and a novel protocol to suppress isotopic scrambling[192] were presented. Such techniques are also critical for NMR in live cells, where new protein delivery systems[193] and NMR studies selecting imino 1H[194], 15N[195],13C[196], or 19F[197] were published. The superb NMR characteristics of 19F continue to inspire its use as reporter spin in (intrinsically disordered) proteins to study their liquid-liquid phase separation[198], conformation[199], and dynamics[200], while applications to RNA were facilitated by recent synthetic advances[201]. Similarly, 77Se was introduced as sulphur analogue (in seleno-methionine) to enable 1H,13C,77Se triple resonance NMR experiments on proteins[202].

NMR methodology continues to advance incrementally and has recently yielded an optimised methyl TROSY experiment with delayed 13C decoupling[203], improved 1H,15N heteronuclear cross-polarization[204], enhanced ADEQUATE type 13C-13C correlation[205], NOAH type concatenation of several heteronuclear experiments[206], and a new J-H,H-filter for spectral simplification[207]. The concept of Pure Shift NMR (i.e., 1H detection with homonuclear nJH,H' decoupling) was applied to TROSY type protein triple resonance experiments[208] and 19F edited FESTA experiments[209], and amended by convection compensation[210]. Finally, NMR spectra processing has seen advances in noise suppression[211] and phasing[212] while non-uniform data sampling (NUS) and processing benefit from recently proposed optimised NUS schedules to reduce aliasing noise[213] and co-processing of similar (serial) spectra[214].

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