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PhD. Thesis

***Proteomics strategies to unveil the biological role of BAG3
through tissue-specific knockout mouse models***

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CHAPTER 1

Background, aim of the research project and thesis outline

Background of the research project

BAG family molecular chaperone regulator 3 (BAG3)

BAG3 was identified as a co-chaperone of the heat shock protein (Hsp) 70, which helps, through the binding to the ATPase domain, the ADP release from the chaperone and the nucleotide cycling. By interacting with Hsp70, BAG3 modulates the activities of this chaperone, including the delivery of client proteins to proteasome. BAG3 can also carry out Hsp70- independent functions, through its interactions with other proteins involved in apoptosis, cytoskeleton dynamics and other pathways (1). BAG3 is a multifunctional protein that plays a crucial role in the cellular stress response through its involvement in various regulatory pathways that control cellular homeostasis under both physiological and pathological conditions. It also participates in numerous intracellular pathways, thereby facilitating their crosstalk and modulating the coordinated response of essential cellular functions, including apoptosis, autophagy, and cell proliferation. It can be secreted by certain cell types, such as cardiomyocytes, pancreatic tumor cells, and hepatocytes (2). Whereas intracellularly BAG3 sustains the levels of anti-apoptotic factors and other molecules, participates in protein quality control, drives the cytoskeleton dynamics, exerts structural and functional roles in myocytes, the discovery of a secreted BAG3 opened a new field of investigation in tumor development and progression, revealing its role in a new signaling pathway, mediated by the BAG3/BAG3R axis, which also includes monocytes and other stromal cells (3).

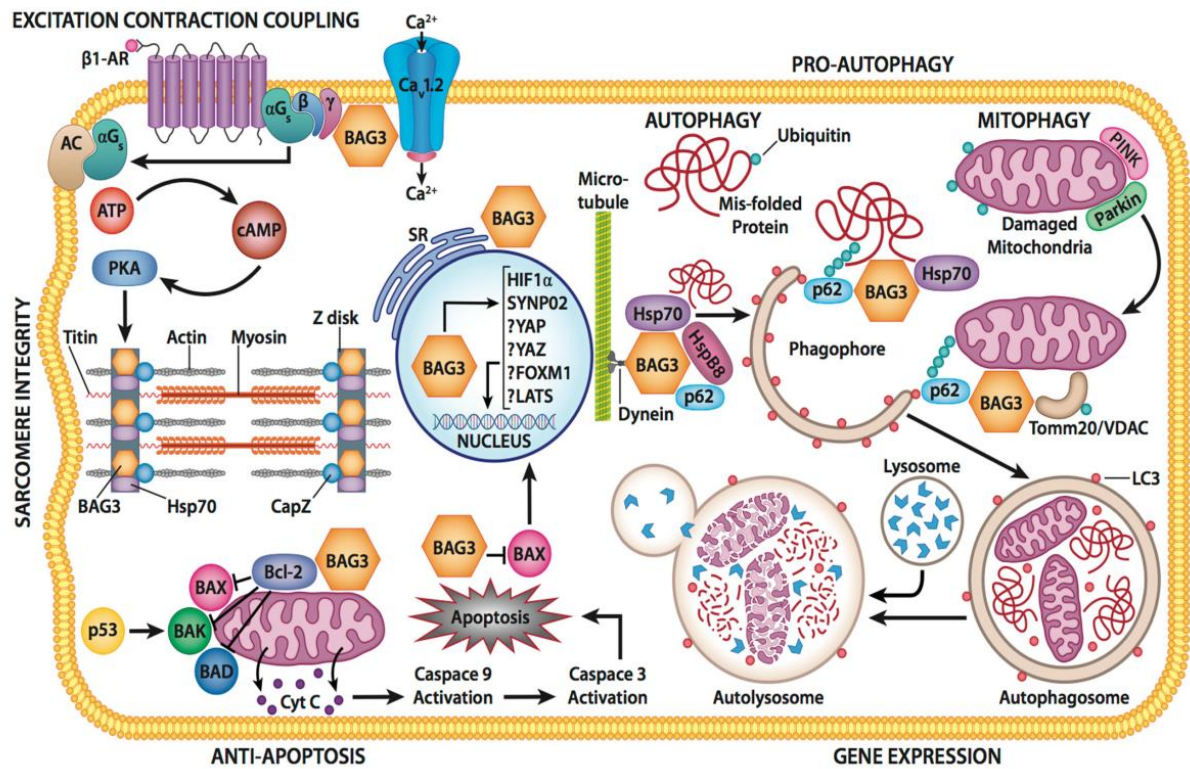


Figure 1: Multiple cellular roles of BAG3 (3).

Investigating the role of BAG3 in Pancreatic carcinoma

Pancreatic carcinoma (PC) is the sixth most common cause of cancer-related deaths in 2022, accounting for nearly 5% of all cancer deaths worldwide (4). Currently, the causes of PC are not yet fully identified, but certain risk factors have been identified and are classified as non-modifiable (age, sex, blood group, family history and genetic susceptibility, diabetes) and modifiable (intestinal microflora, smoking, alcohol, chronic pancreatitis, obesity, dietary factors, infection) (5). At clinical presentations, patients typically present with jaundice due to invasion and obstruction of the common bile duct when the PC is located in the head of the pancreas (6). Approximately 80-85% of patients are diagnosed with unresectable disease. This is largely due to the encasement of major mesenteric vessels or metastatic disease to the liver, peritoneum, or other distant sites. As a result, the 5-year survival rate is less than 5%. Despite advances in diagnostic approaches, perioperative management, radiotherapy techniques, and systemic therapies for advanced disease, there is only modest incremental progress in patient outcomes (7). Only 20% of patients with resectable diseases

survive for 5 years after surgery, and some may still die from the disease afterward (8). The high resistance of pancreatic cancer (PC) to conventional chemotherapy, such as 5-fluorouracil, represents one of the key factors contributing to its poor prognosis, which is further exacerbated by the tumor microenvironment (TME) and alterations in cytokine signaling and metabolic pathways (9). Pancreatic ductal adenocarcinoma (PDAC) accounts for over 90% of all pancreatic tumors and is regarded as a leading cause of cancer-related mortality worldwide, primarily due to late-stage diagnosis. The significant resistance of PDAC to conventional chemotherapy is attributed to its dense functional stroma, composed of extracellular matrix and extensive fibrosis (desmoplasia), which contributes to treatment resistance (10). This stroma exhibits prominent hyperplasia surrounding the tumor tissues, leading to alterations in the normal architecture of pancreatic tissue. It is characterized by the presence of cellular components such as cancer-associated fibroblasts (CAFs), immune cells, and endothelial cells, as well as acellular components like collagen, laminin, and cytokines within the extracellular matrix, creating a physical barrier to drug penetration (8).

The BAG3 protein is secreted by neoplastic cells, and it has been demonstrated that a BAG3-neutralizing monoclonal antibody can slow tumor growth and limit metastatic spread by reducing infiltrating macrophages and survival cytokines. Additionally, BAG3 is found to be overexpressed in various tumor types and plays a crucial role in therapeutic resistance and the interaction between PDAC cells and the stroma (11). BAG3 causes an accumulation of IL-6 in the growth medium of treated HF cells, thereby modulating the TME and acting as a survival factor; pathways involved in proliferation, migration, and enhanced adhesion of cancer cells have also been identified. Therefore, BAG3 represents a potential target for combination therapy as it induces the release of IL-6, CCL2, and HDF from fibroblasts, a mechanism that contributes to tumor progression (12).

Investigating the role of BAG3 in glucose metabolism regulation

Adequate glucose homeostasis is essential for long-term health, and impaired homeostatic regulation can lead to metabolic disorders and an increased risk of cardiovascular diseases, stroke, and type II diabetes. BAG3

downregulation results in increased insulin secretion in response to glucose stimulation and in disruption of the F-actin network. It also binds to SNAP-25 and syntaxin-1, two components of the t-SNARE complex preventing the interaction between SNAP-25 and syntaxin-1. Upon glucose stimulation BAG3 is phosphorylated by FAK and dissociates from SNAP-25 allowing the formation of the SNARE complex, destabilization of the F-actin network and insulin release (3).

Mass spectrometry and proteomic analysis

Mass spectrometry is an analytical technique involving the ionization of molecules followed by fragmentation into ions with different mass-to-charge (m/z) ratios. It is frequently employed for the identification of unknown substances, the quantitative assessment of known compounds, and the elucidation of structural and chemical properties of molecules (13). The first instrument designed to measure the m/z ratio of ionized gaseous atoms was developed by Thompson in 1910. In subsequent years, mass spectrometry proved valuable for the study of various molecules, and between 1913 and 1945, it was primarily utilized for isotopic analysis and atomic weight determination. Initially, MS use was confined to highly specialized laboratories staffed by technically advanced personnel; today, however, mass spectrometry is commonly employed in most research and clinical laboratories (14). A mass spectrometer is thus an instrument capable of ionizing molecules, separating them, and determining their molecular mass by measuring their m/z ratio. Each spectrometer consists of three main components: (a) the ion source, a chamber where ions are generated; (b) an analyzer that separates ions based on their m/z ratio; and (c) a detector that produces an electrical signal from an ion beam (14).

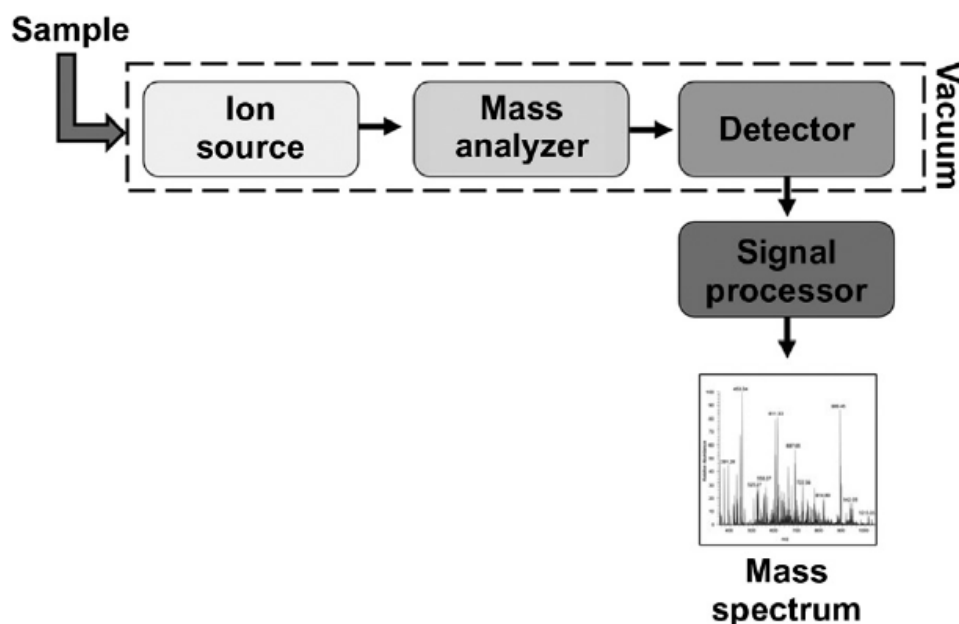


Figure 2: Overview of the Components of a Mass Spectrometer (14).

The introduction of the sample to be analyzed into the mass spectrometer can occur either "on-line" or "off-line." In the former case, it interacts directly with a separation system, such as liquid chromatography or gas chromatography, and everything eluting the chromatographic column enters the mass spectrometer. In the latter case, no separation system is used, and the sample can be introduced directly into the ion source as a gas or vaporized if it is in liquid or solid form (14).

A) Ion Source

The first component of the mass spectrometer is the ion source, where the ionization process takes place, serving as an indispensable preliminary phase. Different ionization techniques exist, and the chosen method determines the type of information obtained from the mass spectrum. Common ionization methods include: (i) Electron Ionization (EI); (ii) Chemical Ionization (CI); (iii) Thermospray and Electrospray Ionization (ESI); and (iv) Matrix-Assisted Laser Desorption/Ionization (MALDI).

- Electron Ionization (EI)

EI is a "hard" ionization technique where the introduced compound is converted into a gaseous form within the ionization chamber and then exposed to an electron beam, resulting in ionization. Ionization occurs via the removal of an electron from the molecule by high-energy electrons, producing a radical cation termed a molecular ion. These ions are then directed to the analyzer.

- Chemical Ionization (CI)

CI is a "soft" ionization technique where the vaporized sample interacts with an ionized reagent gas. The ions produced, termed quasi-molecular ions, have lower energy, resulting in less fragmentation compared to EI. Thus, CI ions are more easily identifiable in the spectrum.

- Thermospray and Electrospray Ionization (ESI)

Both thermospray and ESI are "soft" ionization techniques that generate quasi-molecular ions. In thermospray ionization, the solvent is removed by heating. In ESI, two processes occur: liquid droplets form from the analyte solution, followed by solvent evaporation and ion formation. The analyte molecules are ionized within the charged droplets, which shrink as the solvent evaporates, increasing charge density. Ions are expelled from the droplets by electrostatic repulsion and enter the spectrometer through apertures, where they are accelerated and sent to the analyzer.

- Matrix-Assisted Laser Desorption/Ionization (MALDI)

MALDI is a "soft" ionization method where ions are desorbed and ionized through the absorption of an ultraviolet laser pulse. It effectively ionizes small and large molecules with minimal fragmentation. MALDI-MS is crucial in proteomics for its high sensitivity, rapid analysis, and tolerance to non-volatile salts and buffers commonly used in biological samples (14).

B) Mass Analyzer

The second component of a mass spectrometer is the analyzer, which separates generated ions based on their mass-to-charge (m/z) ratio using electric and/or magnetic fields. Various analyzers exist, each with different resolution capabilities. The most commonly used are: (i) Quadrupole analyzers; (ii) Ion trap analyzers (linear or three-dimensional); (iii) Time-of-Flight (TOF) analyzers; and (iv) Orbitrap analyzers.

- Quadrupole analyzers

Quadrupole analyzers consist of four parallel metal rods. Opposite pairs of rods are subjected to a potential difference combining direct current (DC) and alternating current (AC). Only ions with stable trajectories pass through the rods to reach the detector. By adjusting the applied voltage, ions with specific m/z values exit the quadrupole at different times.

- Ion trap analyzers

Ion traps differ from quadrupoles by using a spherical structure to retain ions within the trap before directing them to the detector. They enable tandem mass spectrometry (MS/MS), where processes occur within the same space at different times. In MS/MS, a precursor ion selected by the first stage (MS1) collides with a gas to generate fragments, which are then analyzed in the second stage (MS2) based on m/z , producing an MS/MS spectrum.

- Time-of-Flight (TOF) analyzers

TOF analyzers rely on the principle that ions with different m/z values accelerated to equal kinetic energy, travel at varying speeds. The analyzer includes a drift tube through which ions travel at different times ($t = a(m/z)^{1/2} + b$). Ions with smaller m/z ratios reach the detector first. TOF analyzers operate in two modes: linear, where the detector is at the end of the tube, or reflectron, where an electrostatic mirror improves ion focus and resolution.

- Orbitrap analyzers

The Orbitrap is an axially symmetric mass analyzer with a central electrode surrounded by outer electrodes. Stable ions rotate around the central electrode with oscillation frequencies dependent on their m/z ratios. Orbitrap uses electric fields to capture and confine ions, offering high-resolution analysis (14).

C) Detector

The detector in a mass spectrometer can vary, with common types being the channeltron and electron multiplier tube (EMT).

- Channeltron

This diode, shaped like a horn, is coated internally with a semiconductor material. Ions striking the surface generate electrons, which undergo significant amplification at the horn's end, creating a signal recorded by the counting system.

- Electron Multiplier Tube (EMT)

Comprising a series of diodes, EMTs multiply the ionic current into a detectable signal. The signal is processed by a computer, supported by software, determining ion abundance based on mass.

The mass spectrum output is a plot of the m/z ratio (x-axis) versus ion abundance or signal intensity (y-axis). In routine applications, spectra are represented as histograms, with abundances normalized to the base peak, the most intense peak. These spectra can be correlated using protein sequence databases for further analysis (14).

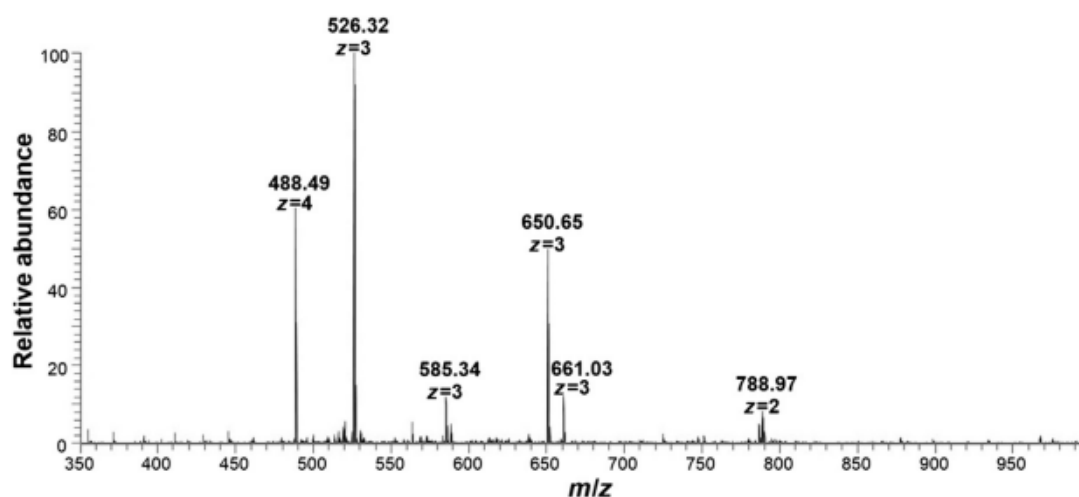


Figure 3: Representative Mass Spectrum of a Complex Peptide Mixture (14).

The term “proteome,” coined by Mark Wilkins in 1994, refers to the entire set of proteins expressed by an organism or biological system—specifically, the proteins synthesized from the genome. The study of the proteome, known as “proteomics,” involves the systematic identification of proteins in relation to their structure, function, activity, abundance, and molecular interactions (15). Proteomics provides essential insights into complex biochemical processes at the molecular level, complementing information obtained from genomics and transcriptomics (16). Through proteome analysis, specific biomarkers can be identified, yielding valuable information on diseases, treatment responses, and other clinical conditions that cannot be discerned through genome analysis alone (17). Various analytical methods are employed in proteomics, with mass spectrometry regarded as a preferred one due to its precision, speed, and sensitivity in identifying and quantifying a large number of proteins. However, given the high complexity of biological matrices and the wide variability in molecular weight and protein content, mass spectrometry often requires combination with separative analytical methods such as liquid chromatography (LC) —particularly high-performance liquid chromatography (HPLC)— capillary electrophoresis (CE), and two-dimensional electrophoresis (2-DE). Chromatography is a common separative technique in “gel-free” proteomic methods, and mass spectrometry combined with “gel-free” techniques can be applied using bottom-up, top-down, or middle-down approaches (14). Given the complexity associated with the acquisition of samples using a proteomics approach, it is

essential to employ dedicated bioinformatics tools for analyzing the raw acquisition data. This step, part of the data processing phase, is equally critical, if not more delicate, than the acquisition itself.

In this PhD thesis project, the following instrumental setup was employed for protein shotgun label-free identification and quantification: LC-MS/MS analysis using the UltiMate™ 3000 UPLC (Thermo Fisher Scientific) chromatographic system with EASY-spray Acclaim™ PepMap™ C18 nanoscale chromatographic column, coupled to the Orbitrap Fusion™ Tribrid™ (Thermo Fisher Scientific) mass spectrometer. Proteomics raw data were processed using Thermo Proteome Discoverer (Thermo Fischer Scientific) to identify differentially expressed proteins (DEPs) in the different experimental conditions, subsequently uploaded into the Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany) tool for Gene Ontology and functional enrichment analysis. The unique feature of IPA is that, in addition to identifying potential biological functions and pathways in which our proteins are involved, it provides information on the modulation of these functions based on all published literature.



Figure 4: Instrumental setup of shotgun label-free proteomic analysis performed in this PhD thesis work.

References

1. Marzullo L, Turco MC, De Marco M. The multiple activities of BAG3 protein: Mechanisms. *Biochimica et Biophysica Acta (BBA) - General Subjects*. agosto 2020;1864(8):129628.
2. Rosati A, Graziano V, De Laurenzi V, Pascale M, Turco MC. BAG3: a multifaceted protein that regulates major cell pathways. *Cell Death Dis*. 7 aprile 2011;2(4):e141–e141.
3. Brenner CM, Choudhary M, McCormick MG, Cheung D, Landesberg GP, Wang JF, et al. BAG3: Nature's Quintessential Multi-Functional Protein Functions as a Ubiquitous Intra-Cellular Glue. *Cells*. 19 marzo 2023;12(6):937.
4. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA A Cancer J Clinicians*. maggio 2024;74(3):229–63.
5. Ilic M, Ilic I. Epidemiology of pancreatic cancer. *WJG*. 2016;22(44):9694.
6. Zhao Z, Liu W. Pancreatic Cancer: A Review of Risk Factors, Diagnosis, and Treatment. *Technol Cancer Res Treat*. 1 gennaio 2020;19:1533033820962117.
7. Mizrahi JD, Surana R, Valle JW, Shroff RT. Pancreatic cancer. *The Lancet*. giugno 2020;395(10242):2008–20.
8. Liang C, Shi S, Meng Q, Liang D, Ji S, Zhang B, et al. Complex roles of the stroma in the intrinsic resistance to gemcitabine in pancreatic cancer: where we are and where we are going. *Exp Mol Med*. dicembre 2017;49(12):e406–e406.
9. Gautam SK, Basu S, Aithal A, Dwivedi NV, Gulati M, Jain M. Regulation of pancreatic cancer therapy resistance by chemokines. *Seminars in Cancer Biology*. novembre 2022;86:69–80.

10. Van Duijneveldt G, Griffin MDW, Putoczki TL. Emerging roles for the IL-6 family of cytokines in pancreatic cancer. *Clinical Science*. 28 agosto 2020;134(16):2091–115.
11. Rosati A, Basile A, D'Auria R, d'Avenia M, De Marco M, Falco A, et al. BAG3 promotes pancreatic ductal adenocarcinoma growth by activating stromal macrophages. *Nat Commun*. 2 novembre 2015;6(1):8695.
12. Dufrusine B, Damiani V, Capone E, Pieragostino D, Dainese E, De Marco M, et al. BAG3 induces fibroblasts to release key cytokines involved in pancreatic cell migration. *J of Cellular Biochemistry*. gennaio 2022;123(1):65–76.
13. T. Alberio, M. Fasano, e P. Roncada, *Proteomica*, 1a ed. Edises, 2021.
14. Thomas SN. Mass spectrometry. In: *Contemporary Practice in Clinical Chemistry* [Internet]. Elsevier; 2019 [citato 10 novembre 2024]. p. 171–85. Disponibile su: <https://linkinghub.elsevier.com/retrieve/pii/B9780128154991000107>
15. M. Ciaccio e G. Lippi, *Biochimica clinica e medicina di laboratorio*, 1a ed. Edises, 2017.
16. Zhang Z, Wu S, Stenoien DL, Paša-Tolić L. High-Throughput Proteomics. *Annual Rev Anal Chem*. 12 giugno 2014;7(1):427–54.
17. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. marzo 2003;422(6928):198–207.

Aims and thesis outline

In the context of exploring the multiple cellular functions of BAG3, we have chosen to focus on its potential as a therapeutic target in pancreatic and hepatocellular carcinoma, as well as its involvement in glucose metabolism, with the aim of identifying relevant signaling pathways. The primary objective of this thesis is to investigate and characterize the physiopathological role of BAG3 in pancreatic cancer, glucose metabolism, and hepatocellular carcinoma, utilizing a proteomic approach. A more detailed workflow is outlined below in the summary of three different studies I contributed to, all of which were part of this PhD thesis project. Finally, during the third year of PhD course, I spent three months in Belgium at the *University of Antwerp*, detailed in the last chapter of this dissertation.

STUDY 1: Proteomic meta-analysis unveils new frontiers for biomarkers research in Pancreatic Carcinoma

This review manuscript has been submitted as:

Federica Di Marco, Maria Concetta Cufaro, Verena Damiani, Beatrice Dufrusine, Erika Pizzinato, Fabio Di Ferdinando, Gianluca Sala, Rossano Lattanzio, Enrico Dainese, Luca Federici, Peter Ponsaerts, Vincenzo De Laurenzi, Ilaria Cicalini and Damiana Pieragostino – *Oncogenesis* 2024

Aim and study design

Study 1 describes the literature review on pancreatic cancer (PC) and the meta-analysis work in which we gathered proteomic research from the past decade. We analyzed PC protein biomarkers, categorizing them as either tissue/cellular markers or circulating markers. We performed the proteomic meta-analysis using the *Ingenuity Pathway Analysis* (IPA) bioinformatic tool to extract new and detailed information from the collected data set. In this work, we present and discuss the integration of results from different published studies on protein biomarkers in a global proteomic meta-analysis to understand which path to pursue scientific research. In particular, cancer signaling, inflammatory response, and cell migration and signaling have emerged as the main pathways described in PC, as well as scavenging of free radicals and metabolic alteration concurrently highlighted new research insights on this disease. Interestingly, from the study of upstream regulators, some were found to be shared by collecting data relating to both biological fluid and tissue biomarkers, side by side: specifically, TNF, LPS, p38 MAPK, AGT, miR-323-5p, and miR-34a-5p. By integrating many biological components with their interactions and environmental relationships, it's possible to achieve an in-depth description of the pathological condition in PC and define correlations between concomitant symptoms and tumor genesis and progression. In conclusion, our work may represent a strategy to combine the results from different studies on various biological samples in a more comprehensive way to understand which path to pursue scientific research.

STUDY 2: From BAG3insKO mouse model generation to proteomic analyses

a. Pancreatic beta-cell specific BAG3 knockout results in chronic hyperinsulinemia inducing insulin resistance

This research manuscript has been published as:

Verena Damiani, Alessia Lamolinara, Ilaria Cicalini, Maria Concetta Cufaro, Francesco Del Pizzo, **Federica Di Marco**, Piero Del Boccio, Beatrice Dufrusine, Michael Hahne, Rossano Lattanzio, Damiana Pieragostino, Manuela Iezzi, Massimo Federici, Maria Caterina Turco, Arianna Maiorana, Carlo Dionisi-Vici and Vincenzo De Laurenzi – *Molecular Metabolism*, 2023 [doi: 10.1016/j.molmet.2023.101752]

b. Pancreatic beta-cell specific BAG3 knockout results in insulin resistance and amyloidosis in mice spleen

This research manuscript is in preparation as:

Federica Di Marco, Verena Damiani, Erika Pizzinato, Rossano Lattanzio, Alessia Lamolinara, Francesco Del Pizzo, Liam McDonnell, Francesco Greco, Peter Ponsaerts, Piero Del Boccio, Vincenzo De Laurenzi and Damiana Pieragostino – 2024

Aim and study design

Study 2 describes the generation of BAG3-specific knockout mouse model through Cre-mediated recombination: BAG3 was silenced in pancreatic β -cells (BAG3insKO) to investigate the physiopathological function of BAG3 in glucose homeostasis. We first performed proteomic analyses on collected organs (liver, muscle and spleen) from the BAG3insKO model at sixty-four weeks of age, comparing the knockout model with the wild-type (WT), and we further performed micro-proteomics to analyze specific regions in the spleens of BAG3insKO and WT mice after Congo red staining. The proteomics results obtained from the liver revealed

the upregulation of cytosolic hydroxymethylglutaryl-CoA synthase (HMGCS1) coupled with the downregulation of the mitochondrial isoform HMGCS2 and acetyl-CoA acetyltransferase 1 (ACAT1), suggesting an increase in cholesterol biosynthesis and a decrease in ketone body formation. These findings are supported by the identification of the following proteins, exclusively found in the livers of BAG3insKO mice, that are involved in cholesterol synthesis: Dhcr24, NSDHL, and Idi-1. The functional proteomic analysis also indicated the downregulation of fatty acid β -oxidation, ethanol degradation, gluconeogenesis, and oxidative phosphorylation in the livers of BAG3insKO mice. Furthermore, the analysis of muscle tissue was also conducted: interestingly, IPA predicted the inhibition of the insulin receptor (INSR), data validated by western blot analysis that showed reduced expression of INSR and of one of its direct targets, the glucose transporter type 4 (GLUT4), in the muscles of BAG3insKO mice compared to WT. These results suggest a condition of muscle insulin resistance. Notably, proteomic profiling of the liver did not show inhibition of the insulin receptor pathway, and no significant reduction of INSR expression is observed in the livers of BAG3insKO mice, suggesting that the liver is still insulin responsive at this stage. Amyloid deposits were observed in the spleens, which we decided to characterize through proteomics and micro-proteomics. BAG3insKO mice exhibit compromised insulin sensitivity, secrete more insulin after glucose stimulation, and become insulin-resistant by 64 weeks of age; these mice also develop poorly defined amyloidosis in the spleen. Due to the challenges in identifying and characterizing protein markers in amyloid plaques, we conducted an initial proteomic analysis to study and describe the phenotype expressed in BAG3insKO mice to confirm and describe the molecular composition of amyloid deposits. A total of 49 proteins were identified and quantified, which are associated with the *amyloid database*. In particular, apolipoprotein A-II (Apo A-II), IgG (H+L) and Serum Amyloid P Component (SAP) are identified as up-regulated proteins among all DEPs. To finally validate proteomics results, we performed an immunohistochemical staining of Apo A-II and IgG (H+L) in our samples, and we found a positive presence in BAG3insKO mice versus WT for both Apo A-II and IgG (H+L). Finally, we carried out a western blot analysis of SAP, which results significantly increased in BAG3insKO mice spleen versus WT; moreover, evaluation of circulating SAP level shows how that protein seems to be deposited in amyloid deposits since it's decreased in BAG3insKO plasma compared to the WT. As regarding

metabolism, main pathways are related to insulin receptor signaling, insulin secretion signaling and signaling by insulin receptor pathways, which globally exhibit a predicted downregulation in BAG3insKO mice spleen. IPA also predicts the inhibition of the insulin receptor (INSR) in all BAG3insKO spleen, presenting the same trend evaluated in our previous work on BAG3insKO muscle. Moreover the “signaling by INSR” canonical pathway is also predicted to be downregulated in all performed proteomic analyses. We further validate this proteomics result by western blot and relative quantification, which confirm a reduced expression of insulin receptor (INSR) in BAG3insKO spleen compared to WT. In conclusion, in the BAG3insKO model, we observed a metabolic shift towards lipogenesis in the liver and a state of hyperinsulinemia in the muscle. The silencing of BAG3 may therefore increase the propensity to develop insulin resistance in the muscle and spleens, where we also identified the onset of amyloidosis with subsequent characterization of the deposits.

STUDY 3: From BAG3albKO mouse model generation to proteomic analysis

Liver-specific BAG3 knockout delays chemically induced hepatocellular carcinoma development

This research manuscript has been submitted as:

Verena Damiani, Erika Pizzinato, Federica Di Marco, Alessia Lamolinara, Francesco Del Pizzo, Aurora Navicella, Paola Lanuti, Rossano Lattanzio, Manuela Iezzi, Michael Hanhe, Damiana Pieragostino and Vincenzo De Laurenzi - *Oncogenesis*, 2024

Aim and study design

Study 3 focuses on the generation of BAG3-specific knockout mouse model through Cre-mediated recombination: BAG3 was silenced in hepatocytes (BAG3albKO) to study its physiopathological role in regulating liver function and hepatocarcinogenesis. In this experiment, mice were treated at eight weeks of age with 100 µg/kg of diethylnitrosamine to induce acute liver injury and subsequently monitor the response in the BAG3albKO model compared to wild-type (WT) mice. The functional analysis comparing the BAG3albKO model to WT indicated a predicted upregulation of fatty acid oxidation and ethanol degradation, leading to an activation of energy metabolism. Additionally, there was a predicted inhibition of necrosis and hepatic steatosis. The functional analysis comparing the BAG3albKO model treated after 8 weeks with 100 µg/kg of diethylnitrosamine to treated WT shows that this condition appears to preserve the organ from treatment-induced hepatocarcinogenesis: the BAG3albKO model reacts to damage with hepatic necrosis while avoiding hepatocarcinogenesis. Together, these findings underscore the multifaceted role of BAG3 in hepatocarcinogenesis pathogenesis and highlight its involvement in tumor initiation and progression.

CHAPTER 2

STUDY 1

Proteomic meta-analysis unveils new frontiers for biomarkers research in Pancreatic Carcinoma

This review manuscript has been submitted as:

Federica Di Marco, Maria Concetta Cufaro, Verena Damiani, Beatrice Dufrusine, Erika Pizzinato, Fabio Di Ferdinando, Gianluca Sala, Rossano Lattanzio, Enrico Dainese, Luca Federici, Peter Ponsaerts, Vincenzo De Laurenzi, Ilaria Cicalini and Damiana Pieragostino – *Oncogenesis* 2024

Integrative proteomic meta-analysis in Pancreatic carcinoma: biomarkers, key pathways, and insights for future research strategies

Pancreatic carcinoma (PC) is the sixth most common cause of cancer-related deaths in 2022, accounting for nearly 5% of all cancer deaths worldwide [1]. Currently, the causes of PC are not yet fully identified [2], but certain risk factors have been identified and are classified as non-modifiable (age, sex, blood group, family history and genetic susceptibility, diabetes) and modifiable (intestinal microflora, smoking, alcohol, chronic pancreatitis, obesity, dietary factors, infection). At clinical presentations, patients typically present with jaundice due to invasion and obstruction of the common bile duct when the PC is located in the head of the pancreas. Alternatively, they may exhibit nonspecific symptoms such as back pain and weight loss. The current methods for establishing the diagnosis of PC include ultrasonography (US), endoscopic ultrasonography (EUS), endoscopic retrograde cholangiography (ERCP), computerized tomography (CT) or magnetic resonance imaging (MRI), with or without guided percutaneous fine-needle biopsy, ascites cytology or exploratory biopsy under laparoscopy or open surgery diagnosis [3]. Approximately 80-85% of patients are diagnosed with unresectable disease. This is largely due to the encasement of major mesenteric vessels or metastatic disease to the liver, peritoneum, or other distant sites. As a result, the 5-year survival rate is less than 5% [4]. Despite advances in diagnostic approaches, perioperative management, radiotherapy techniques, and systemic therapies for advanced disease, there is only modest incremental progress in patient outcomes [4]. Only 20% of patients with resectable diseases survive for 5 years after surgery, and some may still die from the disease afterward. The high resistance of PC to conventional chemotherapy with 5-fluorouracil represents one of the reasons for this poor prognosis [5]. Radiotherapy alone is largely ineffective. Gemcitabine monotherapy was demonstrated to improve median survival by just over 1 month, compared with 5-fluorouracil [6]. Within the last decade, there have been improvements in clinical outcomes with combination chemotherapies with gemcitabine/nab-paclitaxel and 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX), providing median overall survival benefits of 1.8 and 4.3 months, respectively, compared with gemcitabine alone [7], [8]. Although FOLFIRINOX treatment resulted in a lower percentage of patients experiencing reduced quality of life, it also had increased toxicity and adverse events, thus preventing its

administration to patients with multiple comorbidities [9]. Recent research on targeted therapy, immunotherapy, and microbial therapy has shown that these can be used in combination with traditional methods for the treatment of PC, such as surgery, chemotherapy, radiotherapy, and palliative care.

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of PC, and 80-90% of these tumors are invasive; this cancer is characterized by a progression from normal tissue to invasive lesions with specific morphological characteristics. The process starts when acinar cells transform into a ductal phenotype in response to specific stimuli, a phenomenon known as acinar-to-ductal metaplasia (ADM). When combined with an oncogenic "hit," these cells progress to a pathogenic phenotype that eventually develops into Pancreatic Intraepithelial Neoplasia (PanIN), which is graded on a three-tiered scale as PanIN-1 (with A and B subtypes), PanIN-2, and PanIN-3, before becoming invasive PDAC [10]. The majority of PDACs are solid, firm, and infiltrative tumors with poorly defined margins. Microscopically, PDAC is characterized by neoplastic duct-like structures in a dense functional stroma composed of an extracellular matrix and extensive fibrosis (desmoplasia), which is the cause of resistance to therapies [11]. Desmoplastic stroma is characterized by the presence of cellular components, such as cancer-associated fibroblasts (CAFs), immune cells, and endothelial cells, and acellular components, such as collagens, laminin and cytokines in the extracellular matrix (ECM) [12], which is a physical barrier to the penetration of therapeutics [10]. The aggressive nature of PDAC is driven by the inflammatory process and an extensive stromal network in the tumor microenvironment (TME). Pancreatic stellate cells (PSCs) and cancer-associated fibroblasts (CAFs) play a crucial role in promoting the survival, growth, and proliferation of cancer cells. This, in turn, supports cancer metastasis and drug resistance [6]. PDAC tumor growth and progression are enhanced by a pro-inflammatory and immunosuppressive program characterized by the production of cytokines, growth factors, and other signaling molecules in the TME. Therefore, the current scientific and clinical challenge is targeting cancer cells and TME to induce a favorable therapeutic response [6]. Advancements in understanding the molecular mechanisms of PDAC in recent years have led to the identification of six common serum tumor biomarkers: CA19-9, CA242, carcinoembryonic antigen (CEA), CA125, microRNAs, and K-RAS gene mutations in codon 12. These biomarkers are considered the preferred choice for early screening. When combined with CA19-9,

CA125, CEA, and microRNAs increase sensitivity, specificity, and accuracy by 84% compared to using CA19-9 alone [5]. Moreover, new potential biomarkers are identified in circulating cell-free DNA (CfDNA) and mutation-specific circulating cell-free tumor DNA (ctDNA); there is an important correlation between the concentrations of these two factors and treatment response. About the presence of biomarkers, a repertoire of proteins in fibroblasts has been identified after the treatment with conditioned medium from MIA-PaCa2 human pancreatic tumor cell line, such as ITGB3, TGFB1, and TGFB2 (involved in fibroblast movement and apoptosis) and SMAD3, STAT3, and BAG3 (involved in chemotaxis activation and cell adhesion) [13]. In recent years, proteomics based on mass spectrometry (MS) analysis have taken a central role in the investigation of potential biomarkers since they offer the opportunity to identify innovative molecular pathways and quantify the levels of differentially expressed proteins (DEPs) [14]. Particularly, functional proteomics turns out to be useful for combining the examination of protein activation, protein-protein interactions, and activated pathway analyses [15]; it can be used to classify the type of cancer, to study the prognostication and the prediction of response to therapy, and also to discover tool for targeted therapy. In contrast to genomic analysis, which defines potential gene products, proteomics reflects the actual protein expression in response to translational control and degradation or regulation of protein activity through post-translational modifications. It has been shown that protein expression data correlate better with drug sensitivity or resistance than data from other “omics” studies so these analyses can be used to study multifactorial pathologies, such as cancer [16].

In this study, we have gathered proteomic research from the past decade. We have analysed protein biomarkers, categorizing them as tissue/cellular markers or circulating markers. Additionally, we performed a meta-analysis using the Ingenuity Pathway Analysis (IPA) bioinformatic tool to extract new and detailed information from the collected data set.

Results

Distribution and analysis of protein biomarkers across biological fluids and tissues

Table 1 lists the protein markers, distinguishing them not only by biological fluid/cells/tissue of origin but also by their respective trend, biological matrix, tumor type, number of articles, PANTHER protein classification, and reference. We find proteomics and immunohistochemistry studies among the main types of assays used in biomarker discovery. In particular, proteomic tools can be divided into antibody-based (including Western blotting, enzyme-linked immunosorbent assay - ELISA -, immunohistochemistry - IHC -, and protein microarray) and non-antibody-based (including protein mass spectrometry-based technology) [15]. Interestingly, most modulated proteins resulted from studies conducted on biological fluids (74.2%), such as peripheral blood, plasma, serum, urine, and pancreatic juice. In comparison, only 25.8% of proteins are involved in studies conducted on cellular models or sections of tumor tissue. On the one hand, studying a tissue biomarker could be extremely useful in characterizing the tumor type, identifying tumor staging, or following the appropriate therapeutic strategy.

Biomarker	Trend	Sample	Tumor type	# Articles	Panther class.	Ref.
MUC5AC	up	circulating	PanIN1A to late-stage PC	4	-	[17]
S100P	up	DJ	PDAC	1	-	[18]
CEA	up	DJ	PDAC	20	-	[18]
GSN	up	plasma	PC	1	CPs	[19]
LUM	up	plasma	PC	1	-	[19]
TIMP-1	up	plasma	PC	7	-	[19]
CA19-9	up	serum	early-stage PC	24	-	[20]
PROZ	up	serum	early-stage PC	1	-	[20]
TNFRSF6B	up	serum	early-stage PC	1	-	[20]

TFPI	up	plasma	early-stage PDAC (stage IA/IB/IIA, stage IIB)	1	-	[21]
TNC-FN III-C	up	plasma	early-stage PDAC (stage IA/IB/IIA, stage IIB)	1	-	[21]
Survivin	up	serum	PDAC	2	-	[22]
ULBP2	up	serum	PC	2	-	[23]
MIC-1	up	serum	early-stage PC	6	-	[23]
THBS2	up	plasma	PDAC	1	-	[24]
LRG1	up	plasma	PDAC	4	-	[25]
TTR	up	plasma	PDAC	2	-	[25]
Biglycan (BGN)	up	tumour resection	PC - extracellular matrix	1	-	[26]
PEDF	up	tumour resection	PC	1	P-BAMs	[26]
THBS-2	up	tumour resection	PC - TME	1	-	[26]
TGF-β	up	tumour resection	PC - TME	5	-	[26]
βIGH3	up	tumour resection	PC	1	CAMs	[26]
DTNBP1	up	serum	PDAC	1	-	[27]
AGR2	up	PJ	PanIN3 PDAC	3	MIEs	[28]
uPAR	up	urine	PDAC	1	-	[29]
ICAM-1	up	serum	PDAC	1	-	[30]
TIMP-1	up	serum	PDAC	7	-	[30]
ADH	up	serum	early-stage PC	1	-	[31]
C4BPA	up	serum	PDAC	1	-	[32]
CFB	up	plasma	PC	2	-	[33]
IGFBP3	up	plasma	invasive PDAC	2	-	[34]
PIGR	up	plasma	PC	3	-	[35]
OLFM4	up	plasma	PC	1	-	[35]
SYCN	up	plasma	PC	3	-	[35]
COL6A1	up	plasma	PC	1	-	[35]

FLNA	up	tissue	PDAC	1	-	[36]
ITGAV	up	tissue	PDAC	1	CAMs	[36]
TYMP	up	tissue	PDAC	1	MIEs	[36]
ACADM	dow n	tissue	PDAC	1	MIEs	[36]
ACADS	dow n	tissue	PDAC	1	MIEs	[36]
ACAT1	dow n	tissue	PDAC	1	MIEs	[36]
APOA4	up	serum	PC	1	T/CPs	[37]
APOC3	up	serum	PC	1	T/CPs	[37]
IGFBP2	up	serum	PC	3	-	[37]
Nuclear pSTAT3	up	tumour resection	PDAC	1	-	[38]
IL-11	up	plasma	PC	2	ISMs	[39]
LPO (SAPX)	up	OF	PC	1	MIEs	[40]
PPIB (CYPB)	up	OF	PC	1	-	[40]
KRT14	dow n	OF	PC	1	CPs	[40]
Krt13 (Krt1-13)	dow n	OF	PC	1	CPs	[40]
KRT17	dow n	OF	PC	1	CPs	[40]
NGAL	up	plasma	PC	2	T/CPs	[41]
THBS3	up	tissue	PC	1	CAMs	[42]
FLT3	up	tissue	PDAC	1	TSRs	[42]
GPRC5A	up	tissue	PC	1	TSRs	[42]
MYO1F	up	tissue	PC	1	CPs	[42]
GPX2	up	tissue	PC	1	MIEs	[42]
KRT23	up	tissue	PC	1	CPs	[42]
PSCA	up	tissue	PC	1	P- BAMs	[42]
S100A14	up	tissue	PC	1	C-BPs	[42]

LGALS9C	up	tissue	PC	1	-	[42]
MBL2	up	serum	PC	2	-	[43]
MLCK2	up	serum	PC	1	-	[43]
ALDH1A1	up	tissue	PC	1	MIEs	[44]
Pancreatic lipase	up	tissue	PC	1	MIEs	[44]
ANXA1	up	tissue	PC	1	C-BPs	[44]
Proline-hydroxylated α-fibrinogen	up	plasma	PC	1	-	[45]
CXCL7	down	plasma	PC	1	ISMs	[46]
AAT	up	serum	PC	4	-	[47]
C-reactive protein	up	peripheral blood	PC	5	T/CPs	[48]
IL-10	up	peripheral blood	PC	3	ISMs	[48]
IL-23	down	peripheral blood	PC	1	ISMs	[48]
TNFα	up	peripheral blood	PC	1	ISMs	[48]
IL-1ra	up	serum	PDAC	1	ISMs	[49]
IL-6	up	serum	PDAC	5	-	[49]
IL-8	up	serum	PDAC	3	ISMs	[49]
IP-10	up	serum	PDAC	2	-	[49]
MIP-1b	up	serum	PDAC	1	-	[49]
FGF-10/KGF-2	up	serum	PC	1	ISMs	[50]
I-TAC/CXCL11	up	serum	PC	1	ISMs	[50]
OSM	up	serum	PC	1	ISMs	[50]
OA/GPNMB	up	serum	PC	1	ISMs	[50]
SCF	up	serum	PC	1	-	[50]
HSP70	up	serum	PC	2	-	[51]
OPN	up	serum	PDAC	2	ISMs - MIEs	[52]

Apolipoprotein A-I	dow n	serum	PC	2	T/CPs	[53]
Apolipoprotein A-II	dow n	serum	PC	1	T/CPs	[53]
Transthyretin	dow n	serum	PC	4	MIEs	[53]
Tetranectin	dow n	serum	PC	1	ISM	[53]
Apolipoprotein E	up	serum	PC	3	T/CPs	[53]
SYCN	up	serum and plasma	PC	3	-	[54]
REG1B	up	serum and plasma	PC	2	-	[54]
AGR2	up	serum and plasma	PC	3	-	[54]
LOXL2	up	serum and plasma	PC	2	MIEs	[54]

Table 1: Pancreatic cancer biomarkers identified in proteomics and immunohistochemistry studies published approximately in the last ten years with their respective trend, biological matrix, tumor type, number of articles, Panther protein classification, and reference with particular evidence of biomarkers related to PDAC, different stages of PC and PDAC, and TME.

According to “PANTHER Protein classification” (Figure 1A), concerning circulating protein biomarkers, 20.9% of proteins are intracellular signaling molecules (ISM), 9.0% of proteins are transfer/carrier proteins (T/CPs), and metabolite interconversion enzymes (MIEs), 7.5% correspond to cytoskeletal proteins (CPs). Considering cells/tissue proteins biomarkers listed in Table 1 and Figure 1B, the most proteins found (29.2%) are related to metabolite interconversion enzymes (MIEs), 12.5% of proteins are cell adhesion molecules (CAMs), while 8.3% of proteins are related to protein-binding activity modulators (P-BAMs), transmembrane signal receptors (TSRs), calcium-binding proteins (C-BPs), and cytoskeletal proteins (CPs). Proteins involved in the mentioned protein classes are given in the “Panther Class” column of Table 1. All biomarkers used in this meta-analysis are related to pancreatic cancer, in particular fluid and tissue biomarkers linked with PC/PDAC cells or TME are tabulated in the Table 1 -column “tumor type”- with evidence of biomarkers related to PDAC, different stages of PC and PDAC, and TME, since the aggressive nature of PC/PDAC is driven by the inflammatory

process and an extensive stromal network in the TME. Specifically, 59.57% of protein biomarkers are related to PC, biomarkers linked to PDAC represent 26.60% of the total, 10.64% of biomarkers describes particular stages of PC/PDAC and 3.19% of biomarkers are related to TME. The circulating biomarker mucin MUC5AC is up-regulated in stage PanIN1A to late-stage of PC; macrophage inhibitory cytokine-1 (MIC-1), alcohol dehydrogenase (ADH), carbohydrate antigen 19-9 (CA19-9), vitamin K-dependent protein Z (PROZ) and tumor necrosis factor receptor superfamily member 6b (TNFRSF6B) are up-regulated circulating biomarkers related to the early stage of PC. On the other hand, plasma tissue factor pathway inhibitor (TFPI) and tenascin C (TNC-FN) are up-regulated circulating biomarkers related to the early stage of PDAC (stage IA/IB/IIA, stage IIB); anterior gradient-2 (AGR2) and insulin-like growth factor-binding protein-3 (IGFBP3) are up-regulated circulating biomarkers related respectively to stage PanIN3 of PDAC and invasive PDAC. Finally, thrombospondin-2 (THBS-2) and transforming growth factor-beta (TGF- β) are up-regulated tissue biomarkers linked to the TME of PC. As reported in Table 1 -column “# Articles”- it's possible to notice that 62.77% of biomarkers are described in only one meta-analysis article -mostly related to PC and PDAC but also to the early stage of PC, early-stages of PDAC (stage IA/IB/IIA, stage IIB) and TME-; in particular tissue biomarkers as biglycan (BGN), pigment epithelium-derived factor (PEDF) and THBS-2 are related to the TME and the others to PC/PDAC. The 2.13% of biomarkers (CEA and CA19-A) are discussed in more than twenty articles - mostly related to PDAC and early stage of PC- and 35.11% of biomarkers are described in a number of meta-analysis articles between two and seven -related to PDAC, PC, stage PanIN1A and late stage of PC, stage PanIN3 and invasive stage of PDAC.

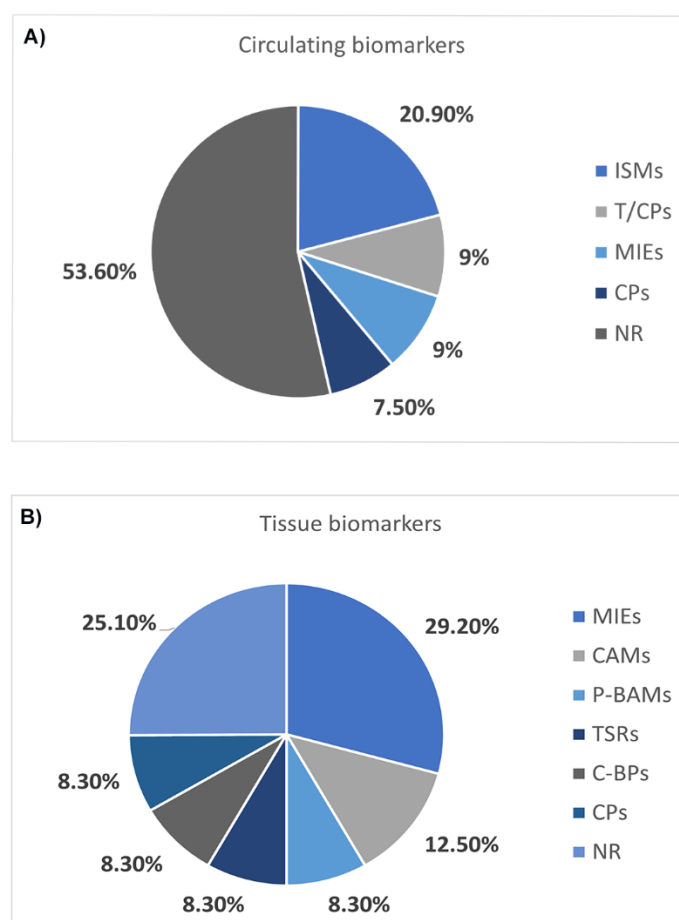


Figure 1: Protein classification according to PANTHER tool for Circulating protein biomarkers (Panel A) and Cells/Tissue protein biomarkers (Panel B). Pie charts show the classification of circulating and tissue biomarkers according to their “Family and Protein Class”: these are grouped into intracellular signaling molecules (ISMs), transfer/carrier proteins (T/CPs), metabolite interconversion enzymes (MIEs), cytoskeletal proteins (CPs), cell adhesion molecules (CAMs), protein-binding activity modulators (P-BAMs), transmembrane signal receptors (TSRs), calcium-binding proteins (C-BPs), and non-relevant (NR) group. This classification was obtained by uploading -separately- the entire dataset of circulating and tissue biomarkers to “PANTHER Protein classification” by “supergrouping of protein families” way.

Functional analysis on fluid biomarkers unveils implications for cancer, inflammation, and lipid metabolism

Among the main pathways we found related to the biomarkers dataset, we considered only the significant “canonical pathways” ($-\log_{10}(\text{p-value}) > 1.3$) where the identified biomarkers could be involved (Figure 2); we find the following interesting functions predicted to be down-regulated ($z\text{-score} < -2$), involved overall in the

inhibition of proliferation and inflammation: retinoic acid receptor (RAR) activation pathway, CDX gastrointestinal cancer signaling, and the adrenergic receptor signaling pathway.

The “RAR activation” pathway leads to potent anti-proliferative and anti-inflammatory properties [57-59]; moreover, RARs are able to repress the activity of transcription factors such as AP-1, which is involved in cell proliferation and survival and in particular in the proliferation of cancer cells [58], and NF-kB, which regulates multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammatory genes [59] and plays a critical role also in cell proliferation and survival [60]. RAR pathway presents an important druggability since this is a target for many drugs in in vitro test and clinical trial. Moreover, the PI3K-alpha inhibitor “idelalisib” terminated the phase 1-clinical trial process in 2024 respectively for metastatic pancreatic adenocarcinoma (NCT03878524) and pancreatic ductal adenocarcinoma (NCT02468557); the PARP (poly ADP-ribose polymerase) inhibitor “niraparib” is in 2023 in the phase 2-clinical trial NCT05442749 for advanced PDAC.

Regarding the inhibition of the “CDX gastrointestinal cancer signaling”, knockouts of CDX genes exhibit increased colorectal cancer and CDX products present important roles in cancer progression and intestinal inflammation [61],[62]. In this scenario, i.e. the drug “ABBV-621” presents TNFSF10 as the target molecule within the “CDX gastrointestinal cancer signaling” and “adrenergic receptor signaling”, and it has completed the phase 1-clinical trial NCT03082209.

Interestingly, the “adrenergic receptor signaling” pathway was found to be inactivated: this pathway is involved in the body’s fight or flight response since norepinephrine (NE) plays a role in mood and sleep regulation, and expression of behavior. Specifically, ADRA1-2 receptors have been found to influence cognitive functions such as working memory, attention, fear, and spatial learning, while ADRB1-2 receptors function in auditory fear, spatial reference, fear memory, and memory retrieval [63]. The inhibition of this pathway and its related implications could be interesting to study and monitor as co-symptomatology of PC. Regarding the pathway’ druggability, the drug “anakinra” targets IL-1A and IL-1B within the adrenergic receptor signaling and it’s in phase 2-clinical trial NCT04926467.

As for the many pathways predicted to be up-regulated (z -score > 2), they are involved in many macro-categories such as cancer signaling, cellular stress and injury, immune response signaling, neurotransmitters, and nervous system and other disease-signaling pathways (Figure 2).

The engagement of pattern recognition receptors (PRRs) results in the activation of specific genes and subsequent triggering of innate immune responses; the NOD-like receptors are members of this family of molecules, helping activate innate immune responses to cellular stress and stress [64], [65]. NOD1 mutations increase susceptibility to inflammatory bowel disease, while NOD2 mutations have been associated with susceptibility to Crohn's disease, Blau syndrome, and other intestinal innate immune defects. Aberrant NOD signaling has long been associated with a range of inflammatory disorders, and its inhibition could be beneficial in the treatment of disorders such as allergic asthma and type 2 diabetes mellitus [66]. Interestingly, the drug “ABBV-621” again is involved in the regulation of this pathway with TNFSF10 as the final therapeutic target. Moreover, as for the adrenergic receptor signaling, “anakinra” is also involved in NOD-like receptors regulation.

Moreover, within the “activin inhibin signaling” pathways, activin A activates the MAPK signaling pathway including P38 mitogen-activated protein kinase (p38-MAPK), further identified as an upregulated upstream regulator, mostly under inflammatory conditions and has a crucial effect on innate and adaptive immune responses and in the pathophysiology of human diseases such as cancer and fibrotic syndromes [67]. Again “anakinra” (NCT04926467) and “idelalisib” (NCT03878524) target this pathway regarding its druggability; moreover, i.e “ASN007” (NCT 03415126) and “avutometinib” (NCT 03875820) as inhibitors of respectively MAPK1-3 and MEKs, are in phase 2 of both clinal trials.

“Cancer cachexia pathway” shows a systemic excess catabolism syndrome that decreases quality of life, ability to tolerate treatment, and eventual survival; the wasting phenotype mostly occurs in skeletal muscle and adipose tissue, but other organs, such as brain, liver, pancreas and heart, are also affected [68]. The activation of such pathways could be of interest for further investigation to deepen the understanding of PC comorbidities and responses to therapies, since loss of body weight, metabolic alterations such as glucose,

protein and lipid metabolism, systemic inflammation, insulin resistance and oxidative stress are pathophysiological changes involved in the progress of cancer cachexia [69]. It could also be interesting to investigate liver and lung function, as hepatic and pulmonary fibrosis pathway appears to be predicted upregulated.

The high mobility group-B1 (HMGB1) has a role in inflammatory responses and tumor metastasis: it's regulated by the activation of inflammatory cells through binding of tumor necrosis factor (TNF), lipopolysaccharide (LPS), further identified as upregulated upstream regulators, and HMGB1 itself and it regulates the activation of MAPK pathways with again p38-MAPK. It also interacts with the extracellular matrix and membrane receptors, affecting cell motility and metastasis [70], [71]. So, targeting the HMGB1 or its receptors, could represent an important potential application in cancer therapeutics: i.e. "ASN007" (NCT03415126) in phase 2-clinical trial targets MAPK1-3 within the modulation of this pathway.

Regarding "inflammation and immune response", triggering receptor expressed on myeloid cells 1 (TREM1) signaling results in the production of proinflammatory cytokines such as TNF and IL-6 [72]; moreover, inflammation and immune response are stimulated by IL-17, IL-33 and IL-6 signaling in a cytokine storm signaling pathway as well as cancer development [73]; in particular, IL-33 signaling results again in activation of MAPK signaling and p38-MAPK [74] and the transcription of the IL-6 gene is also stimulated by tumor necrosis factor (TNF), further identified as upregulated upstream regulator. Additionally, inflammation at the neuronal level also appears to be activated with an excessive cell and tissue damage which results in destruction of normal tissue and chronic inflammation that ultimately results in necrosis of glial cells and neurons [75], [76]; in this case as well, it could be important to further investigate the concomitant symptoms of PC since chronic neuroinflammation is closely related to chronic neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and has also been a focus of research into the pathology underlying psychiatric disorders like depression [77]. Globally, within the tumor microenvironment (TME) pathway, which comprises cancer cell, cytokine environment, extracellular matrix, immune cell subsets, wound healing signaling and other components, the pro-tumorigenic immune response plays a pivotal role in driving immune invasion; the tumor not only manages to escape from the host immune system, but it effectively

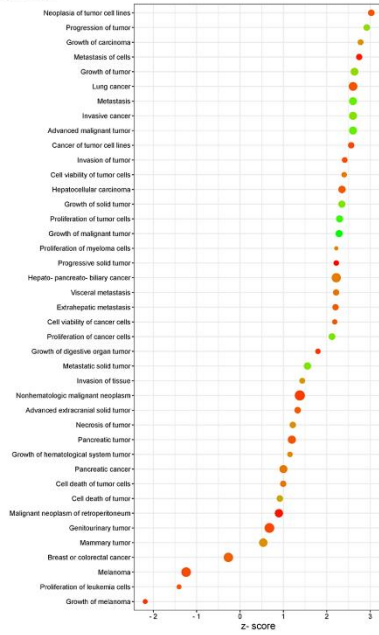
benefits from infiltrating cells by modifying their functions to create microenvironment favorable for tumor progression [78]. For this reason, targeting the inflammatory pathways has become one of the primary objectives in pharmaceutical research on PC.



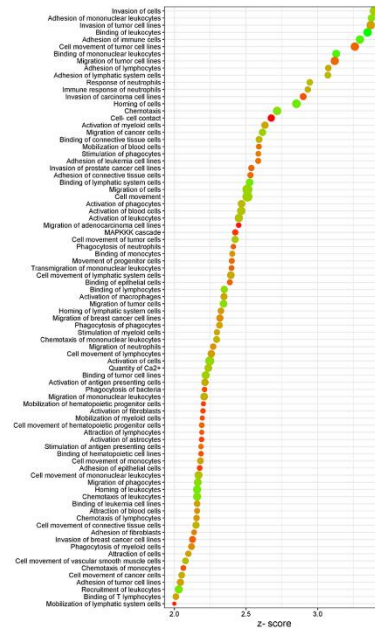
Figure 2: “Canonical pathways” characterization on fluids biomarkers functional analysis: in this bubble blot generated by IPA software, we show the significant pathways previously described. It is possible to notice how each pathway is characterized by a specific “-log10 (p-value)” -only pathways with a value greater than 1.3 are shown and described in the text- and an absolute “z-score value” -regarding the modulation- greater than 2. As described in the legend, orange bubbles are indicative of significantly up-regulated pathways (z-score greater than 2) and down-regulated pathways (z-score lower than -2); the size of bubbles depends on the number of biomarkers that overlap the pathway. This bubble blot has been created using the values of "p-value", "z-score", and “# of molecules” resulting from the functional analysis carried out by IPA software.

Regarding “disease and functions” analysis, the main categories of interest are reported in Figure 3 along with their corresponding z-scores and p-values, describing respectively biofunctions’ modulation and significance: "cancer", "cell signaling and movement", "inflammatory response", "free radical scavenging", and "lipidic metabolism".

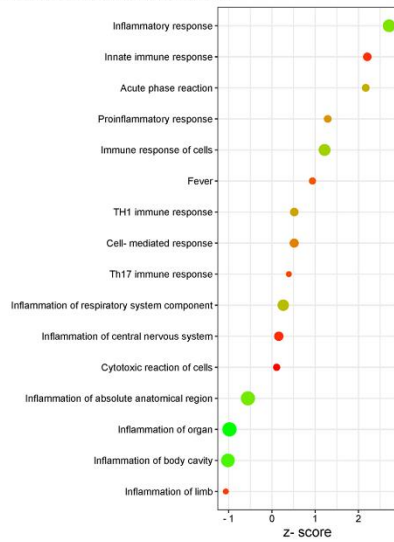
A) Cancer



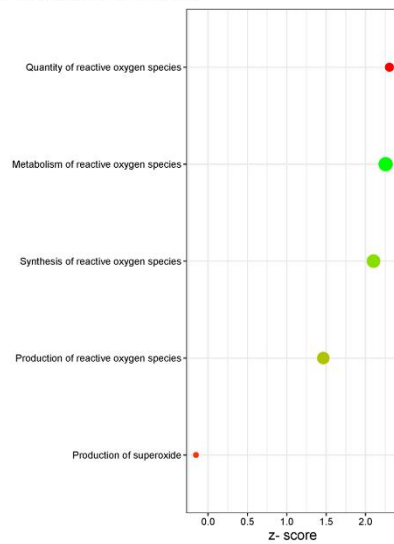
B) Cell Signaling Movement



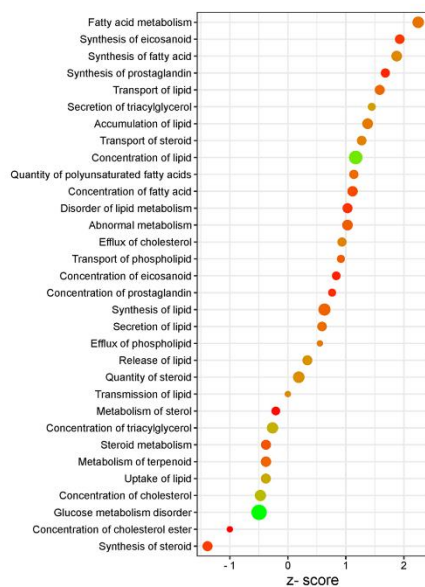
C) Inflammatory Response



D) Free Radical Scavenging

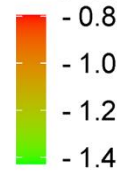


E) Lipid Metabolism



Legend

$-\log_{10}(pvalue)$



count



Figure 3: "Disease and Functions" characterization. We displayed selected entities for each "Disease and Functions" category, also described in biological fluids meta-analysis: A) cancer, B) cell signaling movement, C) inflammatory response, D) free radical scavenging, and E) lipid metabolism. The z-score value shows the modulation (up-regulation for z-score greater than 2 and down-regulation for z-score lower than -2) of each function with a significance of $-\log_{10}$ (p-value) indicated by bubbles color (color scale of red and green). The size of bubbles depends on the number of biomarkers that overlap the described pathway. This bubble blot has been created using the values of "p-value", "z-score", and "# of molecules" resulting from the functional analysis carried out by IPA software.

Since biomarkers have been identified from research papers on PC, "cancer" is the first category we focused on, as a proof of concept (Panel A). Among the functions predicted to be significantly up-regulated (z-score > 2), we find neoplasia of tumor cell lines, metastasis of cells, cancer of tumor cell lines, invasion of tumor, progressive solid tumor, and cell viability of cancer cells. "Pancreatic cancer" and "pancreatic tumor" functions have a z-score value of about 1, so they cannot be considered as predicted functions to be up-regulated with a significant z-score, but the $\log_{10}$ (p-value), on the other hand, is statistically significant. For the "cell signaling and movement" category (Panel B), since biomarkers in the circulating fluid are obviously also implicated in the cell signaling, we could highlight how tumor cell movement and signaling is predicted to be activated by biomarkers such as migration and invasion of tumor cell lines, and cell-cell contact. In this context, we also find a remarkable activation of the immune and inflammatory response in stimulation of phagocytes, monocytes, leukocytes, and lymphocytes. We can also observe a significant activation (z-score > 2) of inflammatory response, innate immune response and acute phase reaction pathways (Panel C) as well as activation of reactive oxygen species' quantity, metabolism, and synthesis (Panel D), confirming an important activation of inflammatory and immune responses, already identified in the "canonical pathways" analysis. As far as lipid metabolism is concerned, the bubble plot (Panel E) shows a significant activation (z-score > 2) of fatty acid metabolism and a fair activation (z-score = 1.9) of eicosanoid synthesis; PC cells need lipids during some conditions such as rapid proliferation, metabolic stress, and many cellular activities; cells acquiring of fatty acids (FAs) and cholesterol depends on lipid uptake from the exogenous environment [79]. It's clear that fatty acid translocase (CD36) is involved in metastasis initiation and proliferation [80]: large tumor size and reduced survival rate are associated with the low expression of CD36 [81]. Regarding cholesterol, its uptake

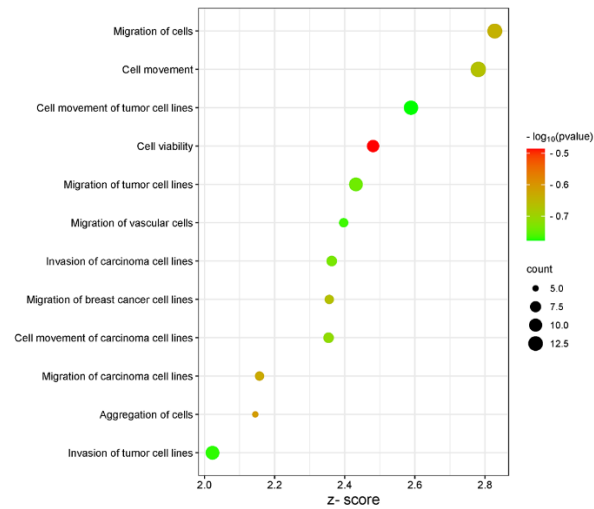
dysregulation contributes to PC carcinogenesis but also cholesterol efflux is critical for cholesterol homeostasis since it contributes to diverse types of tumors. Moreover, despite lipogenesis takes place in hepatocytes and adipocytes, cancer cells can acquire lipids through de novo synthesis because of their high metabolic demand [79].

Insights biomarker pathways explore inflammation and tissue dynamics

When looking at well-characterized metabolic and cell signaling pathways, also known as “canonical pathways”, our analysis showed that the tissue biomarkers are significantly fewer in number and are related to only two distinct upregulated canonical pathways (with a z-score > 1 and a $-\log_{10}(\text{p-value}) > 1.3$). These pathways are “macrophage alternative activation”, which plays a crucial role in innate and adaptive immunity and the inflammatory response, and the “S100 family signaling”, which is linked to cancer signaling pathways. In particular, the immune function is the primary role of S100 proteins, which also are important regulators of macrophage inflammation [82]; cell stress and injury and inflammation promote proinflammatory signaling pathways in turn related to cell differentiation, inflammation, migration, cell survival, proliferation, and tissue repair [83]. Secondly, we again focused on disease and function identification to point out some downstream effects expected to increase or decrease, according to IPA prediction. So, Figure 4 shows the “disease and functions” for each category of interest: in this case, it’s not possible to find the “cancer” category, as a proof of concept in fluid biomarkers, since some pathways are described in a significant way but without any prediction on modulation (not significant z-score). However, we find some downstream effects linked to cell viability and migration of tumor cell lines as significantly upregulated (z-score > 2), such as cell viability, aggregation of cells, migration of cells, cell movement of tumor cell lines, migration of tumor cell lines, invasion of carcinoma cell lines, cell movement of carcinoma cell lines, migration of carcinoma cell lines and invasion of tumor cell lines (Figure 4A). The bubble plot in Figure 4B shows a significant activation of the inflammatory response (z-score > 2) and a significant inhibition of organismal death (z-score < -2). The predicted activation of the inflammatory response and the inhibition of cell death would, however, seem to

confirm the tumor trend, although in a less evident way than in the fluid biomarkers' analysis. Since the IPA research is based on data published in the literature, there is probably less information on tissue studies; this could explain the lower number of correlations found in tissue biomarkers compared to fluids. In this context, it would, therefore, be interesting to further research on tissue biomarkers in PC.

A) Cell Death, Signaling and Movement



B) Inflammatory Response and Organismal Injury

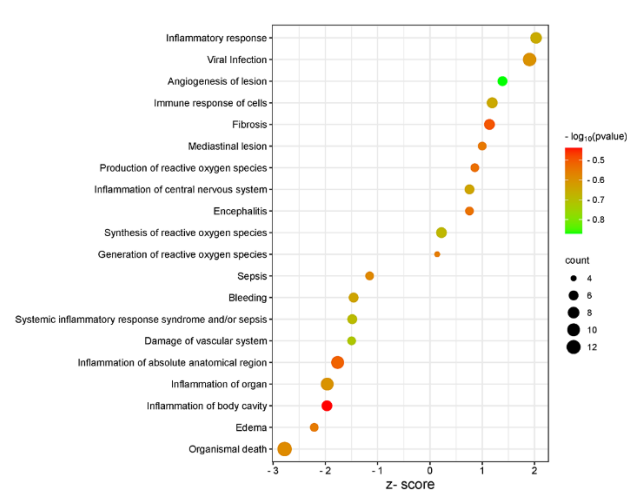


Figure 4: "Disease and Functions" characterization. We displayed selected entities for each "Disease and Functions" category, also described in tissue biomarkers meta-analysis: A) cell death, signaling, and movement and B) inflammatory response and organismal injury. The z-score value shows the modulation (up-regulation for z-score greater than 2 and down-regulation for z-score lower than -2) of each function with a significance of $-\log_{10}(\text{p-value})$ indicated by bubble colors (color scale of red and green). The size of bubbles depends on the number of biomarkers that overlap the described pathway. This bubble blot has been created using the values of "p-value", "z-score", and "# of molecules" resulting from the functional analysis carried out by IPA software.

Exploring upstream regulators to understand transcriptional control and biomarker expression patterns

In the functional enrichment analysis conducted using IPA software, we have identified a cascade of upstream transcriptional regulators that can account for the changes in protein biomarker expression observed in our dataset. This information can provide insights into the biological activities taking place in the analysed fluids and tissues. IPA makes it easy to take this result even further by examining what biological processes,

pathways, and diseases the transcriptional regulators and their targets may control, and how these upstream molecules may regulate one another. Since the upstream analysis aim is to predict some biological regulators above our biomarkers, it may not be affected by the original fluid or tissue; so, we then focused on upstream regulators that are shared between fluid and tissue biomarkers functional analysis with a significant z-score value (z-score > 2 or < -2): we identified seven upstreams that meet these conditions, 470 unique fluid upstreams and 21 unique tissue upstreams (the complete lists are in the Supplementary file “S1 Table: Upstream regulators”). In the following tables (Table 2A – 2B) we list and tabulate the shared seven upstream regulators with their respective information about molecular type, predicted activation state, and activation z-score in both functional analyses; they present the same trend modulation in both functional analyses performed on fluid and tissue protein biomarkers.

Upstream Regulators	Molecular Type	Predicted Activation State	Activation z-score
LPS	Chemical drug	Activated	4.168
TNF	Cytokine	Activated	3.998
P38 MAPK	Group	Activated	2.853
AGT	Growth factor	Activated	2.83
Tetradecanoylphorbol acetate	Chemical drug	Activated	2.571
miR-323-5p (and other miRNAs w/seed GGUGGUC)	Mature microRNA	Inhibited	-2.345
miR-34a-5p (and other miRNAs w/seed GGCAGUG)	Mature microRNA	Inhibited	-2.646

Table 2A: Upstream regulators description and modulation of fluid biomarkers by IPA.

Upstream Regulators	Molecular Type	Predicted Activation State	Activation z-score
LPS	Chemical drug	Activated	2.122
TNF	Cytokine	Activated	2.512
P38 MAPK	Group	Activated	2.2
AGT	Growth factor	Activated	2.085
Tetradecanoylphorbol acetate	Chemical drug	Activated	2.585
miR-323-5p (and other miRNAs w/seed GGUGGUC)	Mature microRNA	Inhibited	-2.236
miR-34a-5p (and other miRNAs w/seed GGCAGUG)	Mature microRNA	Inhibited	-2.449

Table 2B: Upstream regulators description and modulation of tissue biomarkers by IPA.

LPS is predicted to be upregulated and, in turn, increases the stimulation and release of TNF [84], [85] and the activation of P38 MAPK [86] and angiotensinogen (AGT) [87]. It shows a role in the expression of pancreatic cancer cell lines [88], activation and phosphorylation of tumor cell lines [89], and proliferation of pancreatic cancer cell lines [90]. The second upregulated upstream is the TNF, which is involved in the production and release of reactive oxygen species as a confirm of the disease and biofunction predicted to be upregulated as downstream [91], [92]. It's also regulated by P38 MAPK [93], which is the third upregulated upstream and also has a role in the production of reactive oxygen species [94]. AGT, as again upregulated upstream, regulates TNF [95] and presents a positive expression in pancreatic cancer cell lines [96]. Moreover, its product angiotensin II protein, increases the quantity of reactive oxygen species, continuing to confirm our disease and function effects analysis [97]. Tetradecanoyl-phorbol acetate is a drug used in cancer treatment [98] and regulates TNF [99] and P38 MAPK [100]. Its predicted upregulation may be a proof of concept since we are studying protein biomarkers involved in cancer research. The last two upstream regulators are predicted to be downregulated in our original fluids and tissues: the downregulation of human miR-34a-5p is associated with pancreatic cancer in humans [101]. Overall, as another proof of concept, it is related to pancreatic cancer cell lines since in cell culture, human miR-34a-5p mature microRNA increases the arrest in cell cycle

progression of MIA-PaCa-2 cells in cell culture [102] and decreases the viability of MIA PaCa-2 cells [103]. miR-423-5p is regulated by LPS, which increases its expression in cultured THP-1 macrophage cells [104]; according to Volinia et al. [105], it seems to be involved in breast cancer disease so it could be interesting to study its role and its modulation also in PC.

Discussion and conclusions

PC is considered a highly fatal malignancy with an estimated overall 5-year survival rate of patients treated by resection of less than 5%. The efforts of the clinical community in implementing diagnostic approaches and perioperative management collide with the high resistance of PC to conventional chemotherapy treatments, and this represents one of the reasons for this poor prognosis. To date, the “gold standard” for the diagnosis of PC is the fine-needle biopsy performed percutaneously with guidance by image techniques or under direct visualization at surgery. However, as widely discussed in this study, in recent years (16), much progress has been made in the field of biomarker discovery, aimed at the study of functional proteins directly on tissues or in circulating fluids. In fact, current proteomic approaches aim to identify and quantify a high number of proteins from different biological samples, trying to provide the most complete view of the metabolic pathways or pathological processes implicated in the onset and development of PC, with the final purpose being to investigate possible new pharmacological targets. In this study, we have collected protein biomarkers from the last ten years papers, distinguishing them between tissue or cellular markers and circulating markers, and we carried out a meta-analysis through the IPA bioinformatic tool with the aim of providing new and detailed information from the published data. Our meta-analysis revealed that the data currently available in the literature, derived from various biological fluids as well as cells and tissues, are consistent with each other and primarily focused on the description of functions related to cancer and cell migration, as expected. On the other hand, some new evidence has emerged regarding inflammation, activation of free radical production, and lipids metabolism, conditions that could be further explored in the assessment of the onset and progression of PC. It is possible to notice, also as a proof of concept, that we find in fluid biomarkers functional

analysis all pathways related to cancer and inflammation signaling as significantly activated and those related to anti-proliferative and anti-inflammatory functions as significantly inhibited pathways. Moreover, according also to “canonical pathway” analysis, it is interesting to notice that recent studies have investigated the correlation between PC onset and progression and the regulation of lipid metabolism, found to be modulated also in the “Diseases and functions” analysis. Literature data on lipid metabolism are largely related to our results obtained from the functional analysis on fluid biomarkers, better described in the Results section. In this scenario, research on potential therapeutic drugs aims to identify future PC treatment targeting FA synthesis, cholesterol synthesis, lipid catabolism, and transcriptional regulators of lipid metabolism. Globally, this targeted therapy represents a novel and potentially effective strategy for PC treatment but, since it is still necessary to investigate some unclear aspects, further understanding and research are required.

Interestingly, the study of upstream regulators has highlighted some factors already known in the context of PC, such as LPS, AGT, tetradecanoyl-phorbol acetate, miR-34a-5p, and miR-423-5p. At the same time, TNF and p38 MAPK, which are involved in oxidative stress scavenging, have not been directly linked to PC. All these findings could represent new starting points for both the characterization of cancer progression and the study of potential diagnostic biomarkers, allowing a more complete description of clinically relevant information. Moreover, as fully described in the results, all the cited pathways, mostly inflammation, exhibit a significant druggability, which provides valuable insights into the development of additional therapeutic approaches in pancreatic cancer (PC). It's clear that, integrating the large number of biological components with their interactions and environmental relationships, it's possible to reach an in-depth description of the pathological condition in PC and to define correlations between concomitant symptoms and tumor genesis and progression. This meta-analysis, while acknowledging certain limitations, offers valuable insights into specific pathways implicated in prognosis, and druggability. Despite these challenges, this meta-analysis provides a more holistic perspective on existing data. One of the primary benefits of our work is the ability to synthesize and contextualize a wide array of literature data. Individual studies contribute meaningful information, yet when analyzed collectively, they unveil new evidence regarding the pathways involved in various biological processes, such as inflammation. By integrating findings from diverse studies, we hope to stimulate further

investigation into the roles of these pathways, thereby contributing to the advancement of knowledge in the field of proteomics and its applications in clinical settings. Among the significant pathways identified, inflammation, activation of free radical production, and lipids metabolism may represent the main path to pursue in scientific research. Understanding their relationship with PC and determining whether their alterations are a cause or consequence of its onset, could help reveal their potential role in early detection or treatment monitoring. In conclusion, our work represents a strategy to combine results from different studies on various PC biological samples in a more comprehensive way. Finally, exploratory omics analyses can unveil new horizons beyond the scope of literature studies.

Materials and methods

Biomarkers identification and evaluation workflow

To deepen proteomics features and studies in PC, we performed a systematic review of the literature with meta-analysis. In Figure 5 we summarized the inclusion and exclusion criteria used for literature search. We selected article type such as meta-analysis, randomized controlled trial, review and systematic review on the PubMed website from the past decade, searching the keywords “pancreatic cancer”, “PDAC”, “drug” and “biomarkers” (combined with each other using the Boolean operator -AND -OR), obtaining 5156 eligible papers. We then narrowed our search even further by inserting -AND “proteomics”, “immunohistochemistry” as new keywords, resulting in 102 articles. Subsequently, we focused on those that specifically addressed tissue and circulating biomarkers related to diagnosis, prognosis, and overall survival. Following these research parameters, we obtained a total number of 94 PC protein biomarkers (n. 11 down-regulated biomarkers and n. 83 up-regulated) to perform the meta-analysis. Finally, we have classified our biomarkers by “PANTHER -(Protein ANALysis THrough Evolutionary Relationships)- protein classification” basing on their “Family and Protein Class” (supergrouping of protein families). The core of PANTHER is a comprehensive, annotated “library” of gene family phylogenetic trees. All nodes in the tree have persistent

identifiers that are maintained between versions of PANTHER, providing a stable substrate for annotations of protein properties like subfamily and function. Each phylogenetic tree is used to annotate each protein member of the family.

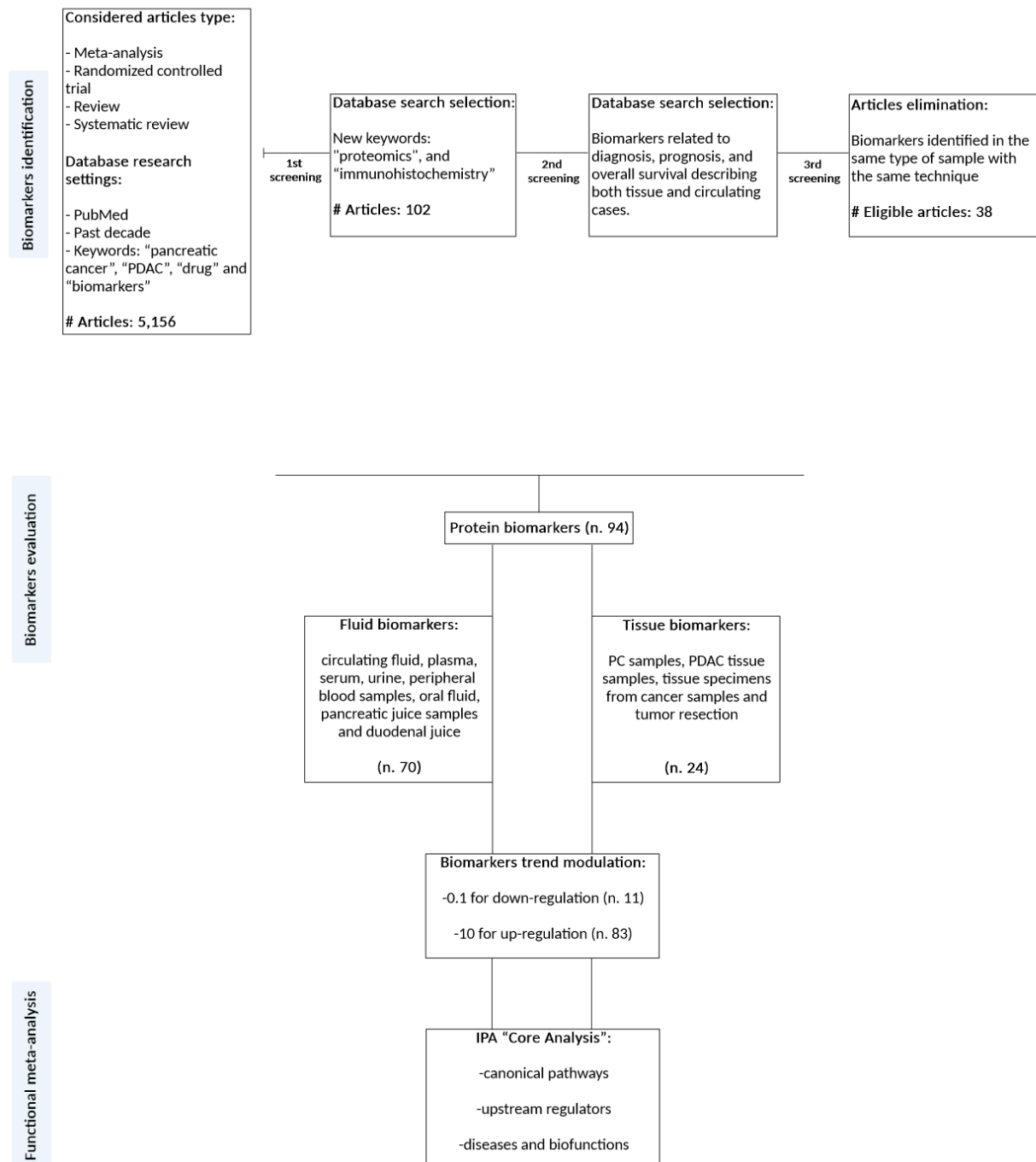


Figure 5: PRISMA chart of meta-analysis workflow consists of biomarkers identification and selection, evaluation, and subsequent functional meta-analysis: we performed a selection process in order to consider only eligible articles, according to our inclusion and exclusion criteria, for the further analysis of circulating and tissue biomarkers.

Functional meta-analysis by Ingenuity Pathway Analysis

As can be seen from Table 1, our selected biomarkers (n. 94) have different origins, so we decided to perform the subsequent functional enrichment analysis through “Ingenuity Pathway Analysis” software (IPA, Qiagen, Hilden, Germany) and categorize the biomarkers based on their origin, since they have distinct functional implications: fluid biomarkers (n. 70) and tissue biomarkers (n. 24). Biomarkers found in plasma, serum, urine, peripheral blood, oral fluid (OF), pancreatic juice (PJ), and duodenal juice (DJ) were categorized as “fluid biomarkers.” Biomarkers identified in pancreatic cancer (PC) samples, pancreatic ductal adenocarcinoma (PDAC) tissue samples, tissue specimens from cancer, and tumor resection were categorized as “tissue biomarkers”. The list of all biomarkers and related fluids/tissues of origin is provided in Table 1, respectively with their trend, biological matrix, tumor type, number of articles, PANTHER protein classification, and reference. Subsequently, we used IPA “Core Analysis” to map statistically protein biomarkers to their functional annotation such as “canonical pathways” analysis, “upstream regulators” analysis and “disease and function effects” networks. As reported in Table 1, we have evaluated the single regulation of each molecular biomarker in the PC fluid or tissue compared with healthy controls. Biomarkers from fluid and tissue studies were uploaded on IPA software as two different matrices composed by protein biomarkers and their modulation trend, as reported in our previous studies [14] (Figure 5). It is important to notice the inherent variability among different proteomic methodologies: the differences in extraction procedures, instruments, and data processing lead to limitations in the comparability of results. So that, the measurements of protein concentrations in proteomics are often expressed as abundance ratios rather than absolute concentrations. In our analysis, biomarkers were classified based only on their modulation status, either upregulated or downregulated, acknowledging the challenge of standardizing these measurements across studies. As described in Claudia Rossi et al. in 2022, we used the same methodology to assess the protein expression of each biomarker: the quantitative data (fold change) from each study were standardized by converting down-regulation values to 0.1 and up-regulation values to 10, reflecting the observed trends. IPA tool returns the results by assigning to each pathway “upstream” and “downstream,” a value of $-\log_{10}(\text{p-value})$ and a z-score value. The $-\log_{10}(\text{p-value})$ measures the statistical overlap between the protein dataset and the function

categories; the significance is attributed to $-\log_{10}(\text{p-value})$ greater than 1.3. Instead, the predicted modulation of each “canonical pathways”, “upstream regulators” and “downstream” network effects is inferred by the z-score generated by IPA system (z-scores ≥ 2.0 means that a molecule or pathway is activated, whereas z-scores ≤ -2.0 means the inhibition of target molecules or pathways). First, we focused on well-characterized metabolic and cell signaling pathways defined as “canonical pathways”. We next studied “diseases and functions,” which allows us to describe and predict the effect of molecular changes in our dataset on biological processes and disease or toxicological functions, trying to predict whether these phenomena are activated or inhibited. Regarding the analysis of “upstream regulators”, IPA can identify the cascade of upstream transcriptional regulators that would cause the observed modulations in biomarker expression.

References

- [1] F. Bray *et al.*, «Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries», *CA Cancer J Clin*, vol. 74, fasc. 3, pp. 229–263, 2024, doi: 10.3322/caac.21834.
- [2] M. Ilic e I. Ilic, «Epidemiology of pancreatic cancer», *World J Gastroenterol*, vol. 22, fasc. 44, pp. 9694–9705, nov. 2016, doi: 10.3748/wjg.v22.i44.9694.
- [3] Z. Zhao e W. Liu, «Pancreatic Cancer: A Review of Risk Factors, Diagnosis, and Treatment», *Technol Cancer Res Treat*, vol. 19, p. 1533033820962117, 2020, doi: 10.1177/1533033820962117.
- [4] J. D. Mizrahi, R. Surana, J. W. Valle, e R. T. Shroff, «Pancreatic cancer», *Lancet*, vol. 395, fasc. 10242, pp. 2008–2020, giu. 2020, doi: 10.1016/S0140-6736(20)30974-0.
- [5] C. Liang *et al.*, «Complex roles of the stroma in the intrinsic resistance to gemcitabine in pancreatic cancer: where we are and where we are going», *Exp Mol Med*, vol. 49, fasc. 12, p. e406, dic. 2017, doi: 10.1038/emm.2017.255.
- [6] H. A. Burris *et al.*, «Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial», *J Clin Oncol*, vol. 15, fasc. 6, pp. 2403–2413, giu. 1997, doi: 10.1200/JCO.1997.15.6.2403.
- [7] T. Conroy *et al.*, «FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer», *N Engl J Med*, vol. 364, fasc. 19, pp. 1817–1825, mag. 2011, doi: 10.1056/NEJMoa1011923.
- [8] D. D. Von Hoff *et al.*, «Randomized phase III study of weekly *nab*-paclitaxel plus gemcitabine versus gemcitabine alone in patients with metastatic adenocarcinoma of the pancreas (MPACT).», *JCO*, vol. 31, fasc. 4_suppl, pp. LBA148–LBA148, feb. 2013, doi: 10.1200/jco.2013.31.4_suppl.lba148.

- [9] T. Conroy *et al.*, «FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer», *N Engl J Med*, vol. 379, fasc. 25, pp. 2395–2406, dic. 2018, doi: 10.1056/NEJMoa1809775.
- [10] G. van Duijneveldt, M. D. W. Griffin, e T. L. Putoczki, «Emerging roles for the IL-6 family of cytokines in pancreatic cancer», *Clin Sci (Lond)*, vol. 134, fasc. 16, pp. 2091–2115, ago. 2020, doi: 10.1042/CS20191211.
- [11] B. Dufrusine *et al.*, «BAG3 induces fibroblasts to release key cytokines involved in pancreatic cell migration», *J Cell Biochem*, vol. 123, fasc. 1, pp. 65–76, gen. 2022, doi: 10.1002/jcb.30172.
- [12] C. Liang *et al.*, «Complex roles of the stroma in the intrinsic resistance to gemcitabine in pancreatic cancer: where we are and where we are going», *Exp Mol Med*, vol. 49, fasc. 12, p. e406, dic. 2017, doi: 10.1038/emmm.2017.255.
- [13] V. Damiani *et al.*, «Proteomics Approach Highlights Early Changes in Human Fibroblasts-Pancreatic Ductal Adenocarcinoma Cells Crosstalk», *Cells*, vol. 11, fasc. 7, p. 1160, mar. 2022, doi: 10.3390/cells11071160.
- [14] C. Rossi *et al.*, «Breast cancer in the era of integrating “Omics” approaches», *Oncogenesis*, vol. 11, fasc. 1, p. 17, apr. 2022, doi: 10.1038/s41389-022-00393-8.
- [15] Y. K. Chae e A. M. Gonzalez-Angulo, «Implications of functional proteomics in breast cancer», *Oncologist*, vol. 19, fasc. 4, pp. 328–335, apr. 2014, doi: 10.1634/theoncologist.2013-0437.
- [16] C. A. Sobsey *et al.*, «Targeted and Untargeted Proteomics Approaches in Biomarker Development», *Proteomics*, vol. 20, fasc. 9, p. e1900029, mag. 2020, doi: 10.1002/pmic.201900029.
- [17] S. Kaur *et al.*, «A Combination of MUC5AC and CA19-9 Improves the Diagnosis of Pancreatic Cancer: A Multicenter Study», *Am J Gastroenterol*, vol. 112, fasc. 1, pp. 172–183, gen. 2017, doi: 10.1038/ajg.2016.482.
- [18] Y. Mori *et al.*, «A minimally invasive and simple screening test for detection of pancreatic ductal adenocarcinoma using biomarkers in duodenal juice», *Pancreas*, vol. 42, fasc. 2, pp. 187–192, mar. 2013, doi: 10.1097/MPA.0b013e3182649979.

- [19] S. Pan *et al.*, «Multiplex targeted proteomic assay for biomarker detection in plasma: a pancreatic cancer biomarker case study», *J Proteome Res*, vol. 11, fasc. 3, pp. 1937–1948, mar. 2012, doi: 10.1021/pr201117w.
- [20] X. Wu *et al.*, «A Panel of Three Biomarkers Identified by iTRAQ for the Early Diagnosis of Pancreatic Cancer», *Proteomics Clin Appl*, vol. 13, fasc. 5, p. e1800195, set. 2019, doi: 10.1002/prca.201800195.
- [21] S. Balasenthil *et al.*, «A Plasma Biomarker Panel to Identify Surgically Resectable Early-Stage Pancreatic Cancer», *J Natl Cancer Inst*, vol. 109, fasc. 8, p. djw341, ago. 2017, doi: 10.1093/jnci/djw341.
- [22] Y.-Q. Ren, H.-Y. Zhang, T. Su, X.-H. Wang, e L. Zhang, «Clinical significance of serum survivin in patients with pancreatic ductal adenocarcinoma», *Eur Rev Med Pharmacol Sci*, vol. 18, fasc. 20, pp. 3063–3068, ott. 2014.
- [23] Y.-F. Zhou *et al.*, «Combined detection of serum UL16-binding protein 2 and macrophage inhibitory cytokine-1 improves early diagnosis and prognostic prediction of pancreatic cancer», *Oncol Lett*, vol. 8, fasc. 5, pp. 2096–2102, nov. 2014, doi: 10.3892/ol.2014.2429.
- [24] J. Kim *et al.*, «Detection of early pancreatic ductal adenocarcinoma with thrombospondin-2 and CA19-9 blood markers», *Sci Transl Med*, vol. 9, fasc. 398, p. eaah5583, lug. 2017, doi: 10.1126/scitranslmed.aah5583.
- [25] J. Park *et al.*, «Diagnostic performance enhancement of pancreatic cancer using proteomic multimarker panel», *Oncotarget*, vol. 8, fasc. 54, pp. 93117–93130, nov. 2017, doi: 10.18632/oncotarget.21861.
- [26] K. Q. McKinney *et al.*, «Discovery of putative pancreatic cancer biomarkers using subcellular proteomics», *J Proteomics*, vol. 74, fasc. 1, pp. 79–88, gen. 2011, doi: 10.1016/j.jprot.2010.08.006.
- [27] X. Guo *et al.*, «Dysbindin as a novel biomarker for pancreatic ductal adenocarcinoma identified by proteomic profiling», *Int J Cancer*, vol. 139, fasc. 8, pp. 1821–1829, ott. 2016, doi: 10.1002/ijc.30227.
- [28] R. Chen *et al.*, «Elevated level of anterior gradient-2 in pancreatic juice from patients with pre-malignant pancreatic neoplasia», *Mol Cancer*, vol. 9, p. 149, giu. 2010, doi: 10.1186/1476-4598-9-149.

- [29] C. Sorio *et al.*, «Elevated urinary levels of urokinase-type plasminogen activator receptor (uPAR) in pancreatic ductal adenocarcinoma identify a clinically high-risk group», *BMC Cancer*, vol. 11, p. 448, ott. 2011, doi: 10.1186/1471-2407-11-448.
- [30] C. Jenkinson *et al.*, «Evaluation in pre-diagnosis samples discounts ICAM-1 and TIMP-1 as biomarkers for earlier diagnosis of pancreatic cancer», *J Proteomics*, vol. 113, pp. 400–402, gen. 2015, doi: 10.1016/j.jprot.2014.10.001.
- [31] A. A. Mohamed *et al.*, «Evaluation of circulating ADH and MIC-1 as diagnostic markers in Egyptian patients with pancreatic cancer», *Pancreatology*, vol. 15, fasc. 1, pp. 34–39, 2015, doi: 10.1016/j.pan.2014.10.008.
- [32] K. Sogawa *et al.*, «Identification of a novel serum biomarker for pancreatic cancer, C4b-binding protein α -chain (C4BPA) by quantitative proteomic analysis using tandem mass tags», *Br J Cancer*, vol. 115, fasc. 8, pp. 949–956, ott. 2016, doi: 10.1038/bjc.2016.295.
- [33] M. J. Lee *et al.*, «Identification of human complement factor B as a novel biomarker candidate for pancreatic ductal adenocarcinoma», *J Proteome Res*, vol. 13, fasc. 11, pp. 4878–4888, nov. 2014, doi: 10.1021/pr5002719.
- [34] T. Yoneyama *et al.*, «Identification of IGFBP2 and IGFBP3 As Compensatory Biomarkers for CA19-9 in Early-Stage Pancreatic Cancer Using a Combination of Antibody-Based and LC-MS/MS-Based Proteomics», *PLoS One*, vol. 11, fasc. 8, p. e0161009, 2016, doi: 10.1371/journal.pone.0161009.
- [35] S. Makawita *et al.*, «Integrated proteomic profiling of cell line conditioned media and pancreatic juice for the identification of pancreatic cancer biomarkers», *Mol Cell Proteomics*, vol. 10, fasc. 10, p. M111.008599, ott. 2011, doi: 10.1074/mcp.M111.008599.
- [36] L. Tao, L. Zhong, Y. Li, D. Li, D. Xiu, e J. Zhou, «Integrated proteomics and phosphoproteomics reveal perturbed regulative pathways in pancreatic ductal adenocarcinoma», *Mol Omics*, vol. 17, fasc. 2, pp. 230–240, apr. 2021, doi: 10.1039/d0mo00125b.

- [37] J. Park *et al.*, «Large-scale clinical validation of biomarkers for pancreatic cancer using a mass spectrometry-based proteomics approach», *Oncotarget*, vol. 8, fasc. 26, pp. 42761–42771, giu. 2017, doi: 10.18632/oncotarget.17463.
- [38] X. Wu *et al.*, «Overcoming chemo/radio-resistance of pancreatic cancer by inhibiting STAT3 signaling», *Oncotarget*, vol. 7, fasc. 10, pp. 11708–11723, mar. 2016, doi: 10.18632/oncotarget.7336.
- [39] C. Ren, Y. Chen, C. Han, D. Fu, e H. Chen, «Plasma interleukin-11 (IL-11) levels have diagnostic and prognostic roles in patients with pancreatic cancer», *Tumour Biol*, vol. 35, fasc. 11, pp. 11467–11472, nov. 2014, doi: 10.1007/s13277-014-2459-y.
- [40] O. Deutsch *et al.*, «Possible proteomic biomarkers for the detection of pancreatic cancer in oral fluids», *Sci Rep*, vol. 10, fasc. 1, p. 21995, dic. 2020, doi: 10.1038/s41598-020-78922-x.
- [41] S. Kaur *et al.*, «Potentials of plasma NGAL and MIC-1 as biomarker(s) in the diagnosis of lethal pancreatic cancer», *PLoS One*, vol. 8, fasc. 2, p. e55171, 2013, doi: 10.1371/journal.pone.0055171.
- [42] M. Ger *et al.*, «Proteomic Identification of FLT3 and PCBP3 as Potential Prognostic Biomarkers for Pancreatic Cancer», *Anticancer Res*, vol. 38, fasc. 10, pp. 5759–5765, ott. 2018, doi: 10.21873/anticancer.12914.
- [43] Y. Rong *et al.*, «Proteomics analysis of serum protein profiling in pancreatic cancer patients by DIGE: up-regulation of mannose-binding lectin 2 and myosin light chain kinase 2», *BMC Gastroenterol*, vol. 10, p. 68, giu. 2010, doi: 10.1186/1471-230X-10-68.
- [44] N. Sanh *et al.*, «Proteomics Profiling of Pancreatic Cancer and Pancreatitis for Biomarkers Discovery», *J Cell Sci Ther*, vol. 9, fasc. 4, p. 287, 2018, doi: 10.4172/2157-7013.1000287.
- [45] T. Yoneyama *et al.*, «Quantitative targeted absolute proteomics-based large-scale quantification of proline-hydroxylated α -fibrinogen in plasma for pancreatic cancer diagnosis», *J Proteome Res*, vol. 12, fasc. 2, pp. 753–762, feb. 2013, doi: 10.1021/pr3008144.

- [46] J. Matsubara *et al.*, «Reduced plasma level of CXC chemokine ligand 7 in patients with pancreatic cancer», *Cancer Epidemiol Biomarkers Prev*, vol. 20, fasc. 1, pp. 160–171, gen. 2011, doi: 10.1158/1055-9965.EPI-10-0397.
- [47] Y. Wang *et al.*, «Screening for serological biomarkers of pancreatic cancer by two-dimensional electrophoresis and liquid chromatography-tandem mass spectrometry», *Oncol Rep*, vol. 26, fasc. 1, pp. 287–292, lug. 2011, doi: 10.3892/or.2011.1278.
- [48] W. Błogowski *et al.*, «Selected cytokines in patients with pancreatic cancer: a preliminary report», *PLoS One*, vol. 9, fasc. 5, p. e97613, 2014, doi: 10.1371/journal.pone.0097613.
- [49] V. E. Shaw *et al.*, «Serum cytokine biomarker panels for discriminating pancreatic cancer from benign pancreatic disease», *Mol Cancer*, vol. 13, p. 114, mag. 2014, doi: 10.1186/1476-4598-13-114.
- [50] C. Torres *et al.*, «Serum cytokine profile in patients with pancreatic cancer», *Pancreas*, vol. 43, fasc. 7, pp. 1042–1049, ott. 2014, doi: 10.1097/MPA.000000000000155.
- [51] S. K. Dutta *et al.*, «Serum HSP70: a novel biomarker for early detection of pancreatic cancer», *Pancreas*, vol. 41, fasc. 4, pp. 530–534, mag. 2012, doi: 10.1097/MPA.0b013e3182374ace.
- [52] K. E. Poruk *et al.*, «Serum osteopontin and tissue inhibitor of metalloproteinase 1 as diagnostic and prognostic biomarkers for pancreatic adenocarcinoma», *Pancreas*, vol. 42, fasc. 2, pp. 193–197, mar. 2013, doi: 10.1097/MPA.0b013e31825e354d.
- [53] K. Felix *et al.*, «Serum protein signatures differentiating autoimmune pancreatitis versus pancreatic cancer», *PLoS One*, vol. 8, fasc. 12, p. e82755, 2013, doi: 10.1371/journal.pone.0082755.
- [54] S. Makawita *et al.*, «Validation of four candidate pancreatic cancer serological biomarkers that improve the performance of CA19.9», *BMC Cancer*, vol. 13, p. 404, set. 2013, doi: 10.1186/1471-2407-13-404.
- [55] J. M. Lehmann, X. K. Zhang, e M. Pfahl, «RAR gamma 2 expression is regulated through a retinoic acid response element embedded in Sp1 sites», *Mol Cell Biol*, vol. 12, fasc. 7, pp. 2976–2985, lug. 1992, doi: 10.1128/mcb.12.7.2976-2985.1992.

- [56] B. Blumberg, D. J. Mangelsdorf, J. A. Dyck, D. A. Bittner, R. M. Evans, e E. M. De Robertis, «Multiple retinoid-responsive receptors in a single cell: families of retinoid “X” receptors and retinoic acid receptors in the *Xenopus* egg», *Proc Natl Acad Sci U S A*, vol. 89, fasc. 6, pp. 2321–2325, mar. 1992, doi: 10.1073/pnas.89.6.2321.
- [57] C. Rochette-Egly, M. P. Gaub, Y. Lutz, S. Ali, I. Scheuer, e P. Chambon, «Retinoic acid receptor-beta: immunodetection and phosphorylation on tyrosine residues», *Mol Endocrinol*, vol. 6, fasc. 12, pp. 2197–2209, dic. 1992, doi: 10.1210/mend.6.12.1283441.
- [58] M. Karin, Z. g Liu, e E. Zandi, «AP-1 function and regulation», *Curr Opin Cell Biol*, vol. 9, fasc. 2, pp. 240–246, apr. 1997, doi: 10.1016/s0955-0674(97)80068-3.
- [59] T. Liu, L. Zhang, D. Joo, e S.-C. Sun, «NF-κB signaling in inflammation», *Sig Transduct Target Ther*, vol. 2, fasc. 1, p. 17023, lug. 2017, doi: 10.1038/sigtrans.2017.23.
- [60] A. Oeckinghaus e S. Ghosh, «The NF-kappaB family of transcription factors and its regulation», *Cold Spring Harb Perspect Biol*, vol. 1, fasc. 4, p. a000034, ott. 2009, doi: 10.1101/cshperspect.a000034.
- [61] J. Yu *et al.*, «CDX2 inhibits the proliferation and tumor formation of colon cancer cells by suppressing Wnt/β-catenin signaling via transactivation of GSK-3β and Axin2 expression», *Cell Death Dis*, vol. 10, fasc. 1, p. 26, gen. 2019, doi: 10.1038/s41419-018-1263-9.
- [62] M. Coskun, J. T. Troelsen, e O. H. Nielsen, «The role of CDX2 in intestinal homeostasis and inflammation», *Biochim Biophys Acta*, vol. 1812, fasc. 3, pp. 283–289, mar. 2011, doi: 10.1016/j.bbadis.2010.11.008.
- [63] J. Zhou, «Norepinephrine transporter inhibitors and their therapeutic potential», *Drugs Future*, vol. 29, fasc. 12, pp. 1235–1244, dic. 2004, doi: 10.1358/dof.2004.029.12.855246.
- [64] J. M. Platnich e D. A. Muruve, «NOD-like receptors and inflammasomes: A review of their canonical and non-canonical signaling pathways», *Arch Biochem Biophys*, vol. 670, pp. 4–14, lug. 2019, doi: 10.1016/j.abb.2019.02.008.

- [65] T. Kawai e S. Akira, «Innate immune recognition of viral infection», *Nat Immunol*, vol. 7, fasc. 2, pp. 131–137, feb. 2006, doi: 10.1038/ni1303.
- [66] L. O. Moreira e D. S. Zamboni, «NOD1 and NOD2 Signaling in Infection and Inflammation», *Front Immunol*, vol. 3, p. 328, 2012, doi: 10.3389/fimmu.2012.00328.
- [67] I. Morianos, G. Papadopoulou, M. Semitekolou, e G. Xanthou, «Activin-A in the regulation of immunity in health and disease», *J Autoimmun*, vol. 104, p. 102314, nov. 2019, doi: 10.1016/j.jaut.2019.102314.
- [68] E. Yeom e K. Yu, «Understanding the molecular basis of anorexia and tissue wasting in cancer cachexia», *Exp Mol Med*, vol. 54, fasc. 4, pp. 426–432, apr. 2022, doi: 10.1038/s12276-022-00752-w.
- [69] H. J. Patel e B. M. Patel, «TNF- α and cancer cachexia: Molecular insights and clinical implications», *Life Sci*, vol. 170, pp. 56–63, feb. 2017, doi: 10.1016/j.lfs.2016.11.033.
- [70] S. Yamada e I. Maruyama, «HMGB1, a novel inflammatory cytokine», *Clin Chim Acta*, vol. 375, fasc. 1–2, pp. 36–42, gen. 2007, doi: 10.1016/j.cca.2006.07.019.
- [71] E. Latz *et al.*, «Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction», *J Biol Chem*, vol. 277, fasc. 49, pp. 47834–47843, dic. 2002, doi: 10.1074/jbc.M207873200.
- [72] A. Bouchon, J. Dietrich, e M. Colonna, «Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes», *J Immunol*, vol. 164, fasc. 10, pp. 4991–4995, mag. 2000, doi: 10.4049/jimmunol.164.10.4991.
- [73] F. Y. Liew, J.-P. Girard, e H. R. Turnquist, «Interleukin-33 in health and disease», *Nat Rev Immunol*, vol. 16, fasc. 11, pp. 676–689, nov. 2016, doi: 10.1038/nri.2016.95.
- [74] S. M. Pinto *et al.*, «A network map of IL-33 signaling pathway», *J Cell Commun Signal*, vol. 12, fasc. 3, pp. 615–624, set. 2018, doi: 10.1007/s12079-018-0464-4.

- [75] M. T. Heneka, M. P. Kummer, e E. Latz, «Innate immune activation in neurodegenerative disease», *Nat Rev Immunol*, vol. 14, fasc. 7, pp. 463–477, lug. 2014, doi: 10.1038/nri3705.
- [76] J. Thundiyil e K.-L. Lim, «DAMPs and neurodegeneration», *Ageing Res Rev*, vol. 24, fasc. Pt A, pp. 17–28, nov. 2015, doi: 10.1016/j.arr.2014.11.003.
- [77] A. H. Miller, V. Maletic, e C. L. Raison, «Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression», *Biol Psychiatry*, vol. 65, fasc. 9, pp. 732–741, mag. 2009, doi: 10.1016/j.biopsych.2008.11.029.
- [78] Y. Fu, S. Liu, S. Zeng, e H. Shen, «From bench to bed: the tumor immune microenvironment and current immunotherapeutic strategies for hepatocellular carcinoma», *J Exp Clin Cancer Res*, vol. 38, fasc. 1, p. 396, set. 2019, doi: 10.1186/s13046-019-1396-4.
- [79] X. Yin *et al.*, «Lipid metabolism in pancreatic cancer: emerging roles and potential targets», *Cancer Commun (Lond)*, vol. 42, fasc. 12, pp. 1234–1256, dic. 2022, doi: 10.1002/cac2.12360.
- [80] G. Pascual *et al.*, «Targeting metastasis-initiating cells through the fatty acid receptor CD36», *Nature*, vol. 541, fasc. 7635, pp. 41–45, gen. 2017, doi: 10.1038/nature20791.
- [81] S. Jia, L. Zhou, T. Shen, S. Zhou, G. Ding, e L. Cao, «Down-expression of CD36 in pancreatic adenocarcinoma and its correlation with clinicopathological features and prognosis», *J Cancer*, vol. 9, fasc. 3, pp. 578–583, 2018, doi: 10.7150/jca.21046.
- [82] C. Xia, Z. Braunstein, A. C. Toomey, J. Zhong, e X. Rao, «S100 Proteins As an Important Regulator of Macrophage Inflammation», *Front Immunol*, vol. 8, p. 1908, 2017, doi: 10.3389/fimmu.2017.01908.
- [83] L. L. Gonzalez, K. Garrie, e M. D. Turner, «Role of S100 proteins in health and disease», *Biochim Biophys Acta Mol Cell Res*, vol. 1867, fasc. 6, p. 118677, giu. 2020, doi: 10.1016/j.bbamcr.2020.118677.
- [84] D. Liu *et al.*, «Nonenzymatic glycation of high-density lipoprotein impairs its anti-inflammatory effects in innate immunity», *Diabetes Metab Res Rev*, vol. 28, fasc. 2, pp. 186–195, feb. 2012, doi: 10.1002/dmrr.1297.

- [85] S. B. A. H. A. Fluitman, D. A. J. P. Denys, C. J. Heijnen, e H. G. M. Westenberg, «Disgust affects TNF-alpha, IL-6 and noradrenalin levels in patients with obsessive-compulsive disorder», *Psychoneuroendocrinology*, vol. 35, fasc. 6, pp. 906–911, lug. 2010, doi: 10.1016/j.psyneuen.2009.12.005.
- [86] X. Feng *et al.*, «Lipopolysaccharide up-regulates the expression of Fcalpha/mu receptor and promotes the binding of oxidized low-density lipoprotein and its IgM antibody complex to activated human macrophages», *Atherosclerosis*, vol. 208, fasc. 2, pp. 396–405, feb. 2010, doi: 10.1016/j.atherosclerosis.2009.07.035.
- [87] C. Klett, W. Hellmann, D. Ganten, e E. Hackenthal, «Tissue distribution of angiotensinogen mRNA during experimental inflammation», *Inflammation*, vol. 17, fasc. 2, pp. 183–197, apr. 1993, doi: 10.1007/BF00916104.
- [88] H. Yin *et al.*, «Gut-derived lipopolysaccharide remodels tumoral microenvironment and synergizes with PD-L1 checkpoint blockade via TLR4/MyD88/AKT/NF-κB pathway in pancreatic cancer», *Cell Death Dis*, vol. 12, fasc. 11, p. 1033, ott. 2021, doi: 10.1038/s41419-021-04293-4.
- [89] C. E. McCoy, S. Carpenter, E. M. Pålsson-McDermott, L. J. Gearing, e L. A. J. O'Neill, «Glucocorticoids inhibit IRF3 phosphorylation in response to Toll-like receptor-3 and -4 by targeting TBK1 activation», *J Biol Chem*, vol. 283, fasc. 21, pp. 14277–14285, mag. 2008, doi: 10.1074/jbc.M709731200.
- [90] H. Nakashima *et al.*, «Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer», *Cancer Res*, vol. 66, fasc. 14, pp. 7041–7049, lug. 2006, doi: 10.1158/0008-5472.CAN-05-4588.
- [91] J. M. Kyriakis e J. Avruch, «Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation», *Physiol Rev*, vol. 81, fasc. 2, pp. 807–869, apr. 2001, doi: 10.1152/physrev.2001.81.2.807.
- [92] X. Chen *et al.*, «Interaction between ferroptosis and TNF-α: Impact in obesity-related osteoporosis», *FASEB J*, vol. 37, fasc. 6, p. e22947, giu. 2023, doi: 10.1096/fj.202201958R.

- [93] F. P. Marchese, A. Aubareda, C. Tudor, J. Saklatvala, A. R. Clark, e J. L. E. Dean, «MAPKAP kinase 2 blocks tristetraprolin-directed mRNA decay by inhibiting CAF1 deadenylase recruitment», *J Biol Chem*, vol. 285, fasc. 36, pp. 27590–27600, set. 2010, doi: 10.1074/jbc.M110.136473.
- [94] Y. Ramiro-Cortés, A. Guemez-Gamboa, e J. Morán, «Reactive oxygen species participate in the p38-mediated apoptosis induced by potassium deprivation and staurosporine in cerebellar granule neurons», *Int J Biochem Cell Biol*, vol. 43, fasc. 9, pp. 1373–1382, set. 2011, doi: 10.1016/j.biocel.2011.06.001.
- [95] T. Mateo *et al.*, «A critical role for TNFalpha in the selective attachment of mononuclear leukocytes to angiotensin-II-stimulated arterioles», *Blood*, vol. 110, fasc. 6, pp. 1895–1902, set. 2007, doi: 10.1182/blood-2007-01-070607.
- [96] V. Lilla *et al.*, «Differential gene expression in well-regulated and dysregulated pancreatic beta-cell (MIN6) sublines», *Endocrinology*, vol. 144, fasc. 4, pp. 1368–1379, apr. 2003, doi: 10.1210/en.2002-220916.
- [97] A. M. Y. Shum e P. Polly, «Cancer cachexia: molecular targets and pathways for diagnosis and drug intervention», *Endocr Metab Immune Disord Drug Targets*, vol. 12, fasc. 3, pp. 247–259, set. 2012, doi: 10.2174/187153012802002910.
- [98] S. Rosewicz, F. Brembeck, A. Kaiser, Z. V. Marschall, e E. O. Riecken, «Differential growth regulation by all-trans retinoic acid is determined by protein kinase C alpha in human pancreatic carcinoma cells», *Endocrinology*, vol. 137, fasc. 8, pp. 3340–3347, ago. 1996, doi: 10.1210/endo.137.8.8754760.
- [99] C. Infante-Duarte, H. F. Horton, M. C. Byrne, e T. Kamradt, «Microbial lipopeptides induce the production of IL-17 in Th cells», *J Immunol*, vol. 165, fasc. 11, pp. 6107–6115, dic. 2000, doi: 10.4049/jimmunol.165.11.6107.
- [100] E. Shankar *et al.*, «A Signaling Network Controlling Androgenic Repression of c-Fos Protein in Prostate Adenocarcinoma Cells», *J Biol Chem*, vol. 291, fasc. 11, pp. 5512–5526, mar. 2016, doi: 10.1074/jbc.M115.694877.
- [101] M. Garofalo e C. M. Croce, «microRNAs: Master regulators as potential therapeutics in cancer», *Annu Rev Pharmacol Toxicol*, vol. 51, pp. 25–43, 2011, doi: 10.1146/annurev-pharmtox-010510-100517.

- [102] D. Lodygin *et al.*, «Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer», *Cell Cycle*, vol. 7, fasc. 16, pp. 2591–2600, ago. 2008, doi: 10.4161/cc.7.16.6533.
- [103] Q. Ji *et al.*, «MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells», *PLoS One*, vol. 4, fasc. 8, p. e6816, ago. 2009, doi: 10.1371/journal.pone.0006816.
- [104] L. Chen, X. Yao, H. Yao, Q. Ji, G. Ding, e X. Liu, «Exosomal miR-103-3p from LPS-activated THP-1 macrophage contributes to the activation of hepatic stellate cells», *FASEB J*, vol. 34, fasc. 4, pp. 5178–5192, apr. 2020, doi: 10.1096/fj.201902307RRR.
- [105] S. Volinia *et al.*, «Breast cancer signatures for invasiveness and prognosis defined by deep sequencing of microRNA», *Proc Natl Acad Sci U S A*, vol. 109, fasc. 8, pp. 3024–3029, feb. 2012, doi: 10.1073/pnas.1200010109.

CHAPTER 3

STUDY 2

From BAG3insKO mouse model generation to proteomic analyses

Pancreatic beta-cell specific BAG3 knockout results in chronic hyperinsulinemia inducing insulin resistance

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The role of BAG3 in insulin secretion and systemic metabolic dysregulation: evidence from beta-cell-specific knockout mouse models

Insulin, secreted by pancreatic beta cells, is a critical hormone in the regulation of glucose metabolism. The release and action of insulin must be finely tuned to meet metabolic demands. Defective insulin secretion by pancreatic β -cells and/or impaired insulin responsiveness in peripheral tissues leads to insulin resistance, ultimately contributing to the development of type 2 diabetes (T2D) (1), (2), (3), (4). Understanding the proteins involved in insulin release and elucidating their mechanisms of action are essential for uncovering the pathogenesis of various metabolic disorders, including congenital hyperinsulinism, metabolic syndrome, T2D, nonalcoholic fatty liver disease (NAFLD), cardiovascular disease, and chronic kidney disease (CKD) (5), (6), (7). BCL2-associated athanogene 3 (BAG3) is a co-chaperone protein that interacts with heat shock protein 70 (HSP70). BAG3 is involved in diverse cellular processes, including development, cytoskeleton organization, apoptosis, and autophagy. Although constitutively expressed in a limited number of tissues, BAG3 is upregulated in various cell types in response to stress stimuli (8), (9), (10), (11). Our previous studies have demonstrated that BAG3 is constitutively expressed in pancreatic beta cells and regulates insulin secretion as a component of the t-SNARE complex, which mediates the trafficking of insulin-containing granules (12). Silencing BAG3 in β -TC-6 mouse insulinoma cells results in reduced intracellular insulin content, likely due to impaired granule retention, accompanied by increased insulin secretion in response to glucose stimulation (12). Furthermore, Youn et al. reported that systemic BAG3 knockout (BAG3KO) mice, which die of severe degeneration of diaphragm, intercostal muscles, cardiac tissue, and skeletal muscles within four weeks of birth, exhibit hypoglycemia and fatty liver. Analysis of hepatic mRNA in these mice revealed alterations in gluconeogenesis, β -oxidation, and lipogenesis, suggesting an adaptive response to malnutrition, consistent with metabolite profiles and reduced peripheral fat depots (13).

In this study, we generated a beta-cell-specific BAG3 knockout mouse model (BAG3 β KO) using Cre-mediated recombination under the control of the insulin 1 promoter. BAG3 β KO mice developed normally, exhibited normal lifespans, and maintained good glucose tolerance but displayed impaired insulin sensitivity. These

mice secreted higher levels of insulin following glucose stimulation and became overtly insulin-resistant by 64 weeks of age. Additionally, BAG3 β KO mice demonstrated histopathological features of CKD and glycogen and lipid accumulation in the liver. Taken together, our findings confirm that BAG3 is a key regulator of insulin secretion. Beta-cell-specific deletion of BAG3 results in hyperinsulinemia, which progressively leads to insulin resistance and a complex metabolic disorder characterized by multiorgan dysfunction.

Results

Generation of beta-cell specific BAG3 knockout mice

Our previous findings demonstrated that silencing BAG3 in insulinoma cells leads to altered insulin secretion. To investigate the role of BAG3 in glucose homeostasis in vivo, we generated a pancreatic beta-cell-specific BAG3 knockout (BAG3 β KO) mouse model. This was achieved using INS1cre mice, which express Cre recombinase under the control of the endogenous Insulin 1 promoter. Unlike the Insulin 2 promoter, the Insulin 1 promoter drives beta-cell-specific expression (14). INS1cre homozygous mice were crossed with BAG3^{fl/fl} mice to produce an F1 heterozygous generation (BAG3^{fl/-}: INS1cre^{+/-}). These mice were then inbred to obtain the F2 generation with the following genotypes: BAG3^{fl/-}: INS1cre^{-/-} and BAG3^{-/-}: INS1cre^{+/-} (Control) and BAG3^{fl/-}: INS1cre^{+/-} and BAG3^{fl/-}: INS1cre^{+/-} (BAG3 β KO), as depicted in Figure 1A. In BAG3 β KO mice, Cre recombinase expression facilitated the deletion of BAG3 floxed exons 2 and 3 (Figure 1B). Genotyping of F1 and F2 generations, performed using genomic DNA isolated from ear punches, confirmed the presence of both floxed and Cre alleles in BAG3 β KO mice (Figure 1C). The absence of BAG3 expression in BAG3 β KO mice was validated through immunofluorescence staining in islets of Langerhans (Figure 1D) and Western blot analysis of isolated islets (Figure 1E). Western blot analysis of other tissues revealed that BAG3 expression remained comparable to control mice (Figure 1F). Importantly, while systemic BAG3 knockout results in death within four weeks due to severe tissue degeneration (13), BAG3 β KO mice exhibited normal fertility, litter size, and lifespan, with no observable macroscopic phenotypic abnormalities (data not shown). Body weight remained consistent between BAG3 β KO and control mice over time (Figure 1G). To assess glucose homeostasis, we

measured blood glucose levels under fed and fasting conditions. No significant differences were observed in blood glucose levels between fed BAG3 β KO and control mice (Figure 1H). However, after 16 hours of fasting, BAG3 β KO mice exhibited significantly lower blood glucose levels compared to controls, as demonstrated by the area under the curve (AUC) analysis (Figure 1I). This reduction in fasting glycemia was particularly evident within the first 12 weeks after birth.

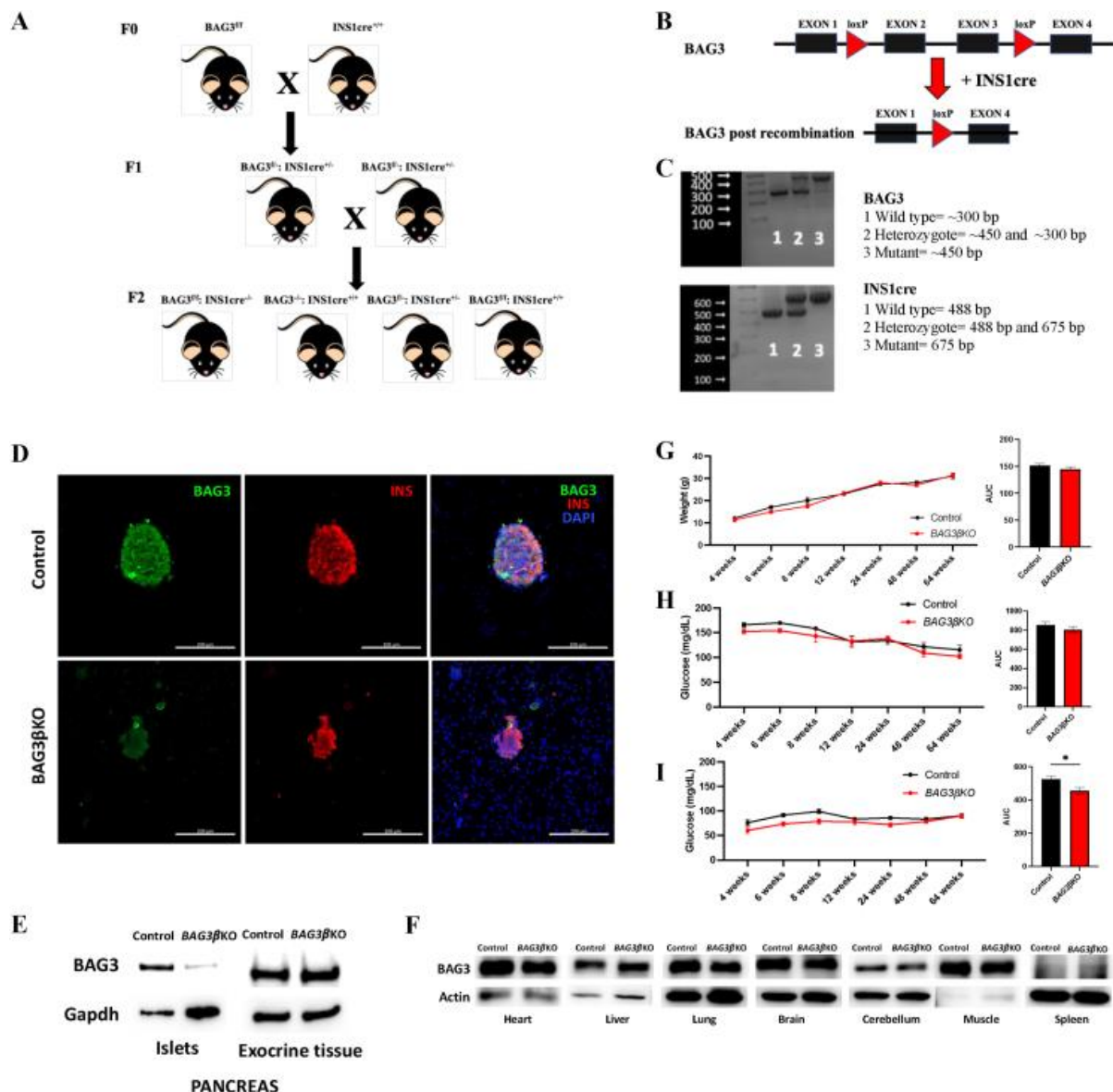


Figure 1: Generation and validation of BAG3 β KO mouse model. (A) Breeding scheme for generating beta-cell specific BAG3 knockout mice. Only the 4 genotypes of interest are shown in the scheme i) BAG3^{fl}; INS1cre^{+/-} and BAG3^{-/-}; INS1cre^{+/-} (Control), ii) BAG3^{fl}; INS1cre^{+/-} and iii) BAG3^{fl}; INS1cre^{+/-} (BAG3 β KO). (B) Schematic image of floxed BAG3 allele and the deletion product after INS1cre recombination; for simplicity, only one allele has been shown. (C) PCR genotyping

results of BAG3 floxed mice and INS1cre recombinase mice from DNA obtained from ear punch samples: 1-Wild type (BAG3^{-/-} and INS1^{cre/-/-}); 2-heterozygote (BAG3^{f/f} and Ins1^{cre+/-}) and 3-Mutant (BAG3^{f/f} and INS1^{cre+/+}). (D) Representative immunofluorescence images of BAG3 and insulin staining in control and BAG3 β KO mice isolated islets. Scale bar: 200 μ m. (E) Western blot analysis of pancreatic islets and exocrine tissue to assess BAG3 specific knockout, while (F) no Cre recombination was found in the heart, liver, lung, brain, cerebellum, muscle, and spleen of BAG3 β KO mice. (G) The growth curve of the body weight and area under the curve (AUC) monitored over time. (H) Fed and (I) fasting blood glucose levels monitored over time and their AUC. Data are presented as mean $\pm$ SEM (n = 6–7 for each group), *p < 0.05.

BAG3 β KO mice show impaired insulin sensitivity and develop insulin resistance

We conducted intraperitoneal glucose tolerance tests (IPGTT) and intraperitoneal insulin tolerance tests (IPITT) in BAG3 β KO mice and control groups at 12, 24, and 64 weeks of age to assess glucose metabolism and insulin sensitivity. The IPGTT results revealed no significant differences between BAG3 β KO and control mice at any of the examined time points (Figure 2A, C, and E). However, IPITT results, which assess the glucose-lowering response to intraperitoneal insulin injection (0.75 U/kg) following 4 hours of fasting, demonstrated notable differences. At 12 weeks of age, BAG3 β KO mice displayed a response similar to that of control mice (Figure 2B). By 24 and 64 weeks, however, BAG3 β KO mice exhibited a significantly attenuated decrease in blood glucose levels compared to controls, indicative of impaired insulin sensitivity (Figure 2D and F). To further investigate, we measured basal fasting insulin levels in BAG3 β KO mice at 12, 24, and 64 weeks. At 12 and 24 weeks, basal insulin levels showed a modest, non-significant increase in BAG3 β KO mice compared to controls (Figure 2G and K). Correspondingly, fasting blood glucose levels and the homeostasis model assessment for insulin resistance (HOMA-IR) index remained comparable between the two groups at these time points (Figures 2H, L, I, and M). At 64 weeks, however, BAG3 β KO mice exhibited significantly elevated fasting insulin levels (Figure 2O) despite similar fasting blood glucose levels (Figure 2P). This resulted in a significantly increased HOMA-IR index in BAG3 β KO mice at this stage (Figure 2Q), indicative of overt insulin resistance. Plasma glucagon concentrations in fasted BAG3 β KO mice were consistent with those of control mice across all time points (Figures 2J, N, and R). Importantly, upon glucose stimulation, BAG3 β KO mice secreted significantly higher levels of insulin compared to controls at all examined ages (Figures 2G, K, and O). Collectively, these findings suggest that BAG3 β KO mice maintain insulin sensitivity up to 12 weeks of age

but develop progressive insulin resistance, becoming overtly insulin-resistant by 64 weeks. This progression is substantiated by elevated fasting insulin levels, increased HOMA-IR, and impaired insulin response in IPITT results.

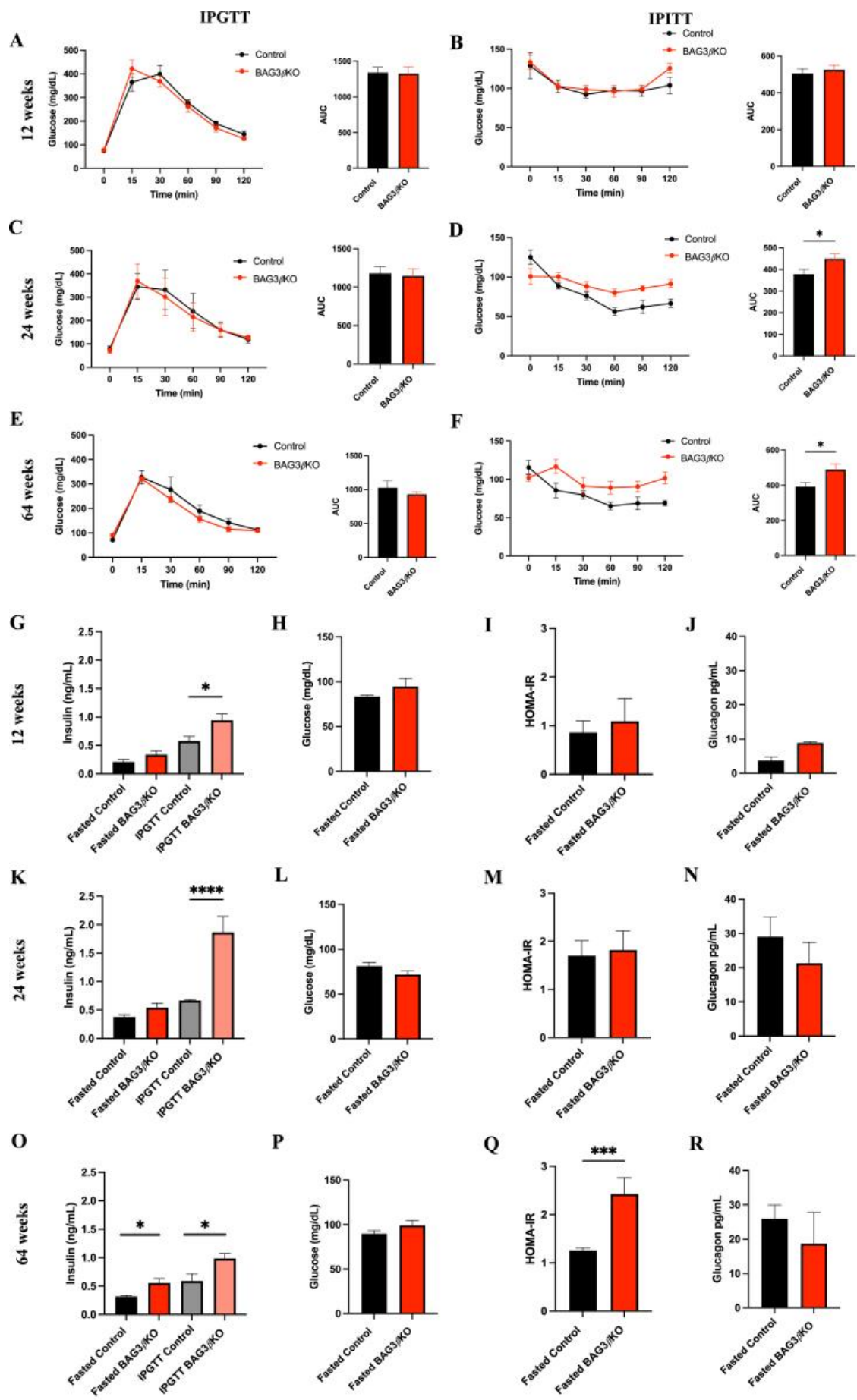


Figure 2: Insulin sensitivity impairment in BAG3 β KO mice leads to insulin resistance. IPGTT (A, C, E) and IPITT (B, D, F) in control vs BAG3 β KO mice on 12 (A, B), 24 (C, D), 64 (E, F) weeks of age, and the respective AUCs. IPGTTs were performed after 16 h of fasting (overnight) and IPITTs after 4 h of fasting. Glucose (2 mg/kg) and insulin (0.75 units/kg) were injected intraperitoneally, and glycemia was measured at different times after injection (n = 4–10 for each group, data are presented as mean values $\pm$ SEM; *p < 0.05.). Circulating insulin (G, K and O) was measured after 16 h of fasting and after a 15-minute glucose challenge (2 g/kg) in control vs BAG3 β KO mice at 12 (G) 24 (K) and 64 (O) weeks of age. Fasting blood glucose levels at 12 (H), 24 (L) and 64 weeks (P). HOMA-IR after 12 (I), 24 (M) and 64 weeks (Q). Fasting glucagon levels at 12 (J), 24 (N), and 64 weeks (R). Data are presented as mean $\pm$ SEM (n = 7–9 for each group); *p < 0.05; **p < 0.01; ***p < 0.0001.

Increased insulin secretion is associated with changes in pancreatic islets morphology

Pancreatic islets adapt to insulin resistance primarily through β -cell hyperplasia and hypertrophy (15). Histological analysis of pancreatic tissue in BAG3 β KO and control mice (Figure 3A, F, and I) revealed that while the number of endocrine islets remained stable across all ages in both groups (Figure 3B), islet size varied significantly with age in BAG3 β KO mice. At 12 weeks of age, the mean islet area in BAG3 β KO mice was smaller, potentially reflecting a developmental deficit resulting from the constitutive deletion of BAG3 in β -cells. However, at later time points, islets appeared to recover normal size and morphology, with islet size more than doubling by 64 weeks of age, likely as an adaptive response to insulin resistance (Figure 3C). A similar trend was observed in the total cell count per islet (Figure 3D). A more detailed analysis of islet dimensions over time revealed an increase in the proportion of larger islets and a corresponding decrease in the number of smaller islets in BAG3 β KO mice. We further performed immunofluorescence staining to identify insulin-secreting β -cells, glucagon-secreting α -cells, and somatostatin-secreting δ -cells. The islet architecture appeared similar in both groups, with β -cells forming the core and α - and δ -cells populating the periphery. In BAG3 β KO mice, the normal cell distribution was largely preserved, although in some islets, rare α - and δ -cells were interspersed with β -cells, resembling the histological features seen in the pancreatic islets of obese and diabetic mice. Additionally, the relative proportions of β -cells and α -cells within the islets varied between BAG3 β KO and control mice (Figure 3E, G). To quantify these differences, we performed image analysis of pancreatic sections stained for insulin and glucagon and plotted the number of positive cells against

the total number of islet cells. The percentage of insulin- and glucagon-producing cells remained constant over time in both groups, with control mice displaying a significantly higher percentage of insulin-producing cells (Figure 3F) and a lower percentage of glucagon-producing cells (Figure 3H) compared to BAG3 β KO mice. Finally, the insulin content of isolated islets appeared reduced in BAG3 β KO mice. Although this result is semi-quantitative, it aligns with previous observations made in the insulinoma model (12).

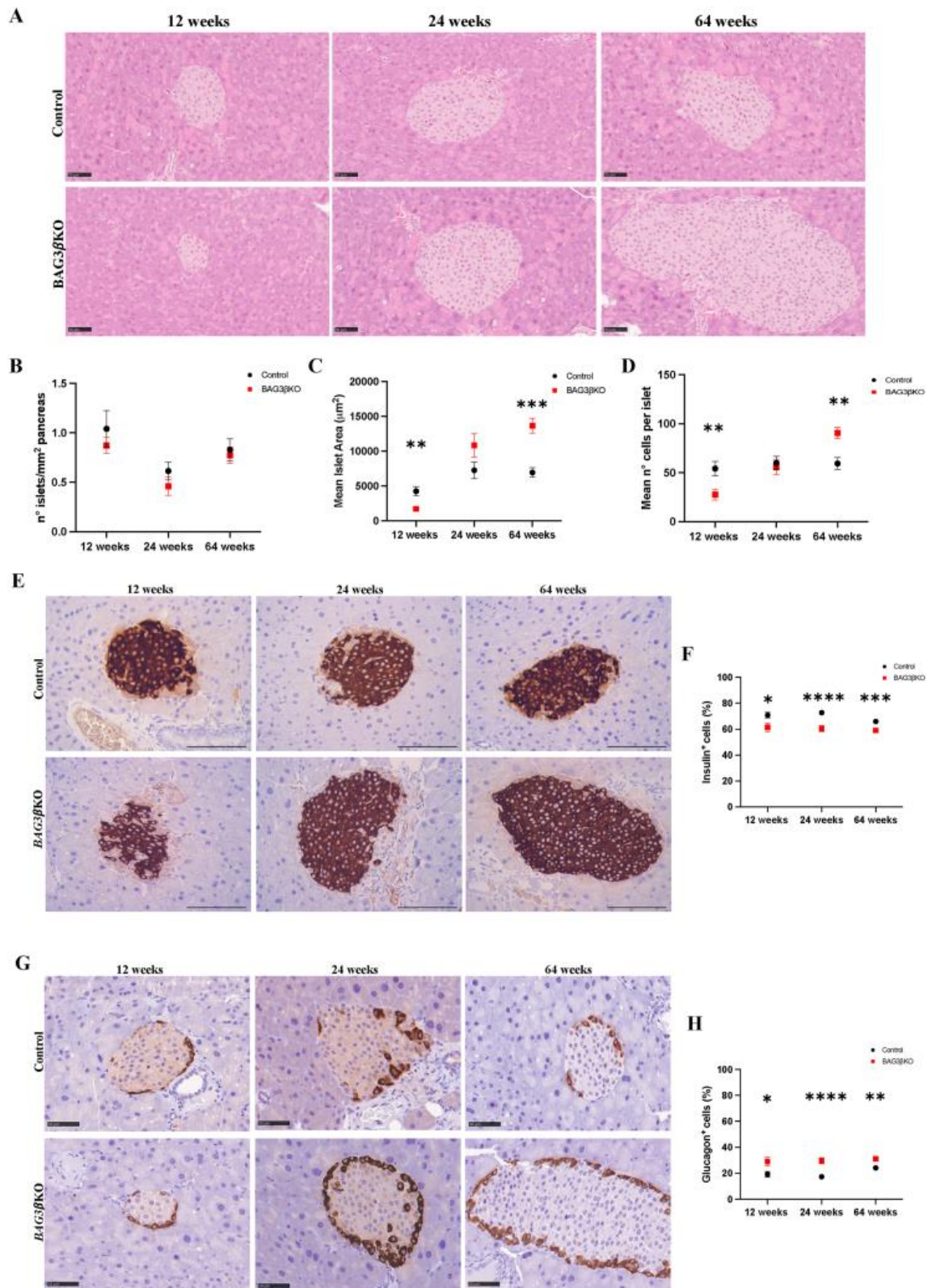


Figure 3: Pancreatic islets morphology evaluation at 12, 24, and 64 weeks of age in BAG3 β KO mice compared to control mice. (A) Representative images of hematoxylin-eosin-stained (H&E) pancreatic sections. Scale bar: 50 μ m. (B) The number of Langerhans islets per section of pancreas. (C) Mean area of islets. (D) Mean number of cells per islet. (E) Representative images of insulin-stained pancreatic sections. Scale bar: 100 μ m (F) Percentage of insulin-positive cells. (G) Representative images of glucagon-stained pancreatic sections. Scale bar: 100 μ m. (H) Percentage of glucagon-positive cells. Data are

presented as mean values $\pm$ SEM; n = 3 for 12 weeks group, n = 8–7 for 24 weeks group, n = 16–14 for 64 weeks group
*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Functional proteomics of the liver and muscle reveals peripheral insulin resistance

To investigate altered metabolic pathways in the muscle and liver of BAG3 β KO mice at 64 weeks of age, when insulin resistance was evident (as indicated by a significantly increased HOMA-IR), we employed a proteomic approach. Pooled homogenates of muscle and liver tissues from both BAG3 β KO and control mice were analyzed using nanoLC-Orbitrap-MS/MS. A total of 1229 proteins were identified in liver samples; among the 1030 common proteins, statistical analysis revealed 295 differentially expressed proteins (DEPs), with 96 up-regulated and 199 down-regulated in BAG3 β KO mice (Figure 4A). Notably, among the DEPs, we observed a significant decrease in the levels of hydroxymethylglutaryl-CoA synthase 2 (HMGCS2), a mitochondrial enzyme involved in ketone body production (p = 0.00078). In contrast, HMGCS1, an isoenzyme involved in sterol biosynthesis and located in the cytoplasm, was significantly increased (p = 0.03) (Figure 4A). To identify altered metabolic pathways, we conducted an Ingenuity Pathway Analysis (IPA) using quantified proteins. The downstream analysis predicted a potent inhibition of gluconeogenesis (z score = -2.745), while efflux of lipids (z score = 2) and hydrogen peroxide concentration (z score = 2.097) were significantly elevated (Figure 4B). Among the canonical pathways predicted to be altered, oxidative phosphorylation was significantly inhibited (z score = -2.47) in BAG3 β KO mice (Figure 4C). This inhibition is expected to increase NADH concentration. To validate this, we quantified the NAD⁺/NADH ratio in hepatic tissue extracts from BAG3 β KO and control mice, confirming that the livers of BAG3 β KO mice had a higher NADH content compared to controls (Figure 4D). The altered NAD⁺/NADH ratio is known to inhibit fatty acid oxidation (16), and consistent with this, fatty acid oxidation was predicted to be significantly repressed (z score = -2.55), which promotes fat accumulation in the liver (Figure 4E). We then used the STRING tool to analyze interactions among the 173 proteins found exclusively in BAG3 β KO livers, revealing interaction clusters involved in oxidation-reduction processes (FDR = 0.00089), intracellular transport (FDR = 0.0012), and regulation of the cellular response to insulin stimulus (FDR = 0.022) (PPI p = 2.53×10^{-09}). Integrating these unique proteins

with the up-regulated DEPs, we identified disease-gene association clusters related to amyloidosis (FDR = 0.0056), lipid metabolism disorders (FDR = 0.0068), and mitochondrial metabolic diseases (FDR = 0.0048) (Figure 4F). In contrast, analysis of the 26 unique proteins in the control group did not reveal any significant interaction clusters. Together, these findings in BAG3 β KO mice suggest that the liver is improperly responding to insulin in conditions (such as starvation) where insulin should not be present, confirming a state of hyperinsulinemia.

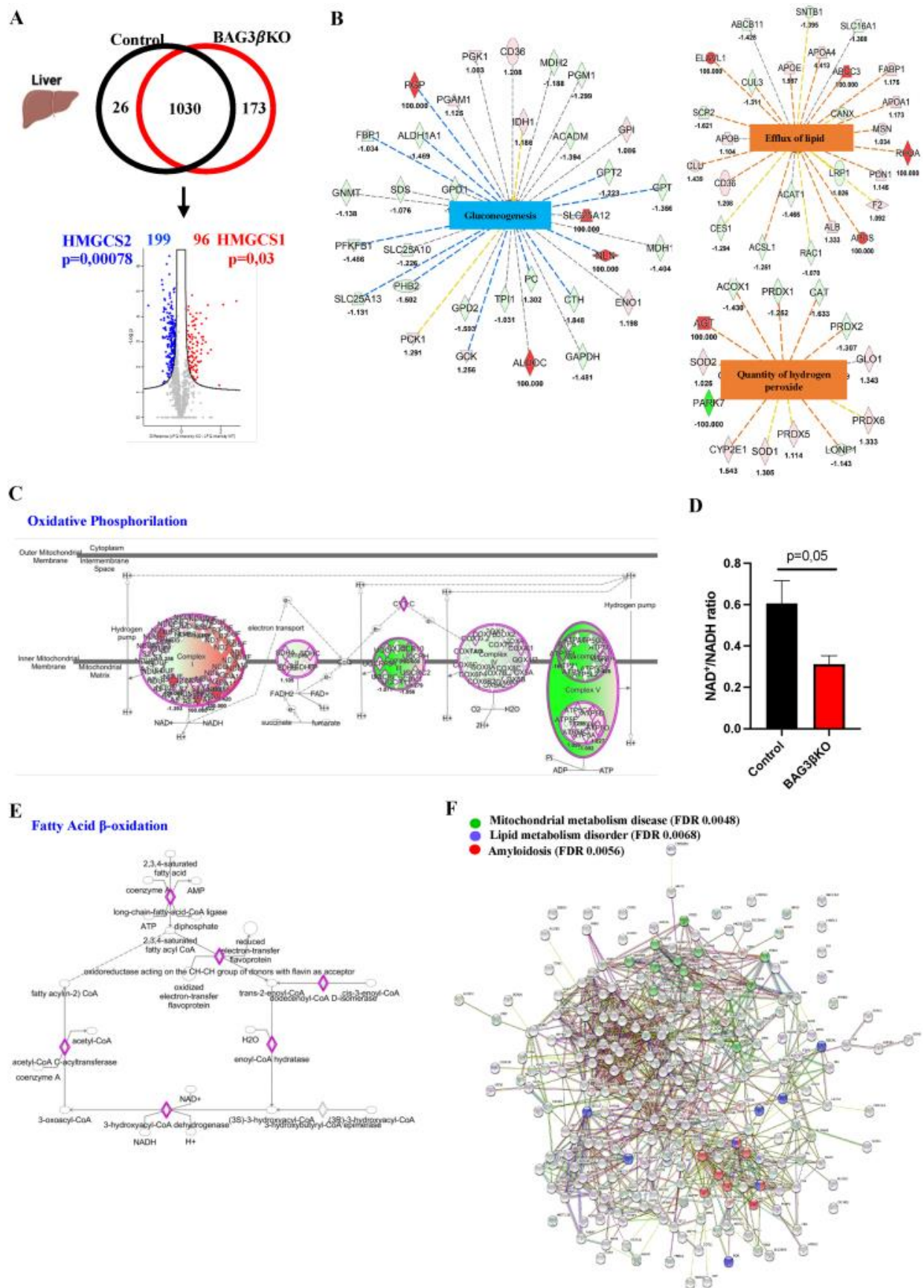


Figure 4: Liver proteomics of BAG3 β KO and Control mice. (A) Venn diagram and volcano plot showing the number of proteins and DEPs detected in livers of 64-weeks old control and BAG3 β KO mice. Down-regulated and up-regulated DEPs

identified by MS/MS analysis are highlighted in blue and red, respectively. HMGC52 and HMGC51 are highlighted. (B) Significantly inhibited and increased downstream functions predicted by IPA analysis of DEPs. Orange nodes and edges indicate activation, while blue nodes and edges indicate inhibition. Yellow edges reveal an inconsistent relationship between the findings and the state of the downstream node; instead, the gray ones indicate no predicted effects (C) Oxidative phosphorylation as decreased canonical pathway predicted by IPA. (D) NAD^+/NADH ratio was calculated from liver extracts of control and BAG3 β KO mice; data are presented as mean values $\pm$ SEM; n = 5 for control and n = 6 for BAG3 β KO mice. (E) Fatty acid oxidation as decreased canonical pathway predicted by IPA. (F) Disease-gene association identified by STRING among the unique proteins found in BAG3 β KO in addition to the significant upregulated DEPs.

Muscle proteomic profiling identified a total of 825 proteins; among the 718 common proteins, 114 were differentially expressed, with 86 up-regulated and 28 down-regulated (Figure 5A). Notably, Ingenuity Pathway Analysis (IPA) predicted inhibition of the insulin receptor (INSR) pathway (z score = -5.404) (Figure 5B; Table S4). This finding was confirmed by Western blot analysis, which showed reduced expression of INSR and its direct target, glucose transporter type 4 (GLUT4), in the muscles of BAG3 β KO mice compared to control mice (Figure 5C). To further investigate the molecular mechanisms, we examined the phosphorylation levels of Protein Kinase B (AKT), a central mediator in the insulin signaling pathway that leads to GLUT4 translocation (17). Western blot analysis revealed a significant reduction in AKT phosphorylation in the muscles of BAG3 β KO mice compared to controls (Figure 5D). Additionally, we assessed the phosphorylation state of extracellular signal-regulated kinase (ERK) 1/2. Interestingly, we observed a significant increase in ERK1/2 phosphorylation in BAG3 β KO muscle tissue (Figure 5D), suggesting a condition of muscle insulin resistance (18). In contrast, proteomic profiling of the liver did not indicate inhibition of the insulin receptor pathway, and no significant reduction in INSR expression was observed in BAG3 β KO liver tissue, suggesting that the liver remains responsive to insulin at this stage.

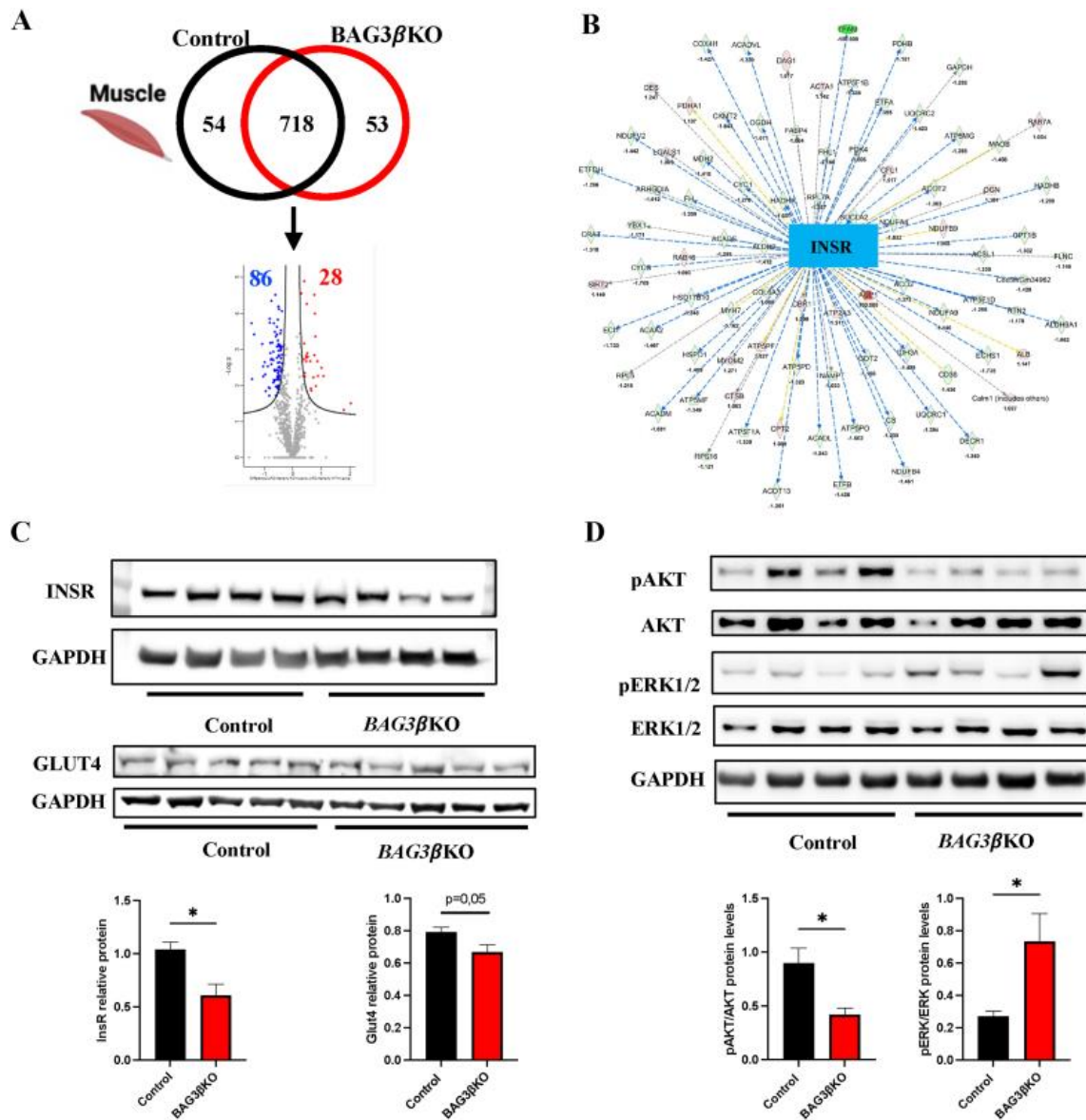


Figure 5: BAG3 β KO mice displayed peripheral but not hepatic insulin resistance. (A) Venn diagram and volcano plot showing the number of proteins and DEPs detected in muscles of 64-weeks old control and BAG3 β KO mice. Down-regulated and up-regulated DEPs identified by MS/MS analysis are highlighted in blue and red, respectively. (B) Significantly inhibited upstream predicted by IPA analysis. (C, D) Representative Western blot of muscle INSR, GLUT4 phospho-AKT/AKT, and phospho-ERK1/2/ERK1/2 expression in control and BAG3 β KO mice. Relative protein expression data are presented as mean values $\pm$ SEM; $n = 7-8$ for each group; * $p < 0.05$.

BAG3 β KO mice show altered circulating branched-chain amino acids (BCAAs) profile

We employed a mass spectrometry-based metabolite profiling platform to assess changes in the blood acyl-carnitine, free carnitine, and amino acid profiles in fasted control and BAG3 β KO mice at 12, 24, and 64 weeks

of age, as summarized in Table 1. Data were analyzed using two-way ANOVA followed by Bonferroni multiple comparisons. No significant changes were observed in acyl-carnitines and free carnitines. In contrast, the concentrations of blood leucine (LEU), isoleucine (ILE), and valine (VAL), which belong to the branched-chain amino acids (BCAAs) group (19), were significantly lower in BAG3 β KO mice at 12 weeks of age (LEU\ILE\PRO-OH $p < 0.0001$; VAL $p = 0.0166$) and 24 weeks of age (LEU\ILE\PRO-OH $p = 0.0344$), consistent with a state of primary hyperinsulinism (20). At 64 weeks of age, however, circulating BCAA concentrations were significantly elevated in BAG3 β KO mice (LEU\ILE\PRO-OH $p < 0.0001$; VAL $p < 0.0001$), in line with the established association between elevated BCAAs and insulin resistance (21), (22). To further confirm these findings, we normalized the data by calculating the ratio of BCAAs to alanine, and the ratio of BCAAs to phenylalanine (an aromatic amino acid). This normalization confirmed that the changes in BCAA levels were significant. Additionally, at 24 weeks of age, BAG3 β KO mice displayed reduced levels of glycine ($p = 0.0008$). However, at 64 weeks, glycine concentrations were also significantly elevated in BAG3 β KO mice (GLY $p = 0.0003$).

	12 weeks			24 weeks			64 weeks		
	Control	KO	p	Control	KO	p	Control	KO	p
ALA	122 $\pm$ 4.5	128.8 $\pm$ 7.2	ns	132.7 $\pm$ 5.	124.2 $\pm$ 9.7	ns	141.9 $\pm$ 13	173.5 $\pm$ 11.6	ns
				92			.3		
ARG	84.2 $\pm$ 1.9	80.4 $\pm$ 3.05	ns	80.07 $\pm$ 3.	72.7 $\pm$ 3.3	ns	85.0 $\pm$ 6.8	94.5 $\pm$ 6.8	ns
	3			24					
CIT	31.8 $\pm$ 1.8	35.3 $\pm$ 2.65	ns	33.7 $\pm$ 1.6	36.02 $\pm$ 3.9	ns	33.5 $\pm$ 3.0	37.72 $\pm$ 2.6	ns
	8			7					

GLN\LYS	360.3 ± 18.65	352.06 ± 20.02	ns	455.5 ± 27.4	470.4 ± 23.3	ns	503.77 ± 4.733	504.65 ± 38.77	ns
GLU	64.1 ± 4.32	62.6 ± 4.04	ns	82.1 ± 4.1	83.4 ± 3.5	ns	98.8 ± 5.8	107.51 ± 9.0	ns
GLY	196.7 ± 9.43	198.8 ± 8.62	ns	243.5 ± 19.3	212.64 ± 18.12	0.0008*	219.62 ± 7.49	265.18 ± 19.6	0.0003*
						**			**
LEU\ILE\	220.2 ± 8.59	180.2 ± 9.54	< 0.0001****	210.96 ± 1.029	186.72 ± 15.72	0.0344*	167.6 ± 14.7	235.6 ± 8.3	< 0.0001**
PRO-OH									**
MET	20.7 ± 1.17	21.7 ± 1.24	ns	27.22 ± 2.8	24.8 ± 1.80	ns	21.4 ± 0.83	21.3 ± 1.6	ns
ORN	23.5 ± 1.15	23.01 ± 0.88	ns	24.6 ± 2.3	26.7 ± 3.5	ns	31.0 ± 1.9	43.8 ± 1.32	ns
PHE	52.80 ± 1.51	54.2 ± 1.99	ns	56.2 ± 2.8	53.42 ± 2.4	ns	56.9 ± 3.9	68.99 ± 2.2	ns
PRO	30.64 ± 1.84	33.90 ± 2.79	ns	35.2 ± 2.3	36.67 ± 2.87	ns	41.66 ± 2.6	49.06 ± 2.63	ns
SA	0.50 ± 0.04	0.61 ± 0.05	ns	0.53 ± 0.05	0.46 ± 0.05	ns	0.45 ± 0.03	0.51 ± 0.04	ns
TYR	38.7 ± 1.71	40.5 ± 5.22	ns	41.9 ± 3.8	44.0 ± 7.3	ns	56.2 ± 3.7	71.32 ± 4.22	ns
VAL	161.9 ± 5.014	144.3 ± 6.57	0.0166*	157.1 ± 6.08	136.8 ± 10.2	ns	125.85 ± 1.60	200.9 ± 10.6	< 0.000

									1**
									**
ASA-Total	0.24 ± 0.0	0.31 ± 0.04	ns	0.13 ± 0.0	0.16 ± 0.03	ns	0.318 ± 0.	0.27 ± 0.03	ns
	3			4			05		
ADO	0.16 ± 0.0	0.15 ± 0.02	ns	0.201 ± 0.	0.23 ± 0.02	ns	0.18 ± 0.0	0.25 ± 0.05	ns
	12			01			2		
C0	10.7 ± 0.8	10.2 ± 0.65	ns	9.83 ± 0.3	9.71 ± 0.68	ns	14.52 ± 1.	15.90 ± 0.65	ns
	8			8			3		
C10	0.04 ± 0.0	0.042 ± 0.00	ns	0.045 ± 0.	0.039 ± 0.002	ns	0.039 ± 0.	0.038 ± 0.002	ns
	02	3		002			002		
C10:1	0.05 ± 0.0	0.05 ± 0.004	ns	0.06 ± 0.0	0.061 ± 0.005	ns	0.049 ± 0.	0.046 ± 0.001	ns
	01			01			002		
C10:2	0.0042 ± 0	0.0052 ± 0.0	ns	0.005 ± 0.	0.005 ± 0.000	ns	0.0037 ± 0	0.003 ± 0.000	ns
	.0002	005		001	7		.0004	4	
C2	26.08 ± 1.	24.49 ± 1.03	ns	22.9 ± 0.6	25.5 ± 1.9	ns	29.68 ± 0.	33.60 ± 0.88	ns
	8			3			89		
C3	1.21 ± 0.1	1.12 ± 0.14	ns	1.33 ± 0.0	0.92 ± 0.12	ns	1.46 ± 0.2	1.52 ± 0.09	ns
	3			4			1		
C3DC\C4	0.32 ± 0.0	0.32 ± 0.03	ns	0.30 ± 0.0	0.30 ± 0.01	ns	0.43 ± 0.0	0.51 ± 0.04	ns
OH	18			2			4		
C4	0.29 ± 0.0	0.33 ± 0.06	ns	0.13 ± 0.0	0.14 ± 0.013	ns	0.53 ± 0.0	0.83 ± 0.07	ns
	4			04			3		
C4DC\C5	0.14 ± 0.0	0.14 ± 0.005	ns	0.14 ± 0.0	0.14 ± 0.007	ns	0.17 ± 0.0	0.17 ± 0.003	ns
OH	04			03			1		

C5	0.07 ± 0.0	0.068 ± 0.00	ns	0.09 ± 0.0	0.08 ± 0.005	ns	0.11 ± 0.0	0.131 ± 0.008	ns
	04	6		07			1		
C5:1	0.001 ± 0.	0.002 ± 0.00	ns	0.0032 ± 0	0.0028 ± 0.00	ns	0.003 ± 0.	0.003 ± 0.000	ns
	0002	04		.001	05		0004	5	
C5DC\C6	0.073 ± 0.	0.069 ± 0.00	ns	0.073 ± 0.	0.065 ± 0.003	ns	0.08 ± 0.0	0.106 ± 0.006	ns
OH	005	7		004			08		
C6	0.077 ± 0.	0.079 ± 0.00	ns	0.101 ± 0.	0.081 ± 0.004	ns	0.11 ± 0.0	0.129 ± 0.007	ns
	007	6		006			08		
C6DC	0.044 ± 0.	0.06 ± 0.006	ns	0.044 ± 0.	0.039 ± 0.003	ns	0.067 ± 0.	0.043 ± 0.003	ns
	004			003			007		
C8	0.046 ± 0.	0.045 ± 0.00	ns	0.055 ± 0.	0.049 ± 0.005	ns	0.054 ± 0.	0.053 ± 0.003	ns
	004	4		003			002		
C8:1	0.012 ± 0.	0.012 ± 0.00	ns	0.011 ± 0.	0.011 ± 0.001	ns	0.010 ± 0.	0.011 ± 0.000	ns
	001	05		0002			001	7	
D-ADO	0.012 ± 0.	0.004 ± 0.00	ns	0.01 ± 0.0	0.011 ± 0.005	ns	0.012 ± 0.	0.012 ± 0.002	ns
	005	2		04			002		
C12	0.06 ± 0.0	0.065 ± 0.00	ns	0.071 ± 0.	0.053 ± 0.002	ns	0.060 ± 0.	0.061 ± 0.006	ns
	04	4		003			002		
C12:1	0.036 ± 0.	0.039 ± 0.00	ns	0.042 ± 0.	0.035 ± 0.002	ns	0.037 ± 0.	0.034 ± 0.000	ns
	002	2		003			001	6	
C14	0.22 ± 0.0	0.26 ± 0.02	ns	0.26 ± 0.0	0.21 ± 0.01	ns	0.22 ± 0.0	0.24 ± 0.028	ns
	11			1			1		
C14:1	0.11 ± 0.0	0.13 ± 0.006	ns	0.15 ± 0.0	0.12 ± 0.003	ns	0.125 ± 0.	0.12 ± 0.01	ns
	07			08			003		

C14:2	0.033 ± 0.003	0.035 ± 0.002	ns	0.05 ± 0.003	0.039 ± 0.001	ns	0.031 ± 0.001	0.033 ± 0.003	ns
C14OH	0.018 ± 0.001	0.021 ± 0.002	ns	0.02 ± 0.001	0.019 ± 0.002	ns	0.0227 ± 0.001	0.026 ± 0.003	ns
C16	1.53 ± 0.1	1.6 ± 0.08	ns	1.59 ± 0.0	1.54 ± 0.13	ns	1.31 ± 0.0	1.48 ± 0.11	ns
C16:1	0.17 ± 0.008	0.21 ± 0.009	ns	0.24 ± 0.002	0.201 ± 0.007	ns	0.22 ± 0.002	0.228 ± 0.02	ns
C16:1OH\	0.05 ± 0.002	0.057 ± 0.003	ns	0.076 ± 0.006	0.0604 ± 0.003	ns	0.056 ± 0.004	0.053 ± 0.006	ns
C16OH	0.078 ± 0.002	0.076 ± 0.007	ns	0.061 ± 0.005	0.054 ± 0.002	ns	0.072 ± 0.001	0.066 ± 0.010	ns
C18	0.27 ± 0.002	0.29 ± 0.015	ns	0.32 ± 0.0018	0.292 ± 0.03	ns	0.24 ± 0.001	0.260 ± 0.02	ns
C18:1	0.56 ± 0.032	0.62 ± 0.03	ns	0.77 ± 0.004	0.69 ± 0.03	ns	0.70 ± 0.004	0.801 ± 0.08	ns
C18:1OH	0.06 ± 0.002	0.059 ± 0.006	ns	0.06 ± 0.006	0.055 ± 0.005	ns	0.06 ± 0.001	0.06 ± 0.01	ns
C18:2	0.27 ± 0.001	0.29 ± 0.01	ns	0.43 ± 0.002	0.38 ± 0.01	ns	0.24 ± 0.002	0.30 ± 0.029	ns
C18:2OH	0.023 ± 0.001	0.027 ± 0.003	ns	0.03 ± 0.004	0.029 ± 0.002	ns	0.023 ± 0.003	0.024 ± 0.005	ns
C18OH	0.017 ± 0.001	0.019 ± 0.002	ns	0.019 ± 0.002	0.015 ± 0.001	ns	0.018 ± 0.002	0.020 ± 0.003	ns

C20	0.19 ± 0.0	0.32 ± 0.03	ns	0.026 ± 0.	0.027 ± 0.002	ns	0.012 ± 0.	0.018 ± 0.003	ns
	13			002			001		
C20:0-LPC	1.25 ± 0.0	0.88 ± 0.11	ns	2.38 ± 0.2	1.82 ± 0.27	ns	0.93 ± 0.0	0.998 ± 0.07	ns
	5			5			8		
C22	0.007 ± 0.	0.007 ± 0.00	ns	0.012 ± 0.	0.013 ± 0.001	ns	0.008 ± 0.	0.009 ± 0.001	ns
	001	05		002			0004		
C22:0-LPC	0.91 ± 0.0	0.71 ± 0.05	ns	1.42 ± 0.0	1.53 ± 0.04	ns	0.66 ± 0.0	0.65 ± 0.06	ns
	4			9			4		
C24	0.01 ± 0.0	0.010 ± 0.00	ns	0.017 ± 0.	0.018 ± 0.001	ns	0.011 ± 0.	0.014 ± 0.001	ns
	01	07		003	3		001		
C24:0-LPC	1.60 ± 0.0	1.29 ± 0.09	ns	2.46 ± 0.2	2.41 ± 0.21	ns	1.16 ± 0.1	1.31 ± 0.08	ns
	5						1		
C26	0.003 ± 0.	0.005 ± 0.00	ns	0.0032 ± 0	0.0042 ± 0.00	ns	0.007 ± 0.	0.008 ± 0.000	ns
	0008	1		.0008	1		001	5	
C26:0-LPC	0.37 ± 0.0	0.38 ± 0.019	ns	0.503 ± 0.	0.37 ± 0.04	ns	0.26 ± 0.0	0.30 ± 0.03	ns
	5			04			4		
LEU-ILE-	1.82 ± 0.1	1.42 ± 0.11	0.037*	1.60 ± 0.0	1.54 ± 0.17	ns	1.19 ± 0.0	1.38 ± 0.09	ns
PRO-	1			9			5		
OH/ALA									
VAL/ALA	1.34 ± 0.0	1.14 ± 0.092	ns	1.19 ± 0.0	1.13 ± 0.11	ns	0.89 ± 0.0	1.18 ± 0.08	0.03
	7			5			6		8*
LEU-ILE-	4.17 ± 0.1	3.35 ± 0.24	0.018*	3.76 ± 0.1	3.49 ± 0.2	ns	2.95 ± 0.1	3.42 ± 0.08	0.02
PRO-	4			1			6		2*
OH/PHE									

VAL/PHE	3.07 ± 0.1	2.68 ± 0.19	ns	2.80 ± 0.0	2.56 ± 0.14	ns	2.22 ± 0.1	2.91 ± 0.13	0.00
	01			5			5		93*
									*

Table 1: Metabolomic signature of BAG3 β KO mice. Values are mean $\pm$ SEM; n = 5 for 12 weeks group, n = 5 for 24 weeks group, n = 4–6 for 64 weeks group.

BAG3 β KO mice show histopathological in liver and kidney

The regulation of glycogen synthesis and degradation is crucial for maintaining blood glucose homeostasis (23). To investigate glycogen storage, we performed Periodic Acid-Schiff (PAS) staining on liver sections from control and BAG3 β KO mice. After 16 hours of fasting, glycogen was nearly absent from the liver of both groups at 12 weeks of age, with only scattered PAS-positive hepatocytes near the central vein beginning to re-accumulate glycogen. At 24 weeks, glycogen recovery was more pronounced in pericentral hepatocytes of both groups, with a modest, non-significant increase in BAG3 β KO mice. By 64 weeks of age, glycogen content was significantly higher in both groups, but more pronounced in BAG3 β KO mice. Notably, 62.5% of control mice and 43% of BAG3 β KO mice showed similar glycogen accumulation in pericentral hepatocytes. However, 37.5% of control mice and 57% of BAG3 β KO mice exhibited glycogen accumulation in periportal hepatocytes (Figure 6A), with the BAG3 β KO group showing significantly more pronounced periportal glycogen storage (Figure 6B), indicating altered glycogen metabolism consistent with insulin sensitivity differences in the liver. Further proteomic analysis of liver proteins involved in glycogen metabolism revealed no significant changes in the expression of key enzymes such as glycogen synthase, glycogen phosphorylase, and 1,4- α -glucan-branching enzyme, suggesting that the observed differences in periportal glycogen accumulation are likely to be due to differential enzymatic activity. PAS staining also revealed a more prominent microvesicular steatosis in the livers of BAG3 β KO mice compared to controls (Figure 6C). At 24 weeks of age, no steatosis was observed in control mice, while 43% of BAG3 β KO mice exhibited steatosis at levels 1 and 2 (Figure 6D, upper histogram). At 64 weeks, 25% of control mice and 50% of BAG3 β KO mice showed steatosis at levels 1 and 2 (Figure 6D,

lower histogram). Gene expression analysis of key lipid metabolism regulators and circulating lipid levels in 64-week-old BAG3 β KO mice confirmed these findings, showing overexpression of SREBP1 but not SREBP2, and downregulation of CYP7a1 (Figure 6E). Plasma lipid analysis revealed significantly lower triglyceride levels in BAG3 β KO mice, with no differences in total cholesterol, high-density lipoprotein (HDL), or low-density lipoprotein (LDL) concentrations (Figure 6F). These results further support the altered insulin responsiveness in BAG3 β KO mice.

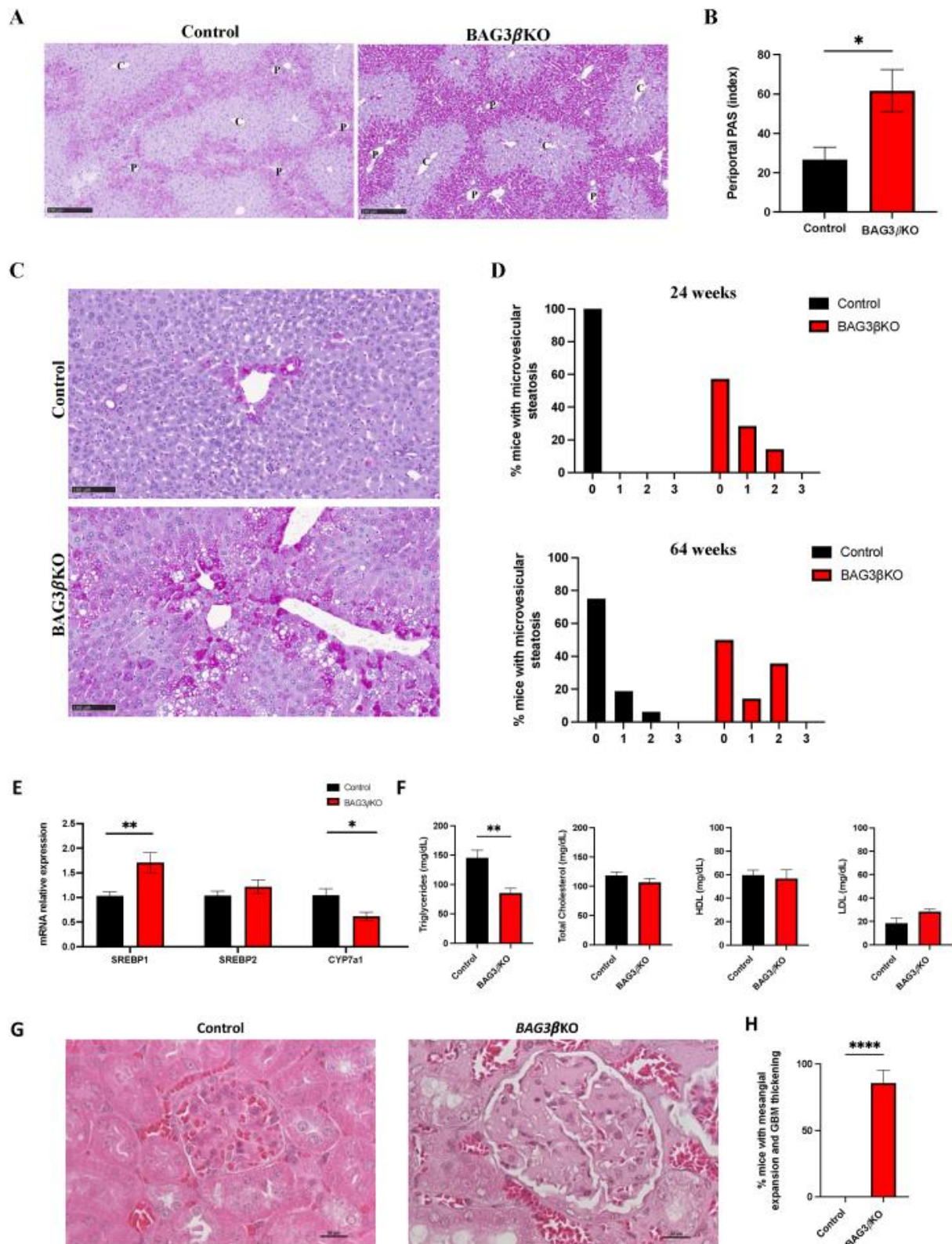


Figure 6: Histopathological evaluation of liver and kidney in the new BAG3 β KO mouse model. (A) Representative images of PAS-stained livers of 64 weeks old BAG3 β KO and control mice: portal triad (P), central vein (C). Scale bar: 250 μ m. (B) Quantification of liver glycogen accumulation around the portal triads in BAG3 β KO mice compared to control mice. Data are presented as mean values $\pm$ SEM; n = 16–14 for 64 weeks group; *p < 0.05. (C) PAS-stained liver tissue sections of control and BAG3 β KO mice at 64 weeks of age showing microvesicular liver steatosis in a BAG3 β KO mouse. Scale bar: 100 μ m.

(D) Histograms showing the percentage of BAG3 β KO and control mice presenting microvesicular steatosis at 24 and 64 weeks of age. Microvesicular steatosis was scored from 0 to 3 based on the percentage of the total area affected: 0 (<5%), 1 (5–33%), 2 (34–66%) and 3 (>66%). € mRNA levels evaluated by real-time PCR for SREBP1, SREBP2, and CYP7a. Data are presented as mean values $\pm$ SEM; n = 4 for each group; *p < 0.05; **p < 0.01. (F) Levels of plasma triglycerides, total cholesterol, HDL, and LDL in 64 weeks old BAG3 β KO and control mice. Data are presented as mean values $\pm$ SEM; n = 7 for each group; **p < 0.01. (G) Representative H&E-stained kidney sections from control and BAG3 β KO mice showing mesangial matrix expansion and thickening of the glomerular basement membrane in 64 weeks old BAG3 β KO mouse. Scale bar: 20 μ m. (H) Percentage of mice, showing mesangial matrix expansion and/or thickening of the glomerular basement membrane. Data are presented as mean values $\pm$ SEM; n = 14–16 for each group; ****p $\leq$ 0.0001.

We also observed morphological alterations in the kidneys of BAG3 β KO mice. While renal glomeruli and tubules appeared histologically normal in BAG3 β KO mice at 12 and 24 weeks of age (data not shown), significant changes were evident at 64 weeks of age. At this time point, 12 out of 15 (85.7%) BAG3 β KO mice exhibited mesangial matrix expansion and/or thickening of the glomerular basement membrane (Figure 6G, H). These changes are characteristic of chronic kidney disease (CKD), a common complication associated with insulin resistance (24), (25). Additionally, we observed tubular atrophy and tubulointerstitial fibrosis in one BAG3 β KO mouse, further suggesting renal involvement in the context of aging and insulin resistance.

Discussion and conclusions

In our previous work, we demonstrated that BAG3, as part of the SNARE complex, plays a critical role in insulin secretion. To explore this further, we knocked out BAG3 specifically in pancreatic β -cells of mice. BAG3 β KO mice breed normally and exhibit a regular growth rate, similar to their control counterparts, but show increased circulating insulin levels. Under fasting conditions, circulating insulin progressively increases in BAG3 β KO mice compared to controls as they age. Interestingly, glucose levels in BAG3-deficient mice are normal under fed conditions but are lower than controls at 12 weeks of age, which suggests an adaptive response to elevated insulin levels, consistent with our previous findings in an insulinoma model (12). Over time, compensation mechanisms seem to take place, and by 24 weeks, glucose levels in starved BAG3 β KO mice are similar to controls. The IPGTT confirms that BAG3 β KO mice can maintain normal glucose

homeostasis. However, the IPITT test reveals impaired insulin response starting at 24 weeks of age, indicating the onset of insulin resistance, likely as a compensatory response to increased insulin secretion. By 64 weeks, the HOMA-IR index is elevated in BAG3 β KO mice, strongly indicating insulin resistance. In addition to its role in the SNARE complex, BAG3 loss may also impair autophagy, contributing to the observed phenotype (26). Indeed, we detected reduced levels of p62/SQSTM1, a key player in autophagy (27). Autophagy is crucial for maintaining the structure, mass, and function of pancreatic β -cells (27), (28), and it is known to be regulated by BAG3 in conjunction with the ubiquitin-binding protein p62/SQSTM1 (29). The decrease in p62/SQSTM1 levels in BAG3 β KO mice suggests an impaired autophagic process, although further studies are needed to fully understand this potential mechanism. Proteomic analysis of the muscle and liver tissues revealed striking differences between BAG3 β KO and control mice, suggesting that, up to 64 weeks of age, the liver remains responsive to insulin, while the muscle has developed resistance. Insulin regulates liver metabolism primarily by promoting glycogen synthesis, enhancing lipogenic gene expression, and inhibiting gluconeogenesis (30), (31), (32). Proteomic analysis of BAG3 β KO mice livers indicated inhibition of gluconeogenesis, β -oxidation, oxidative phosphorylation, and ketone body production, while lipid efflux and sterol biosynthesis were predicted to be activated. These findings suggest that the liver in BAG3 β KO mice still appropriately responds to insulin, even under fasting conditions, aligning with the observation of elevated basal insulin levels. In contrast, proteomic profiling of muscle cells indicated reduced activity of the insulin receptor (INSR) pathway, with decreased INSR and GLUT4 expression and diminished AKT phosphorylation. Additionally, increased phosphorylation of ERK1/2 in BAG3 β KO muscle suggests a shift towards insulin resistance (33). The combination of chronic hyperinsulinemia and impaired insulin action likely contributes to the abnormal accumulation of glycogen and lipids in the liver, alongside reduced circulating triglyceride levels. It is well-documented that diabetic livers accumulate more glycogen than healthy livers, even under fasting conditions (34). Accordingly, we observed elevated periportal glycogen accumulation in BAG3 β KO mice, possibly due to structural alterations in glycogen storage mechanisms under fasting conditions (35). Lipid metabolism in the liver is primarily regulated by the sterol regulatory element-binding proteins (SREBPs), which control the expression of enzymes involved in the synthesis of cholesterol, fatty acids, triglycerides, and phospholipids

(36), (37). In BAG3 β KO mice, we observed increased expression of SREBP1, which regulates fatty acid and triglyceride synthesis, without a corresponding increase in SREBP2, which primarily regulates cholesterol biosynthesis. Moreover, Cyp7a1, a gene encoding the rate-limiting enzyme in bile acid synthesis, was downregulated. CYP7A1 overexpression in the liver is known to prevent obesity, fatty liver, and insulin resistance in mice fed a high-fat diet (38). These findings suggest that BAG3 β KO mice may exhibit early signs of non-alcoholic fatty liver disease (NAFLD). Our proteomic data also indicate activation of Complex I in the mitochondria, but inhibition of other oxidative phosphorylation complexes, leading to a decreased NAD⁺/NADH ratio. This altered redox balance impairs fatty acid oxidation, promoting fat accumulation in the liver. This condition creates a vicious cycle where liver fat accumulation exacerbates mitochondrial oxidative stress due to the NAD⁺/NADH imbalance. This supports studies suggesting that increasing NAD⁺ levels could have therapeutic effects on metabolic disorders like type 2 diabetes, metabolic syndrome, and NAFLD (39). Congenital hyperinsulinism (CHI) is characterized by unregulated insulin secretion from pancreatic β -cells, leading to hyperinsulinemic hypoglycemia, and is associated with mutations in key genes involved in insulin secretion (40), (41). Interestingly, defects in BAG3 could be involved in the pathogenesis of CHI, as the phenotype of BAG3 β KO mice shows primary hyperinsulinism early in life (42). The increased insulin production in response to insulin resistance likely drives the expansion of pancreatic islets in BAG3 β KO mice, consistent with studies showing that pancreatic islets undergo morphological and metabolic changes to maintain normoglycemia (15), (43), (44). Furthermore, at 12 weeks of age, BAG3 β KO mice show reduced levels of circulating branched-chain amino acids (BCAAs), which are consistent with primary hyperinsulinism due to insulin-mediated inhibition of gluconeogenesis (45). In contrast, as the mice become insulin resistant, their circulating BCAAs levels rise, further supporting the development of insulin resistance (19). BAG3 β KO mice also show elevated circulating glycine, in contrast to the reduced levels of glycine typically associated with obesity, type 2 diabetes, and NAFLD (46). However, despite the insulin-resistant state in BAG3 β KO mice, they do not develop obesity, type 2 diabetes, or clear NAFLD.

The metabolic abnormalities in BAG3 β KO mice also result in kidney alterations, including mesangial matrix expansion and thickening of the glomerular basement membrane, which are key features of diabetic

nephropathy and chronic kidney disease (CKD), particularly in insulin resistance (47). Interestingly, severe insulin resistance (SIR) is associated with CKD features in type 2 diabetes patients (48). Thickening of the glomerular basement membrane and mesangial expansion are common pathological features of CKD, which can result in glomerulosclerosis, tubulointerstitial fibrosis, and loss of normal renal architecture. These findings suggest that insulin resistance can contribute to CKD even in the absence of diabetes (49), (50), (51).

In conclusion, we demonstrated that selective knockout of BAG3 in pancreatic β -cells impairs insulin secretion both under basal conditions and in response to glucose administration. At an early stage, this leads to primary hyperinsulinism, characterized by lower plasma glucose levels. Over time, however, peripheral insulin resistance develops. As insulin resistance progresses, higher insulin levels are required to enhance cellular glucose uptake and inhibit hepatic glucose production, preventing hyperglycemia. This creates a vicious cycle of escalating insulin levels. Chronic metabolic dysfunction results in organ damage, as evidenced by impaired glycogen and lipid accumulation in the liver, along with renal alterations leading to glomerulopathy. Our findings underscore the crucial role of BAG3 in insulin secretion and highlight its involvement in the development of insulin resistance in BAG3 β KO mice. The silencing of BAG3 in β -cells causes an early phase of primary hyperinsulinism, followed by the onset of insulin resistance. These results position BAG3 as a potential novel gene involved in the pathogenesis of congenital hyperinsulinism. Moreover, the BAG3 β KO mouse serves as a unique model for studying the pathogenetic mechanisms underlying multi-organ damage associated with prolonged exposure to elevated insulin levels and insulin resistance.

Materials and methods

Animals and maintenance

All animal experimentation procedures were performed in compliance with protocols approved by the Institutional Animal Healthcare of the University of Chieti and the guidelines established by the Italian Ministry of Health (Protocol No. 204/2022-PR). Mice were housed in a pathogen-free environment maintained at 22 °C with a 12-hour light/dark cycle and had libitum access to food and water. Beta cell-specific BAG3

knockout mice were generated using Cre-loxP-mediated recombination technology. Specifically, homozygous BAG3 floxed mice (BAG3^{tm1c(EUCOMM)Hmgu}; BAG3^{flox/flox}), kindly provided by Dr. Michael Hanhe, were crossed with mice expressing Cre recombinase under the control of the mouse insulin 1 promoter [B6(Cg)-Ins1^{tm1.1(cre)Thor/J}; INS1cre^{+/+}; stock no. 026801, The Jackson Laboratory]. For experiments, control mice were either homozygous for the floxed BAG3 allele without the INS1cre allele (BAG3^{fl/fl}) or carried only the INS1cre allele (INS1cre^{+/+}), whereas beta cell-specific BAG3 knockout mice carried two floxed BAG3 alleles and the INS1cre allele simultaneously (BAG3^{βKO}). Mice were grouped into separate cages based on genotype at 4 weeks of age, and body weight was monitored weekly. Age-matched male control and BAG3^{βKO} mice were used for the experiments, as it is well-established that female mice are protected from metabolic decline (52).

DNA extraction and genotyping and islets isolation

Genomic DNA was extracted from a small ear punch and amplified using polymerase chain reaction (PCR) with the Xpert directXtract PCR Kit. Genotyping for INS1cre was performed as detailed on The Jackson Laboratory's website (<http://jaxmice.jax.org/>). Genotyping for BAG3 was carried out using the following primers: BAG3-471 forward – ACTATCTGCTGTCTACACTGC and flanked LoxP BAG3-459 reverse – TGAGCGGCACTTTAAGGTTT. PCR products were resolved on a 2% agarose gel, producing either a wild-type DNA product (≈300 bp) or a wild-type DNA product with the upstream LoxP site (mutant product ≈450 bp). Genotyping was performed using the MJ Mini 48-Well Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following amplification protocol: 95 °C for 3 min (1 cycle); 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 2 s (40 cycles); 72 °C for 3 min (1 cycle), followed by soaking at 4 °C.

Mouse pancreatic islets were isolated as previously described with minor modifications (53). Briefly, a 1.2 mg/mL collagenase solution was injected into the common bile duct of a euthanized animal. The pancreas was then excised and digested at 37 °C for 15 minutes. Subsequently, islets were recovered, purified using a Histopaque gradient, handpicked, and used for subsequent experiments.

Immunofluorescence analysis

Freshly isolated islets were subjected to cytocentrifugation on glass slides for 3 minutes at 500 rpm. The cytopsin preparations were then fixed with 4% paraformaldehyde and incubated overnight with rabbit polyclonal anti-BAG3 (1:200 dilution; NBP2-27398, Novus Biologicals, Milan, Italy) and mouse monoclonal anti-Insulin B (C-12) (1:1000 dilution; sc-377,071, Santa Cruz Biotechnology) antibodies. Afterward, the slides were incubated with anti-rabbit Alexa Fluor 488 (A-11008, Thermo Fisher Scientific, Waltham, MA, USA) and anti-mouse Alexa Fluor 546 (A-11030, Thermo Fisher Scientific) secondary antibodies for 1 hour at room temperature (RT). For immunofluorescence staining of pancreatic tissues, slides were deparaffinized, serially rehydrated, and subjected to antigen retrieval for 10 minutes in a microwave using the EnVision FLEX Target Retrieval Solution Low pH buffer (K8005, Dako). The slides were then incubated overnight with mouse monoclonal anti-Insulin B (C-12) antibody (1:1000 dilution; sc-377,071, Santa Cruz Biotechnology), rabbit polyclonal anti-Glucagon antibody (1:200 dilution; PA5-88091, Thermo Fisher Scientific), and rat monoclonal anti-Somatostatin antibody (1:300 dilution; MA5-16987, Thermo Fisher Scientific). Following incubation, the slides were stained with anti-mouse Alexa Fluor Plus 647 (A-32728, Thermo Fisher Scientific), anti-rabbit Alexa Fluor 546 (A-11010, Thermo Fisher Scientific), and anti-rat Alexa Fluor 488 (A-11006, Thermo Fisher Scientific) secondary antibodies for 1 hour at RT. Nuclei were counterstained with DAPI for 15 minutes, and images were acquired using a Zeiss LSM800 confocal microscope.

RNA extraction and qPCR

Total RNA was extracted from islets or livers using the RNeasy kit. RNA concentration was determined by measuring absorbance using a NanoDrop spectrophotometer. The RNA was then reverse-transcribed into cDNA using TaqMan™ Reverse Transcription Reagents. Quantitative PCR was performed using SsoAdvanced Universal SYBR Green Supermix on a Bio-Rad CFX96 Touch System. Transcript levels were normalized to actin and are presented as ratios relative to control levels.

Western blotting

Tissues were homogenized using ice-cold lysis buffer (50 mM TRIS-HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 1 mM EDTA, 1 mM MgCl₂, 10% Glycerol) supplemented with a protease inhibitor cocktail and a phosphatase inhibitor cocktail. Protein concentrations were quantified using the Bradford method. Sample aliquots containing 20–30 µg of protein were loaded onto 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for detection. The membranes were incubated overnight at 4 °C with primary antibodies: anti-BAG3 (1:2000; Novus Biologicals, Milan, Italy), anti-INSR (1:1000), anti-AKT (1:1000), anti-phospho-AKT (1:1000), anti-ERK1/2 (1:1000), anti-phospho-ERK1/2, and anti-GAPDH (1:2000; all from Cell Signaling Technology, Danvers, MA, USA), anti-GLUT4 Rabbit polyclonal Ab (1:1000; Antibodies.com, Cat. #95872), anti-p62/SQSTM1 (1:1000; BD Biosciences, San Jose, CA, USA), anti-Insulin B (C-12) (1:500; Santa Cruz Biotechnology), and anti-β-actin (1:40,000; Sigma-Aldrich, Milan, Italy). The membranes were then washed and incubated with a secondary antibody (1:20,000 dilution; Bio-Rad, Milan, Italy) for 1 hour at room temperature. Bound antibodies were detected using the enhanced chemiluminescent (ECL) method.

Blood glucose measurements and metabolic studies

Blood glucose levels were measured under both fed and fasting conditions using a Contour Next glucometer from tail vein blood samples. An intraperitoneal glucose tolerance test (IPGTT) was performed following an overnight fast. Blood glucose levels were measured at baseline (time 0) and at 15, 30, 60, 90, and 120 minutes post-glucose injection (2 g/kg body weight). For the intraperitoneal insulin tolerance test (IPITT), mice were fasted for 4 hours prior to receiving an intraperitoneal injection of insulin (0.75 U/kg body weight). Blood glucose levels were measured at time 0 and at 15, 30, 60, 90, and 120 minutes after insulin injection. Both IPGTTs and IPITTs were conducted on mice at 12, 24, and 64 weeks of age.

Plasma insulin and glucagon measurements and homeostasis model assessment of insulin resistance

Plasma insulin and glucagon concentrations were quantified using enzyme-linked immunosorbent assays (ELISA), following the manufacturer's protocols (Crystal Chem).

To evaluate the onset of insulin resistance, the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was used. HOMA-IR was calculated using the following formula: fasting insulin ($\mu\text{U/dL}$) $\times$ fasting blood glucose (mmol/L) / 22.5 (54).

Biochemical analysis

Mice at 12, 24, and 64 weeks of age were fasted overnight. Blood samples were collected by cardiac puncture from euthanized mice into Microvette® 200 Lithium Heparin tubes. A 100 μL aliquot of the lithium-heparinized blood was centrifuged for 10 minutes at 4 °C to separate plasma. Triglycerides, total cholesterol, HDL, and LDL levels were measured using a VChemys Automatic Biochemical Analyzer following the manufacturer's instructions.

Metabolomic analysis

Mice at 12, 24, and 64 weeks of age were fasted overnight. A drop of blood was obtained from the tail vein, spotted onto absorbent filter paper, and air-dried at room temperature for at least 2 hours. Dried blood spot (DBS) disks, approximately 3.2 μL of whole blood, were punched into 3.2 mm-diameter disks and subsequently extracted for the determination of 13 amino acids (AAs), 34 acylcarnitines (ACs), free carnitine, adenosine (ADO), deoxyadenosine (D-ADO), and 4 lyso-phosphatidylcholines (LPCs) using the NeoBase 2 Non-Derivatized MSMS Kit (Perkin Elmer Life and Analytical Sciences, Turku, Finland), as previously described (55), (56). Specifically, 125 μL of extraction solution containing an isotopically labeled internal standard were added to the DBS disks, which were then extracted at 45 °C for 30 minutes with mixing at 700 rpm. Following extraction, 100 μL of the solution were transferred into a 96-well plate and analyzed using a

targeted metabolomics approach by Flow-Injection Analysis Tandem Mass Spectrometry (FIA-MS/MS). The FIA-MS/MS system consists of an Acquity UPLC I-Class coupled with a Xevo TQD tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The system operated in positive electrospray ionization mode with multiple reaction monitoring (MRM) acquisition. The capillary voltage was set to 3.50 kV, the source temperature was 120 °C, the desolvation temperature was 350 °C, and the collision cell gas pressure was 3.0×10^{-3} mbar Argon. A 10 μ L sample was injected into the ion source, and the run time was 1.1 minutes, injection-to-injection. Data were processed using MassLynx V4.2 and NeoLynx Software.

NAD⁺/NADH ratio measurement

Total NAD⁺ and NADH levels were measured following the manufacturer's protocol (Abcam). Briefly, 25 μ L of NAD/NADH control buffer or NADH extraction buffer was added to the whole liver lysate sample, and the mixture was incubated at 37 °C for 15 minutes. Subsequently, 75 μ L of NAD/NADH reaction mixture was added to each sample, and the samples were incubated at room temperature for 1 hour. Fluorescence signals were measured using a Synergy H1 microplate reader at an excitation/emission wavelength of 540/590 nm.

Histological and immunohistochemical analysis

Mice at 12, 24, and 64 weeks of age were fasted overnight and euthanized. Necropsy was performed, and all organs were harvested, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5- μ m thickness. Sections were stained with Hematoxylin and Eosin for histological analysis. Slides were independently evaluated by two pathologists to assess the presence of histological changes in the tissues. Immunohistochemical staining on formalin-fixed paraffin-embedded (FFPE) tissues was carried out using the following primary antibodies: mouse monoclonal anti-Insulin B (C-12) antibody (1/1000 dilution; Santa Cruz Biotechnology, sc-377,071) and rabbit polyclonal anti-Glucagon antibody (1/200 dilution; Thermo Fisher Scientific, PA5-88091). Antigen retrieval was performed by microwaving the slides for 10 minutes in EnVision FLEX Target Retrieval Solution Low pH buffer. The sections were then incubated overnight with the primary

antibodies. EnVision anti-rabbit (Dako, K4003) or anti-mouse (Dako, K4001) IgG were used as secondary antibodies for 30 minutes, followed by chromogenic detection with 3-3' tetrahydrochloride diaminobenzidine. After chromogen incubation, the slides were counterstained with Hematoxylin. Images were acquired using a Leica DMRD optical microscope.

For the histological assessment of glycogen deposition and lipid accumulation, Periodic Acid Schiff (PAS) staining was performed on liver sections using the PAS Kit, according to the manufacturer's instructions (Bio-Optica). For histological and immunohistochemical evaluations, whole-slide sections were scanned with a Nanozoomer scanner and analyzed using QuPath v0.3.2 software. The following tools were applied: the area annotation tool for islet area, the positive cell detection tool for insulin and glucagon quantification (% of positive cells), and the pixel classifier tool for PAS evaluation. Microvesicular steatosis was assessed and scored from 0 to 3 based on the percentage of the total area affected, using the following categories: 0 (<5%), 1 (5–33%), 2 (34–66%), and 3 (>66%), according to the NASH Clinical Research Network Steatosis Grading Score (57). The insulin and glucagon secretion index were calculated using the following formula: % of positive cells × area of islets × number of islets.

Label-free proteomic analysis and bioinformatics

Liver and muscle tissues from BAG3insKO and WT mice were processed using the FASP (Filter Aided Sample Preparation) method following mechanical homogenization with a turrax mixer (1 min, highest speed) and ultrasonication on ice at 80% amplitude in a lysis buffer (6 M urea in 100 mM Tris/HCl, pH 7.5). The homogenates were then centrifuged to remove tissue debris. A total of 50 µg of protein from each sample, quantified using the Bradford Protein Assay with Bovine Serum Albumin as the calibration standard, were digested with trypsin. For protein identification and quantification, tryptic digests from each liver and muscle sample were analyzed in triplicate by LC-MS/MS using an UltiMate™ 3000 UPLC (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto a Trap Cartridge C18 (0.3 mm ID, 5 mm L, 5 µm PS, Thermo Fisher Scientific) and

separated on an EASY-spray Acclaim™ PepMap™ C18 nanoscale chromatographic column (75 µm ID, 25 cm L, 2 µm PS, Thermo Fisher Scientific). Proteomics data from liver and muscle samples were processed using MaxQuant version 1.6.10.50. The UniProt database "released 2019_11, taxonomy Mus Musculus, 21,990 entries" was used with Andromeda for peptide identification and search. Parameters for data processing were set as outlined in our previous work (58). A univariate statistical analysis was performed, visualizing results in a Volcano Plot (FDR = 0.003 and S0 = 0.1), to identify differentially expressed proteins between experimental conditions (p-value threshold = 0.05). Protein ratios (BAG3insKO livers/Control) were uploaded to Ingenuity Pathway Analysis (IPA) for Gene Ontology and functional enrichment analysis (z-scores ≥ 2.0 for activation and z-scores ≤ -2.0 for inhibition of transcriptional regulators or downstream effects, based on published literature). For robust protein quantification, proteins with an abundance ratio variability of less than 50% and identified by at least two peptides were considered quantified. Protein–protein interaction (PPI) networks were analyzed using the STRING database.

Statistical analysis

Experimental data are presented as means $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism v.9. The normality of the data distribution was assessed using the Shapiro–Wilk test. Depending on the distribution, either Student's t-test or the Mann–Whitney U test was applied for comparisons between two groups. For comparisons involving more than two groups, analysis of variance (ANOVA) or the Kruskal–Wallis test was used, followed by post-hoc Tukey or Dunn's tests, respectively. Statistical significance is indicated in the figures with asterisks as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

References

1. Fujimoto WY. The importance of insulin resistance in the pathogenesis of type 2 diabetes mellitus. *The American Journal of Medicine*. aprile 2000;108(6):9–14.
2. Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, et al. Pathophysiology of Type 2 Diabetes Mellitus. *IJMS*. 30 agosto 2020;21(17):6275.
3. Groop L. Pathogenesis of type 2 diabetes: the relative contribution of insulin resistance and impaired insulin secretion. *Int J Clin Pract Suppl*. ottobre 2000;(113):3–13.
4. Roden M, Shulman GI. The integrative biology of type 2 diabetes. *Nature*. 5 dicembre 2019;576(7785):51–60.
5. Yan T, Huang Y, Wu JHY, Zhuang XD, Pan XF. Editorial: Insulin Resistance, Metabolic Syndrome, and Cardiovascular Disease. *Front Cardiovasc Med*. 22 giugno 2022;9:959680.
6. Perdomo CM, Garcia-Fernandez N, Escalada J. Diabetic Kidney Disease, Cardiovascular Disease and Non-Alcoholic Fatty Liver Disease: A New Triumvirate? *JCM*. 10 maggio 2021;10(9):2040.
7. Seo DH, Suh YJ, Cho Y, Ahn SH, Seo S, Hong S, et al. Advanced Liver Fibrosis Is Associated with Chronic Kidney Disease in Patients with Type 2 Diabetes Mellitus and Nonalcoholic Fatty Liver Disease. *Diabetes Metab J*. 31 luglio 2022;46(4):630–9.
8. Marzullo L, Turco MC, De Marco M. The multiple activities of BAG3 protein: Mechanisms. *Biochimica et Biophysica Acta (BBA) - General Subjects*. agosto 2020;1864(8):129628.
9. Rosati A, Graziano V, De Laurenzi V, Pascale M, Turco MC. BAG3: a multifaceted protein that regulates major cell pathways. *Cell Death Dis*. 7 aprile 2011;2(4):e141–e141.
10. Sherman MY, Gabai V. The role of Bag3 in cell signaling. *J of Cellular Biochemistry*. gennaio 2022;123(1):43–53.

11. Behl C. Breaking BAG: The Co-Chaperone BAG3 in Health and Disease. *Trends in Pharmacological Sciences*. agosto 2016;37(8):672–88.
12. Iorio V, Festa M, Rosati A, Hahne M, Tiberti C, Capunzo M, et al. BAG3 regulates formation of the SNARE complex and insulin secretion. *Cell Death Dis*. 12 marzo 2015;6(3):e1684–e1684.
13. Youn DY, Lee DH, Lim MH, Yoon JS, Lim JH, Jung SE, et al. Bis deficiency results in early lethality with metabolic deterioration and involution of spleen and thymus. *American Journal of Physiology-Endocrinology and Metabolism*. dicembre 2008;295(6):E1349–57.
14. Thorens B, Tarussio D, Maestro MA, Rovira M, Heikkilä E, Ferrer J. Ins1 Cre knock-in mice for beta cell-specific gene recombination. *Diabetologia*. marzo 2015;58(3):558–65.
15. Mezza T, Muscogiuri G, Sorice GP, Clemente G, Hu J, Pontecorvi A, et al. Insulin Resistance Alters Islet Morphology in Nondiabetic Humans. *Diabetes*. 1 marzo 2014;63(3):994–1007.
16. Fan L, Cacicedo JM, Ido Y. Impaired nicotinamide adenine dinucleotide (NAD⁺) metabolism in diabetes and diabetic tissues: Implications for nicotinamide-related compound treatment. *J of Diabetes Invest*. novembre 2020;11(6):1403–19.
17. Hernandez R, Teruel T, Lorenzo M. Akt mediates insulin induction of glucose uptake and up-regulation of GLUT4 gene expression in brown adipocytes. *FEBS Letters*. 13 aprile 2001;494(3):225–31.
18. Leto D, Saltiel AR. Regulation of glucose transport by insulin: traffic control of GLUT4. *Nat Rev Mol Cell Biol*. giugno 2012;13(6):383–96.
19. Ramzan I, Ardavani A, Vanweert F, Mellett A, Atherton PJ, Idris I. The Association between Circulating Branched Chain Amino Acids and the Temporal Risk of Developing Type 2 Diabetes Mellitus: A Systematic Review & Meta-Analysis. *Nutrients*. 20 ottobre 2022;14(20):4411.
20. Chaussain JL, Georges P, Gendrel D, Donnadieu M, Job JC. Serum branched-chain amino acids in the diagnosis of hyperinsulinism in infancy. *The Journal of Pediatrics*. dicembre 1980;97(6):923–6.

21. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat Rev Endocrinol.* dicembre 2014;10(12):723–36.
22. Yoon MS. The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism. *Nutrients.* 1 luglio 2016;8(7):405.
23. Nawaz A, Zhang P, Li E, Gilbert RG, Sullivan MA. The importance of glycogen molecular structure for blood glucose control. *iScience.* gennaio 2021;24(1):101953.
24. Spoto B, Pisano A, Zoccali C. Insulin resistance in chronic kidney disease: a systematic review. *Am J Physiol Renal Physiol.* 1 dicembre 2016;311(6):F1087–108.
25. Steffes MW, Østerby R, Chavers B, Mauer SM. Mesangial Expansion as a Central Mechanism for Loss of Kidney Function in Diabetic Patients. *Diabetes.* 1 settembre 1989;38(9):1077–81.
26. Stürner E, Behl C. The Role of the Multifunctional BAG3 Protein in Cellular Protein Quality Control and in Disease. *Front Mol Neurosci.* 21 giugno 2017;10:177.
27. Riahi Y, Wikstrom JD, Bachar-Wikstrom E, Polin N, Zucker H, Lee MS, et al. Autophagy is a major regulator of beta cell insulin homeostasis. *Diabetologia.* luglio 2016;59(7):1480–91.
28. Ebato C, Uchida T, Arakawa M, Komatsu M, Ueno T, Komiya K, et al. Autophagy Is Important in Islet Homeostasis and Compensatory Increase of Beta Cell Mass in Response to High-Fat Diet. *Cell Metabolism.* ottobre 2008;8(4):325–32.
29. Gamerding M, Hajieva P, Kaya AM, Wolfrum U, Hartl FU, Behl C. Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. *EMBO J.* 8 aprile 2009;28(7):889–901.
30. Patel BM, Goyal RK. Liver and insulin resistance: New wine in old bottle!!! *European Journal of Pharmacology.* novembre 2019;862:172657.
31. Cherrington AD, Edgerton D, Sindelar DK. The direct and indirect effects of insulin on hepatic glucose production in vivo. *Diabetologia.* 19 agosto 1998;41(9):987–96.

32. Petersen MC, Shulman GI. Mechanisms of Insulin Action and Insulin Resistance. *Physiological Reviews*. 1 ottobre 2018;98(4):2133–223.
33. Fujishiro M, Gotoh Y, Katagiri H, Sakoda H, Ogihara T, Anai M, et al. Three Mitogen-Activated Protein Kinases Inhibit Insulin Signaling by Different Mechanisms in 3T3-L1 Adipocytes. *Molecular Endocrinology*. 1 marzo 2003;17(3):487–97.
34. Oliveira DSD, Amado CAB, Martini MC, Suzuki-Kemmelmeier F, Bracht A. Glycogen levels and energy status of the liver of fasting rats with diabetes types 1 and 2. *Braz arch biol technol*. settembre 2007;50(5):785–91.
35. Wang L, Liu Q, Wang M, Du Y, Tan X, Xu B, et al. Effects of fasting on liver glycogen structure in rats with type 2 diabetes. *Carbohydrate Polymers*. giugno 2020;237:116144.
36. Shimano H, Sato R. SREBP-regulated lipid metabolism: convergent physiology — divergent pathophysiology. *Nat Rev Endocrinol*. dicembre 2017;13(12):710–30.
37. DeBose-Boyd RA, Ye J. SREBPs in Lipid Metabolism, Insulin Signaling, and Beyond. *Trends in Biochemical Sciences*. maggio 2018;43(5):358–68.
38. Li T, Owsley E, Matozel M, Hsu P, Novak CM, Chiang JYL. Transgenic expression of cholesterol 7 α -hydroxylase in the liver prevents high-fat diet-induced obesity and insulin resistance in mice†. *Hepatology*. agosto 2010;52(2):678–90.
39. Xie N, Zhang L, Gao W, Huang C, Huber PE, Zhou X, et al. NAD⁺ metabolism: pathophysiologic mechanisms and therapeutic potential. *Sig Transduct Target Ther*. 7 ottobre 2020;5(1):227.
40. Maiorana A, Dionisi-Vici C. Hyperinsulinemic hypoglycemia: clinical, molecular and therapeutical novelties. *J of Inher Metab Disea*. luglio 2017;40(4):531–42.
41. Galcheva S, Demirbilek H, Al-Khawaga S, Hussain K. The Genetic and Molecular Mechanisms of Congenital Hyperinsulinism. *Front Endocrinol*. 26 febbraio 2019;10:111.

42. Maiorana A, Lepri FR, Novelli A, Dionisi-Vici C. Hypoglycaemia Metabolic Gene Panel Testing. *Front Endocrinol.* 29 marzo 2022;13:826167.
43. Mezza T, Cinti F, Cefalo CMA, Pontecorvi A, Kulkarni RN, Giaccari A. β -Cell Fate in Human Insulin Resistance and Type 2 Diabetes: A Perspective on Islet Plasticity. *Diabetes.* 1 giugno 2019;68(6):1121–9.
44. Kehm R, König J, Nowotny K, Jung T, Deubel S, Gohlke S, et al. Age-related oxidative changes in pancreatic islets are predominantly located in the vascular system. *Redox Biology.* maggio 2018;15:387–93.
45. Lu J, Xie G, Jia W, Jia W. Insulin resistance and the metabolism of branched-chain amino acids. *Front Med.* marzo 2013;7(1):53–9.
46. Alves A, Bassot A, Bulteau AL, Pirola L, Morio B. Glycine Metabolism and Its Alterations in Obesity and Metabolic Diseases. *Nutrients.* 16 giugno 2019;11(6):1356.
47. Tervaert TWC, Mooyaart AL, Amann K, Cohen AH, Cook HT, Drachenberg CB, et al. Pathologic Classification of Diabetic Nephropathy. *Journal of the American Society of Nephrology.* aprile 2010;21(4):556–63.
48. Ahlqvist E, Storm P, Käräjämäki A, Martinell M, Dorkhan M, Carlsson A, et al. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. *The Lancet Diabetes & Endocrinology.* maggio 2018;6(5):361–9.
49. Artunc F, Schleicher E, Weigert C, Fritsche A, Stefan N, Häring HU. The impact of insulin resistance on the kidney and vasculature. *Nat Rev Nephrol.* dicembre 2016;12(12):721–37.
50. Dave N, Wu J, Thomas S. Chronic Kidney Disease-Induced Insulin Resistance: Current State of the Field. *Curr Diab Rep.* luglio 2018;18(7):44.
51. De Cosmo S, Menzaghi C, Prudente S, Trischitta V. Role of insulin resistance in kidney dysfunction: insights into the mechanism and epidemiological evidence. *Nephrology Dialysis Transplantation.* 1 gennaio 2013;28(1):29–36.

52. Stone AC, Noland RC, Mynatt RL, Velasquez SE, Bayless DS, Ravussin E, et al. Female Mice Are Protected from Metabolic Decline Associated with Lack of Skeletal Muscle HuR. *Biology (Basel)*. 17 giugno 2021;10(6):543.
53. Carter JD, Dula SB, Corbin KL, Wu R, Nunemaker CS. A Practical Guide to Rodent Islet Isolation and Assessment. *Biol Proced Online*. dicembre 2009;11(1):3.
54. Tahapary DL, Pratisthita LB, Fitri NA, Marcella C, Wafa S, Kurniawan F, et al. Challenges in the diagnosis of insulin resistance: Focusing on the role of HOMA-IR and Tryglyceride/glucose index. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. agosto 2022;16(8):102581.
55. Rossi C, Cicalini I, Zucchelli M, Di Ioia M, Onofrij M, Federici L, et al. Metabolomic Signature in Sera of Multiple Sclerosis Patients during Pregnancy. *IJMS*. 14 novembre 2018;19(11):3589.
56. Rossi C, Cicalini I, Rizzo C, Zucchelli M, Consalvo A, Valentinuzzi S, et al. A False-Positive Case of Methylmalonic Aciduria by Tandem Mass Spectrometry Newborn Screening Dependent on Maternal Malnutrition in Pregnancy. *IJERPH*. 20 maggio 2020;17(10):3601.
57. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease†. *Hepatology*. giugno 2005;41(6):1313–21.
58. Madonna R, Moscato S, Polizzi E, Pieragostino D, Cufaro MC, Del Boccio P, et al. Connexin 43 and Connexin 26 Involvement in the Ponatinib-Induced Cardiomyopathy: Sex-Related Differences in a Murine Model. *Int J Mol Sci*. 28 maggio 2021;22(11):5815.

Pancreatic beta-cell specific BAG3 knockout results in insulin resistance and amyloidosis
in mice spleen

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BAG3 deficiency in beta-cells: investigating its role in insulin secretion dysregulation, amyloidosis onset, and peripheral insulin resistance in the spleen

As already described, Bcl-2-associated athanogene 3 (BAG3) is a multifunctional protein involved in several cellular processes, including protein folding, degradation, apoptosis regulation, cytoskeleton dynamics, and insulin secretion. Its dysregulation has been associated with several pathological conditions, including insulin resistance (1), (2), (3). Insulin, secreted by pancreatic β -cells, plays a pivotal role in the regulation of glucose metabolism. Its secretion and action require precise modulation to accommodate metabolic demands. Dysfunction in insulin secretion by pancreatic β -cells and/or reduced insulin sensitivity in peripheral tissues leads to insulin resistance, a key contributor to the pathogenesis of type 2 diabetes (T2D) (4), (5), (6), (7). Elucidating the proteins involved in insulin secretion and their mechanistic roles is essential for understanding the development of metabolic disorders, including congenital hyperinsulinism, metabolic syndrome, T2D, nonalcoholic fatty liver disease (NAFLD), cardiovascular disease, and chronic kidney disease (CKD) (8), (9), (10). Our previous studies have identified BAG3 as a constitutive protein in pancreatic β -cells, where it regulates insulin secretion as a component of the t-SNARE complex, which facilitates the trafficking of insulin-containing granules (11). Silencing BAG3 in β -TC-6 mouse insulinoma cells leads to reduced intracellular insulin content, likely due to impaired granule retention, coupled with enhanced insulin secretion in response to glucose stimulation (11). Additionally, systemic BAG3 knockout (BAG3KO) mice exhibit early postnatal lethality, primarily due to severe degeneration of the diaphragm, intercostal muscles, cardiac tissue, and skeletal muscles. These mice also develop hypoglycemia and fatty liver. Transcriptomic analyses of hepatic tissue in BAG3KO mice revealed altered pathways of gluconeogenesis, β -oxidation, and lipogenesis, indicative of an adaptive response to malnutrition. These findings align with metabolic profiling data showing reduced peripheral fat depots (12). Collectively, our findings establish BAG3 as a critical regulator of insulin secretion. The β -cell-specific deletion of BAG3 results in hyperinsulinemia, which subsequently leads to insulin resistance and a multifaceted metabolic disorder characterized by systemic dysfunction across multiple organs. We demonstrated the onset of peripheral insulin resistance in muscle tissue in our latest work (1) and decided to investigate potential metabolic alterations in other extra-pancreatic organs, such as the spleen. In

this context, the histological analysis of spleens from 64-week-old BAG3 β KO mice using hematoxylin and eosin staining revealed that most BAG3 β KO mice exhibited an enlargement of the red pulp, attributed to a fibrotic process indicative of splenic amyloidosis. This finding was further confirmed by Congo red staining observed under polarized light; as no evidence of fibrosis was detected in younger mice at earlier ages, we decided to further characterize amyloid deposits, we performed two distinct proteomic approaches.

Amyloidosis encompasses a group of complex conditions characterized by the extracellular deposition of amyloid fibrils, derived from various precursor proteins, that may lead to progressive organ dysfunction and systemic impairment. Common features of all amyloid fibrils are the beta-pleated sheet configuration and the immunohistochemical tinctorial behavior even though they vary with respect to the primary structure of the protein involved (13). In humans, forty-two different chemical types of amyloid proteins originating from different precursor proteins have been described so far. The clinical manifestation of amyloidosis is different, reflecting the broad spectrum of precursor proteins and the range of organs that can be affected. While some patients may exhibit systemic symptoms influencing multiple organs, others may present localized deposits affecting a singular organ such as the kidney, heart, spleen, and liver (14), (15). The most common type of amyloidosis is systemic light chain (AL) amyloidosis, which is caused by the deposition of immunoglobulin light chains produced by a clonal population of plasma cells (16). The diagnosis of AL amyloidosis typically involves the detection of monoclonal immunoglobulin light chains in the serum or urine, followed by the identification of amyloid deposits in tissue biopsies using Congo red staining and immunohistochemistry (17), (18). Other types of amyloidosis, such as transthyretin (ATTR) amyloidosis and amyloid-A (AA amyloidosis), can be diagnosed based on the identification of specific amyloid proteins in tissue biopsies using immunohistochemistry and mass spectrometry (19), (20), (21).

In animals, amyloidosis has been reported in a wide range of species, including mice, dogs, cats, horses, and non-human primates (22). Mouse models of amyloidosis have been instrumental in understanding the underlying mechanisms of amyloid diseases, as well as in developing potential treatments. Several mouse models have been developed to study different forms of amyloidosis, including Alzheimer's disease, Parkinson's disease, type 2 diabetes (T2D), and systemic amyloidosis such as AA amyloidosis (23), (24). The

development of transgenic animal models of amyloidosis has provided valuable tools for studying the pathogenesis of amyloidosis and for testing novel therapeutic strategies.

Characterizing amyloidosis has been a challenging task for scientists, owing to the complex nature of these disorders. One of the key challenges in studying amyloidosis is the diversity of the amyloid proteins involved, each of which can form different types of amyloid fibrils with distinct biochemical and biophysical properties. Moreover, the clinical manifestations of amyloidosis can vary widely depending on the type and location of the amyloid deposits, making it difficult to establish clear diagnostic criteria and treatment strategies. The importance of characterizing amyloidosis lies in its multifaceted nature and the substantial overlap of its manifestations with those of other diseases. Misdiagnosis or delayed recognition can lead to ineffective treatments and poor outcomes, highlighting the necessity for precise characterization. Advances in imaging techniques, histopathology, biochemical assays, and genetic testing have revolutionized the approach to diagnosing and characterizing amyloidosis. These techniques allow for the identification of specific amyloid fibril proteins and pinpoint their systemic or localized pattern of deposition. Histological analysis remains a cornerstone, with biopsy specimens subjected to Congo red staining and polarization microscopy, revealing the characteristic amyloid birefringence. The emergence of mass spectrometry-based proteomics has added to the diagnostic accuracy and overall understanding of amyloidosis (14).

In this study we compare two different approaches, shotgun label free proteomics and laser captured micro proteomics, to characterize splenic amyloidosis raised up in mice bearing peripheral insulin resistance, impaired glycogen and lipid accumulation in the liver and renal glomerulopathy (1).

Results

BAG3 β KO mice developed splenic amyloidosis modifying tissue architecture

We have previously shown that tissue specific BAG3 knockout in pancreatic beta cells leads to impaired insulin secretion with the development of peripheral insulin resistance, impaired glycogen and lipid accumulation in

the liver and renal changes leading to renal glomerulopathy over time. By analysing the spleens of 64-week-old BAG3 β KO mice by hematoxylin and eosin, we found 9 out of 14 (64.3%) BAG3 β KO mice with an increased size of the red pulp due to the development of a fibrotic process suggestive of splenic amyloidosis, confirmed by Congo red staining visualized under polarized light (Figure 1A e 1B), while at earlier stages of age the fibrotic process was absent in all mice. In BAG3 β KO mice's spleen, atrophy of the white pulp is evidenced by smaller germinal centers with significantly reduced numbers of B (B220⁺ cells) and T lymphocytes (CD3⁺ cells), and the red pulp also shows a significant reduction in the macrophage population (F4/80⁺ cells) due to the presence of amyloid deposition as shown in Figure 1C-D.

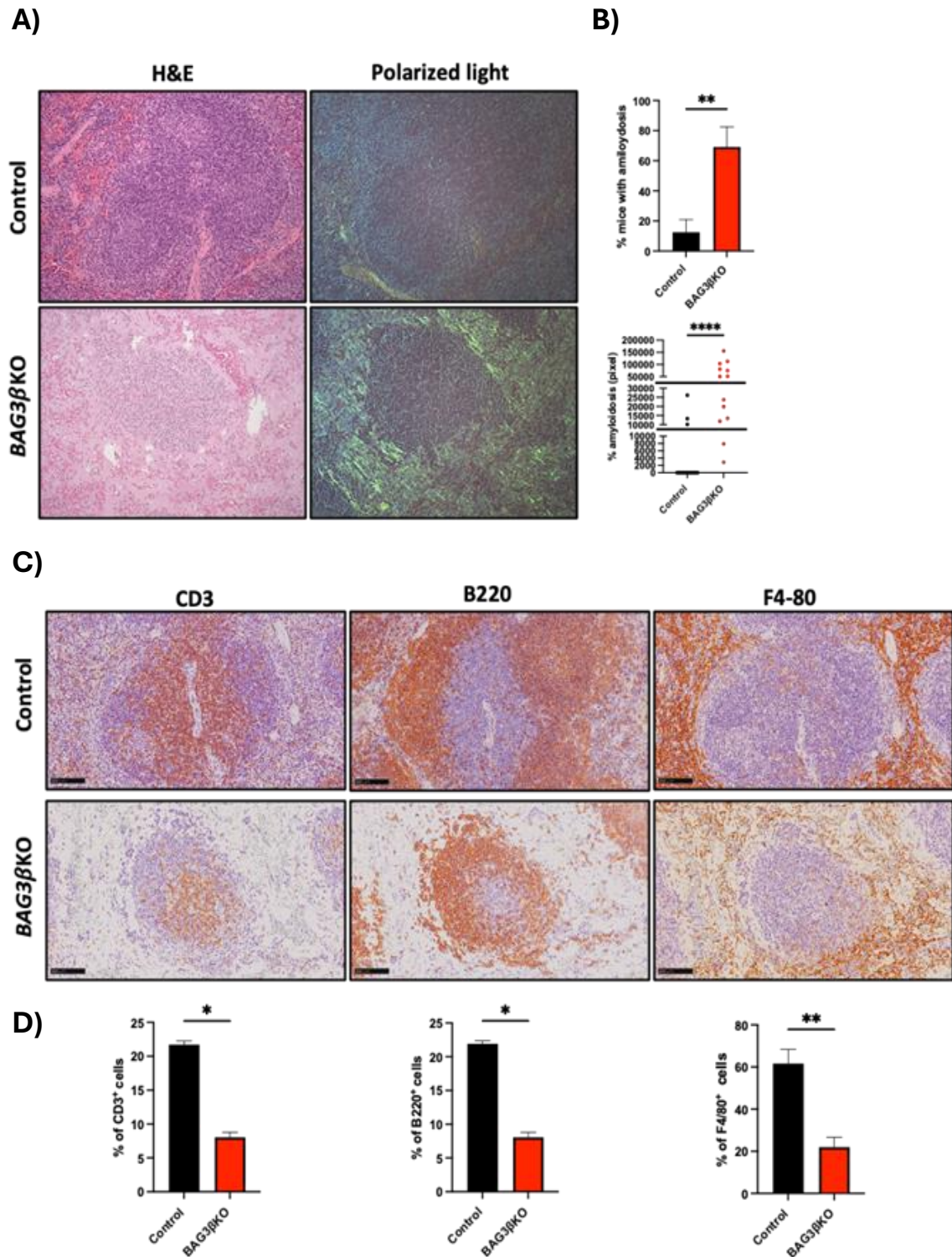


Figure 1: A) Representative images of immunohistochemically H&E-stained and Congo-red stained spleen sections visualized under polarized light. B) Percentage of mice harboring splenic amyloidosis. C) Stratification of the mice according to the quantification of the amyloidosis, expressed in pixels. Data are presented as mean values $\pm$ SEM; $n=13-16$ for each group; ** $p \leq 0.01$; **** $p \leq 0.0001$. D) Representative images anti-B220, anti-CD3 and anti-F4/80-stained spleen

sections of control and BAG3 β KO mice at 64 weeks of age and their relative quantification. Data are presented as mean values $\pm$ SEM; n=3-6 for each group; *p<0.05; **p $\leq$ 0.01.

Quantitative label-free shotgun proteomic and micro-proteomic analyses

We have therefore tried to characterize amyloid deposits using two approaches: label-free shotgun proteomic on spleens as a whole and micro-proteomic analyses with laser dissection on Congo Red+ regions and Congo Red- regions, following the study design reported in Figure 2.

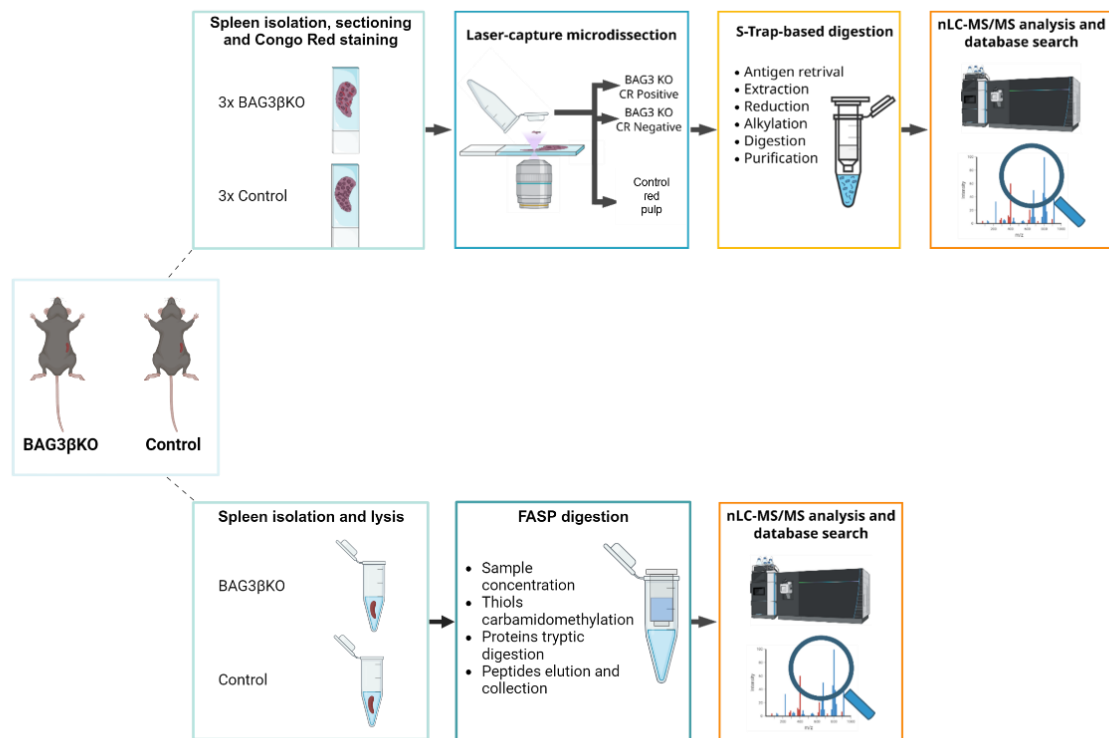
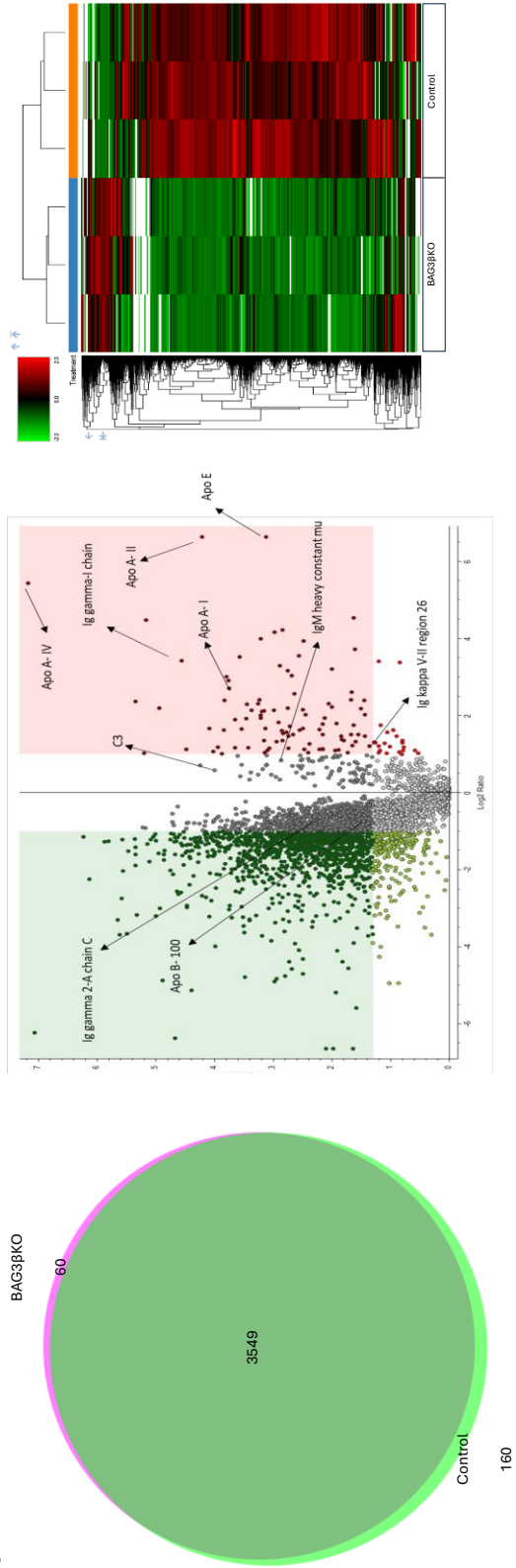


Figure 2: Schematic representation of LCM-micro-proteomics and label-free shotgun proteomics workflows. FFPE spleens from three BAG3 β KO mice and three control mice were sectioned and stained with Congo Red (CR). Laser capture microdissection was used to isolate CR-positive areas from BAG3 β KO mice, CR-negative areas from BAG3 β KO mice, and the corresponding areas of control mice. The micro-dissected samples were then extracted and prepared for quantitative proteomics analysis using S-TrapTM Micro columns and analyzed by nLC-MS/MS. Spleens isolated from BAG3 β KO and control mice were lysed and digested by FASP protocol, so tryptic peptides were acquired by nLC-MS/MS (Image generated by Biorender.com).

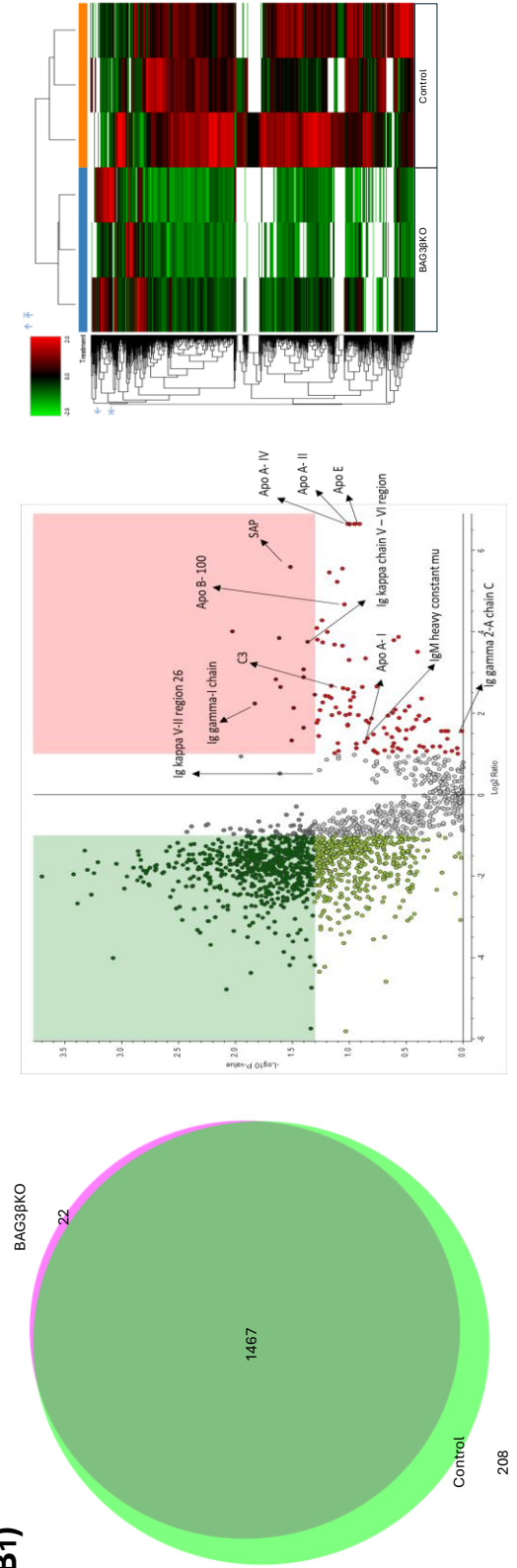
At first, we performed the label-free shotgun proteomics analysis in which we compared the BAG3 β KO mice spleen to the control, highlighting DEPs in the two experimental conditions and to characterizing the spleens of the two different models. The Venn diagrams in Figure 3 display the unique and shared proteins between the experimental groups in the four different proteomic analyses. In particular, the label-free shotgun analysis presents 3549 shared proteins between BAG3 β KO and control spleens; we can see 60 unique proteins in BAG3 β KO murine model and 160 unique proteins in the control (Figure 3A). On the one hand, the micro-proteomic analysis performed on BAG3 β KO Congo Red⁺ regions compared to control highlights 1467 shared proteins, 22 unique proteins in BAG3 β KO Congo Red⁺ regions and 208 unique proteins in the control (Figure 3-B1). On the other hand, the micro-proteomic analysis of BAG3 β KO Congo Red⁻ regions and control plots 1694 shared proteins, 49 unique proteins in BAG3 β KO Congo Red⁻ regions and 61 unique proteins in the control (Figure 3-B2). Finally, the micro-proteomics comparing BAG3 β KO Congo Red⁺ and Congo Red⁻ regions shows 838 shared proteins, 2 unique proteins in BAG3 β KO Congo Red⁺ and 35 unique proteins in BAG3 β KO Congo Red⁻ (Figure 3-B3). Looking at quantitative data, as reported in the Volcano Plot of Figure 3A, there are 96 significantly upregulated proteins represented as red spots on the right side and 1213 significantly downregulated proteins represented as green spots on the left side. Among these, we focused on the modulation of proteins involved in splenic amyloidosis process: Ig gamma 2-A chain C, Apo B-100, C3, Apo A-IV, Ig gamma-I chain, Apo A-I, Apo A-II, Apo E, IgM heavy constant mu and Ig kappa V-II region 26. The relative protein modulation trends, identified in all proteomic investigations, are reported in Volcano Plots and Heatmaps in Figure 3. In detail, as regards the analysis performed on BAG3 β KO Congo Red⁺ regions compared to the control (Figure 3-B1), we can observe 10 significantly upregulated, again represented by red spots on the right side, and 509 significantly downregulated proteins represented by green spots on the left side. In this analysis, we focused on Ig kappa V-II region 26, Ig gamma-I chain, C3, Apo B-100, Apo A-I, SAP, IgM heavy constant mu, Ig kappa chain V – VI region, Apo A-IV, Apo A-II, Apo E and Ig gamma 2-A chain C as the most interesting amyloidogenic proteins. To better identify DEPs and therefore characterize amyloid deposits, we performed the last two micro-proteomic analyses on BAG3 β KO Congo Red⁻ regions compared to the control. Volcano Plot in Figure 3-B2 shows 6 significantly upregulated proteins, represented by red

spots on the right side, and 153 significantly downregulated proteins (green spots on the left side); in this case it's possible to notice an expression change in some proteins such as IgM heavy constant mu, Ig kappa V-II region 26 and Apo A-I in each comparison. Finally, the Volcano Plot in Figure 3-B3 highlights 2 significantly upregulated proteins and 101 significantly downregulated proteins. Moreover, it's possible to notice how Ig gamma-I chain, Apo B-100, C3, SAP, Ig kappa V-II region 26, IgM heavy constant mu, Ig gamma 2-A chain C, Apo A-I, Apo A-II, Apo E and Apo A-IV show an increased trend (not-significant data) in BAG3 β KO mice Congo Red+ vs Congo Red- areas.

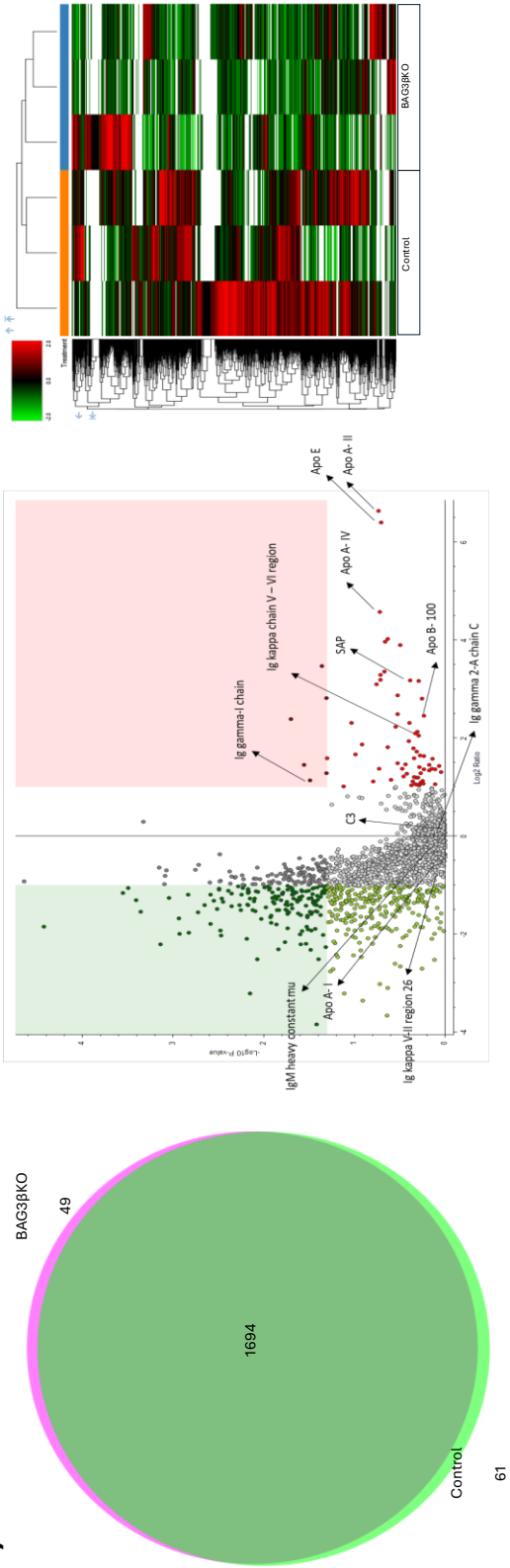
A)



B1)



B2)



B3)

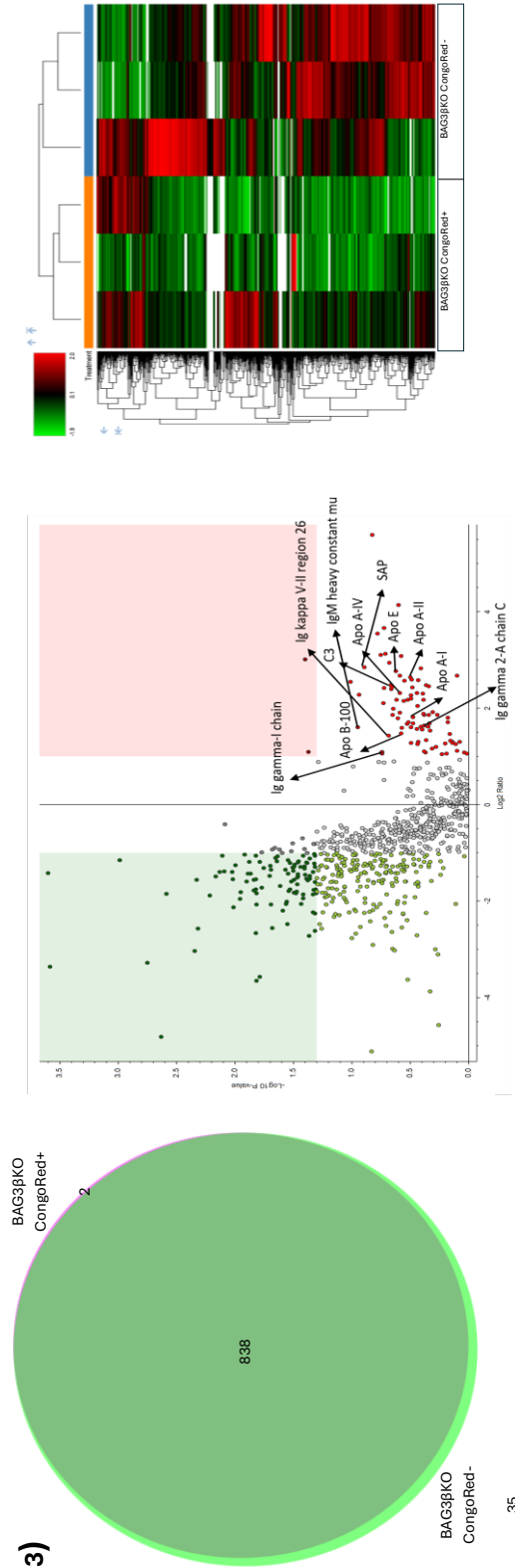


Figure 3: Venn diagrams display the unique and shared proteins between the experimental groups in label-free proteomic analysis and in micro-proteomic analyses. Volcano Plot and respective Heatmap show the proteins expression trend in A) label-free shotgun proteomic analysis on BAG3 β KO mice spleen compared to control; B1) BAG3 β KO Congo Red+ regions compared to control; B2) BAG3 β KO Congo Red- regions compared to control and B3) BAG3 β KO Congo Red+ regions compared to BAG3 β KO Congo Red- regions. Volcano Plots represent in the red/green squares the significantly upregulated/downregulated proteins (cut-off value: p-value = 0.05 and log2 fold change = 2). Heatmaps describe the analytical replicates cluster within the two mice models for each analysis.

Proteomics characterization of amyloid deposits

Firstly, based on the Gene Ontology enrichment analysis by FunRich tool, we observed that protein datasets identified and quantified in all proteomic analyses (BAG3 β KO vs Control; BAG3 β KO_CongoRed+ vs Control; BAG3 β KO_CongoRed- vs Control; BAG3 β KO_CongoRed+ vs BAG3 β KO_CongoRed-) are related to amyloidosis (Figure 4).

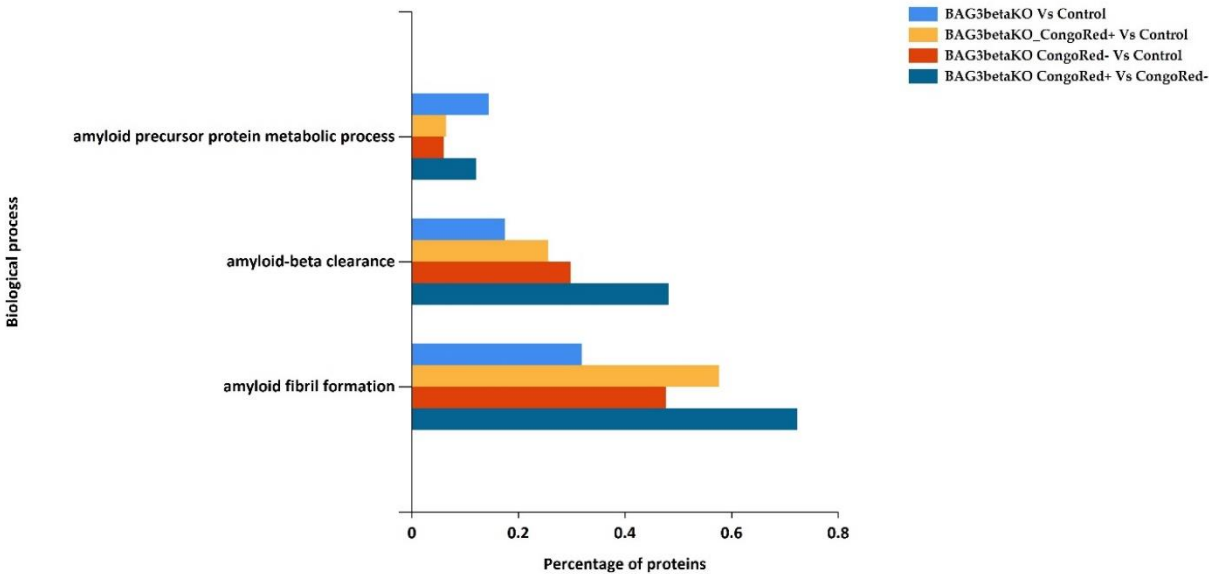
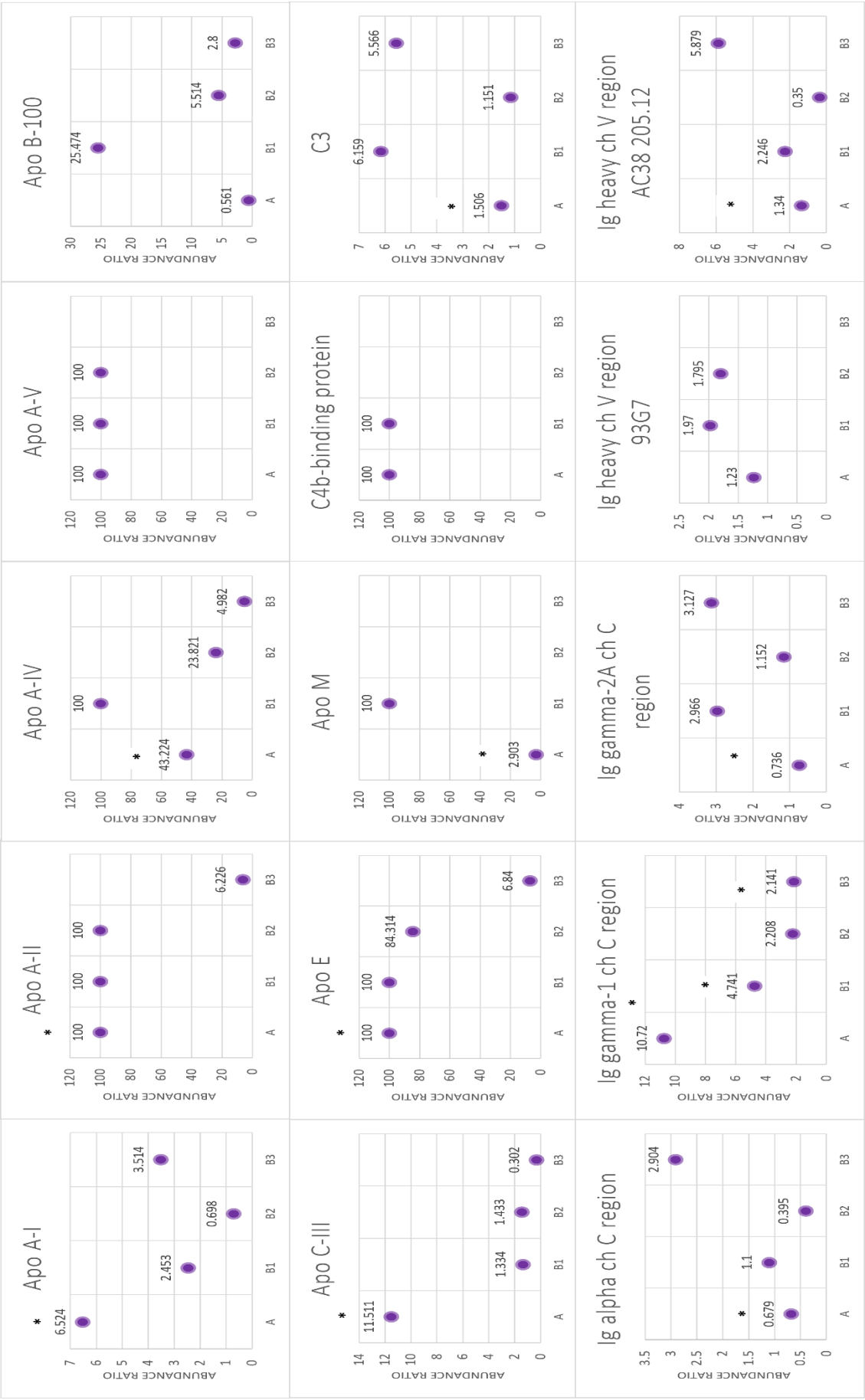


Figure 4: FunRich biological amyloidosis processes related to different proteomics datasets.

Focusing on proteins involved in amyloidosis process, we selected the following proteins displayed in Figure 5 in alphabetical order: Apo A-I, Apo A-II, Apo A-IV, Apo A-V, Apo B-100, Apo C-III, Apo E, Apo M, C4b-binding protein , C3, Ig alpha ch C region, Ig gamma-1 ch C region, Ig gamma-2A ch C region, Ig heavy ch V

region 93G7, Ig heavy ch V region AC38 205.12, Ig k ch V-II region 26-10, Ig k ch V-III region PC7175, Ig k ch V-IV region S107B, IgG receptor FcRn sub p51, Immunoglobulin heavy const mu, Immunoglobulin J ch, Immunoglobulin k ch var 12-41, Immunoglobulin k const, Serum amyloid P-component and Transthyretin. First, Apo A-I is significantly upregulated in BAG3 β KO mouse model compared to control in the global proteomic analysis and presents the same expression trend in BAG3 β KO Congo Red⁺ comparisons; on the other hand, no significant modulation in BAG3 β KO Congo Red⁻ analysis is observed. Regarding Apo A-II, it's considered an exclusive presence (abundance ration of about 100) in the BAG3 β KO mouse when compared to control, while an overexpression trend was observed comparing BAG3 β KO Congo Red⁺ with negative areas; as for Apo A-IV, it's considered a unique protein in BAG3 β KO Congo Red⁺ regions compared to the control with an abundance ratio of about 100. On the other hand, Apo A-V appears to be exclusively expressed in the BAG3 β KO mouse models in all proteomic analyses carried out except in the BAG3 β KO Congo Red⁺ compared to the negative regions. Apo B-100 presents no expression modulation in the global proteomic analysis, but it's over-expressed in the three micro-proteomic analyses with no significant abundance ratio p-value. Apo C-III is significantly upregulated in the BAG3 β KO mouse model compared to the control condition and is significantly downregulated in the BAG3 β KO Congo Red⁺ compared to the negative regions analysis. Apo E is considered a unique protein in the global proteomic analysis and in the analysis on Congo Red⁺ regions compared to the control and presents an upregulation trend in other micro-proteomic analyses. Finally, Apo M is upregulated in the global proteomic analysis, and it's exclusively expressed in BAG3 β KO Congo Red⁺ compared to the control; on the other hand, there is not detected in other micro-proteomic analyses. Also, C4b-binding protein is a unique protein in global proteomics and in micro-proteomic analyses performed on BAG3 β KO Congo Red⁺ areas. As for the trend in protein expression of the immunoglobulin classes, Ig alpha chain C region is downregulated in the micro-proteomic analysis of BAG3 β KO Congo Red⁻, while the Ig gamma-1 chain C region presents a significant upregulation in all analyses performed; moreover, Ig gamma-2A chain C region is upregulated exclusively within analyses on Congo Red⁺ regions. Globally, Ig heavy chain V region 93G7 and Ig kappa chain V-IV region S107B display a not significant modulation. Ig heavy chain V region AC38 205.12 presents an upregulation in the analysis on Congo Red⁺ regions and a downregulation in

the Congo Red- regions analysis. On the one hand, Ig kappa chain V-II region 26-10 charts an upregulation in the global proteomic analysis on the BAG3 β KO mouse model compared to the control and in the BAG3 β KO Congo Red+ compared to the negative regions. On the other hand, Ig kappa chain V-III region PC7175 presents a significant upregulation in the global proteomic analysis and no modulation/detection in the further micro-proteomic analyses. IgG receptor FcRn sub p51, as well as Immunoglobulin kappa constant, displays a downregulation in the micro-proteomic analysis on Congo Red- regions; on the other hand, Immunoglobulin kappa constant charts an upregulation in the BAG3 β KO Congo Red+ compared to the negative regions. All together Immunoglobulin heavy constant mu, Immunoglobulin J chain and Immunoglobulin kappa chain variable 12-41 show an upregulation in the Congo Red+ areas. Finally, Serum amyloid P-component results upregulated in the BAG3 β KO mouse model compared to the control condition in all four proteomics analyses. Interestingly, Transthyretin is upregulated only in the BAG3 β KO Congo Red+ areas compared to the negative and the control, seeming to be deposited in that spleen's area.



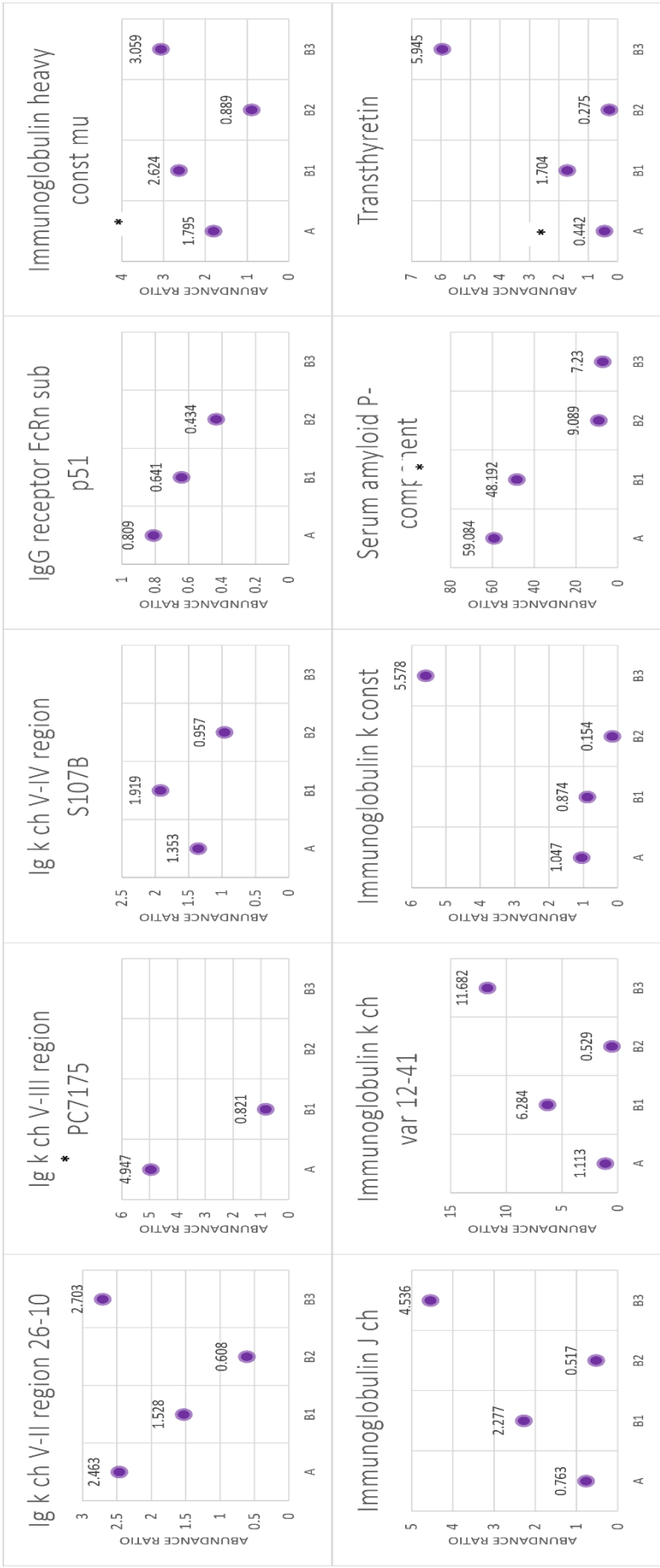
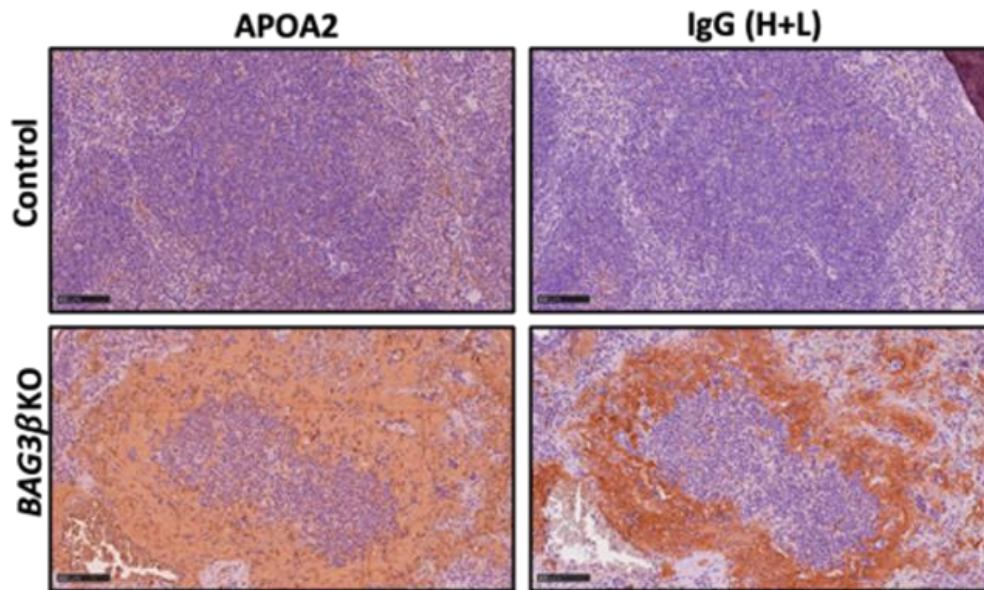


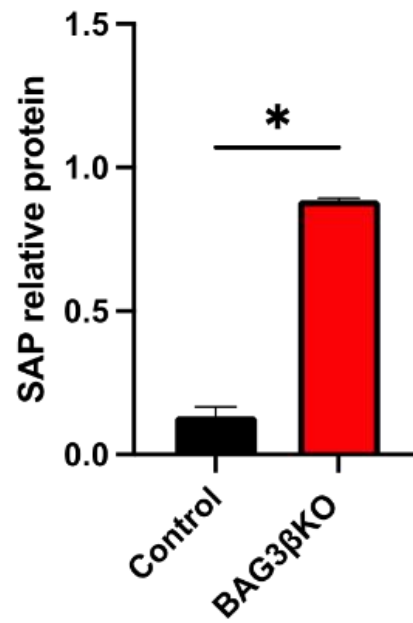
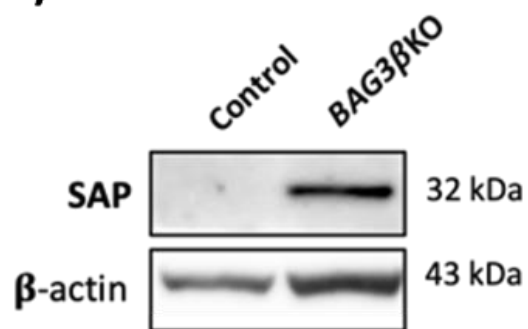
Figure 5: Graphical representation of the abundance ratio trend for each protein across the four performed proteomic analyses: A) label-free shotgun proteomic analysis on BAG3 β KO mice spleen compared to control; B1) BAG3 β KO Congo Red+ regions compared to control; B2) BAG3 β KO Congo Red- regions compared to control and B3) BAG3 β KO Congo Red+ regions compared to BAG3 β KO Congo Red- regions. It is possible to notice that abundance ratio values characterized by a p-value < 0.05 are highlighted with an asterisk in the various plots depicted.

To further confirm the amyloidogenic process activation in the BAG3 β KO Congo Red⁺ regions, Gene Ontology and enrichment analysis by IPA highlights an activation (z-score value: 1.807) of the “amyloid fiber formation” canonical pathway in only Congo Red⁺ versus Congo Red⁻ regions. Immunohistochemical staining corroborates an accumulation of both Apo A-II and IgG (H+L) in BAG3 β KO mice versus controls, as shown in Figure 6A. Finally, we carried out a western blot analysis of SAP, which results significantly increased in BAG3 β KO mice spleen versus controls, as described in Figure 6B with also the relative quantification; moreover, evaluation of circulating SAP level shows in Figure 6C how that protein seems to be deposited in amyloid deposits since it's decreased in BAG3 β KO plasma compared to the control.

A)



B)



C)

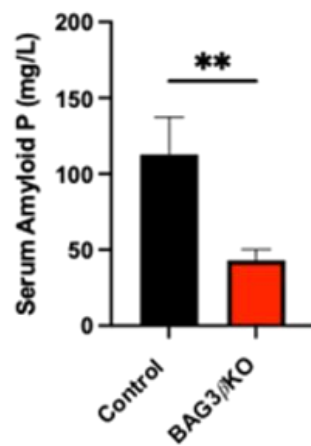


Figure 6: A) Immunohistochemical staining of Apo A-II and IgG (H+L) confirms a positive presence in BAG3 β KO model compared to the control condition. B) Western blot analysis and relative quantification on spleen and C) evaluation of circulating SAP level. Data are presented as mean value $\pm$ SEM; n=4 animals for each experimental condition; *p <0.05; **p<0.01.

Functional Gene Ontology and enrichment analysis unveil changes in BAG3 β KO splenic metabolism

We finally decided to perform a functional Gene Ontology and enrichment analysis to highlight also interesting changes in BAG3 β KO splenic metabolism by using “canonical pathways analysis” and “upstream regulators” tools in IPA. Functional results unveil the same modulation trend for many metabolic pathways in BAG3 β KO spleens such as branched-chain amino acid catabolism, ethanol degradation, fatty acid oxidation, fatty acyl-CoA biosynthesis, gluconeogenesis I, glucose metabolism, glutamate and glutamine metabolism, glycogen degradation and metabolism, glycolysis I, ketogenesis, ketolysis, and oxidative phosphorylation. In the “upstream regulators” functional analysis, IPA predicts the inhibition of the insulin receptor (INSR) in all BAG3 β KO spleen, as shown in Figure 7A, presenting the same trend evaluated in our previous work on BAG3 β KO muscle (1); moreover the “signaling by INSR” canonical pathway is also predicted to be downregulated in all performed proteomic analyses. So that, in Figure 7B, we further validate this proteomics result by western blot and relative quantification, which confirm a reduced expression of insulin receptor (INSR) in BAG3 β KO spleen compared to control mice, proving that BAG3 β KO mouse model results in a significant insulin resistance both in spleen and muscle.

A)

Analysis	INSR z-score value
BAG3KO Congo Red+ Vs BAG3KO Congo Red-	-4.886
BAG3KO Congo Red- Vs Control	-2.94
BAG3KO Congo Red+ Vs Control	-5.31
BAG3KO Vs Control	-7.531

Analysis	Signaling by INSR
BAG3KO Congo Red+ Vs BAG3KO Congo Red-	-2
BAG3KO Congo Red- Vs Control	-1.897
BAG3KO Congo Red+ Vs Control	-3
BAG3KO Vs Control	-4.123

B)

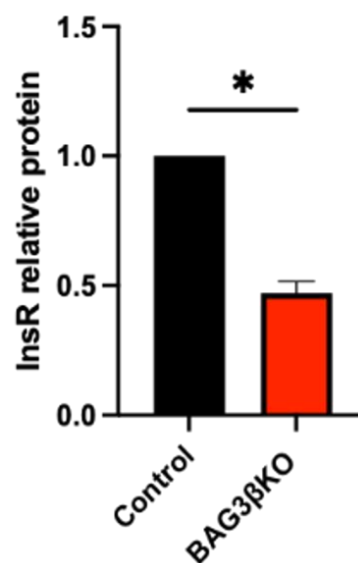
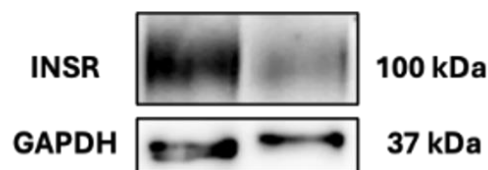


Figure 7: A) Upstream regulators analysis shows the predicted inhibition of INSR in all performed proteomic analyses (z-scores < 2); in particular, the network below shows the significantly inhibited INSR predicted by IPA analysis in proteomics of BAG3 β KO spleen versus control (z-score value: -7.531). B) Western blot analysis and quantification of INSR describes its decrease in BAG3 β KO spleen. Data are presented as mean value $\pm$ SEM; n=4 animals for each experimental condition; *p < 0.05; **p < 0.01.

Discussion and conclusions

Using our previous work as a background to explain the role of BAG3 in glucose homeostasis and insulin secretion in liver and muscle's pancreatic beta-cells knockout (1), in this paper we again investigated BAG3's role in the spleen of the same mouse model. Since chronically altered metabolic condition leads in time to amyloid deposits in 64-weeks-old BAG3 β KO mice' spleens, we then decided to focus on proteomics characterization of amyloid deposits and identification and/or confirmation of any metabolic alterations.

Regarding the first aim of this work, we initially confirmed that amyloidosis modifies spleen tissue architecture in our mouse model. To further characterize amyloid deposits, we performed two different approaches to proteomic analysis such as label-free proteomics and laser-capture micro-proteomics, since they represent innovative methodologies for the in-depth analysis and characterization of proteins in biological samples, which can be particularly invaluable for studying amyloidosis. On the one hand, label-free proteomics is a method for identifying and quantifying proteins without the use of molecular labels or dyes. Unlike label-based approaches which require the tagging of proteins or peptides, label-free approaches typically rely on mass spectrometry to analyze the protein composition of a sample directly. In the context of amyloidosis, label-free proteomics can be used to study the protein composition of amyloids in affected tissues. On the other hand, laser-capture microdissection (LCM) followed by micro-proteomics is a highly precise approach used to isolate specific cells or microscopic regions from tissue sections. Once the desired cells are isolated, micro-proteomics—proteomic analysis on a microscale—can be applied. LCM allows for the selective examination of individual cells within a tissue that may be affected by amyloid deposits. This is potential for biomarker discovery, since analysis of the proteins in regions of tissue affected by amyloidosis may lead to the identification of novel biomarkers for early detection and prognosis of the disease. Both label-free proteomics and laser-capture micro-proteomics are instrumental in advancing our understanding of amyloidosis. By providing detailed protein expression profiles and enabling the study of specific cellular changes associated with amyloid deposition, these allow for a more comprehensive characterization of the disease, facilitating better diagnostic, prognostic, and therapeutic strategies. Globally, we were able to characterize amyloid deposits' composition by both two proteomics approaches with validation on the overexpression of Apo A-II, IgG (H+L) and SAP in BAG3 β KO mice spleens. Moreover, we could also demonstrate the activation of biological processes related to amyloidosis by functional gene ontology and enrichment analyses performed by FunRich and IPA, which confirms the amyloidogenic process activation in BAG3 β KO Congo Red⁺ areas compared to the negative. As regards the metabolic picture within BAG3 β KO mice, we unveil changes also in splenic metabolism, confirming the same metabolic trend previously described in liver and muscle (1). Finally, we also confirmed the insulin resistance onset in 64-weeks-old BAG3 β KO mice spleen as well as in muscle,

assuming therefore that the knockout of BAG3 in pancreatic beta-cells is related to the onset of marked insulin resistance in some extra-pancreatic organs: in the functional analysis we found out the predicted inhibition of INSR, as an upstream regulator, as well as the same predicted modulation trend for insulin receptor signaling, insulin secretion signaling and signaling by insulin receptor pathways, further validated by western blot and quantification analysis. Finally, we again showed that specific knockout of BAG3 in pancreatic β -cells leads to alteration of insulin secretion, in turn related to the onset of insulin resistance in spleen and muscle. Moreover, this chronically impaired metabolic condition leads to organ damage, in particular in spleen, where we described the development of amyloid deposits.

Our findings highlight the complex role of BAG3 in regulating insulin secretion, systemic metabolism, and tissue homeostasis, while also uncovering novel connections to splenic amyloidosis. The results of this study raise several intriguing possibilities worth further exploration. First, the observed amyloidogenic process in BAG3 β KO mice, particularly the accumulation of amyloid deposits in the spleen, might be indicative of a broader systemic impact of BAG3 deletion on protein homeostasis. The upregulation of amyloidogenic proteins suggests that BAG3 may play a protective role in preventing aberrant protein aggregation. This finding aligns with BAG3's known function in autophagy and chaperone-mediated protein degradation. The potential link between BAG3 and amyloid fiber formation offers a unique perspective on how BAG3 dysregulation could contribute to diseases characterized by amyloid deposition, such as systemic amyloidosis, and even non-amyloidogenic proteinopathies.

Second, the simultaneous alteration in metabolic pathways suggests that the spleen could serve as a metabolically active organ under certain pathological conditions. This challenges the conventional view of the spleen primarily as an immune organ and positions it as a participant in systemic energy homeostasis. The predicted inhibition of insulin receptor signaling in the spleen raises the question of whether splenic insulin resistance contributes to the systemic metabolic dysfunction seen in BAG3 β KO mice. Moreover, the observed changes in metabolic pathways overlap with similar findings in BAG3 β KO muscle, as reported previously (1). This parallel suggests a coordinated multi-organ dysfunction in response to BAG3 deletion, potentially

mediated by impaired insulin signaling and altered protein homeostasis. The hypothesis that BAG3 may act as a central regulator of metabolic and proteostatic networks warrants further investigation.

Materials and methods

Mouse models generation and maintenance

All animal experimental programs were conducted according to protocols approved by the Institutional Animal Healthcare of the University of Chieti and in accordance with the institutional guidelines of the Italian Ministry of Health (Protocol No. 204/2022-PR). Mice were housed in a pathogen-free environment (22 °C, 12-h/12-h light/dark cycle), where they accessed freely to food and water. The generation of beta cell-specific BAG3 knockout mice (BAG3 β KO) and the mating strategies adopted have been described in our previous work (1). Briefly, BAG3 β KO were obtained by crossing BAG3 homozygous floxed mice with mice expressing Cre recombinase under the mouse insulin 1 promoter. Aged-matched control and BAG3 β KO male mice were used for the experiments (Figure 8).



Figure 8: Schematic representation of BAG3 β KO mice timeline: once the mouse model was generated, spleens were isolated after 64 weeks for subsequent analyses (Image generated by Biorender.com).

Immunohistochemistry (IHC) analysis

Spleens were harvested and fixed in 10% neutral buffered formalin (05-01005Q, Bio-Optica), embedded in paraffin and then sectioned at 5- μ m thickness. Sections were stained with Hematoxylin (05-06002/L, Bio-Optica) and Eosin (05-M11007, Bio-Optica) for histological analysis and were independently evaluated by two pathologists. Immunohistochemical staining on formalin fixed paraffin-embedded (FFPE) tissues were performed using the following primary antibodies: rat anti-mouse B220 antibody (550286, BD Pharmingen), rabbit anti-mouse CD3 antibody (ab16669, Abcam), rabbit anti-mouse F4/80 antibody (#70076, Cell Signaling), rabbit anti-mouse APOA2 (16845-1-AP, Proteintech), followed by the appropriate secondary antibodies. For IgG immunostaining, biotin-SP goat anti-mouse IgG (H+L) (115-065-003, Jackson) was used as secondary antibody. Immunostainings were developed with HRP methods and the DAB Chromogen system (Dako). After chromogen incubation, slides were counterstained in Hematoxylin (Bio-Optica). For histological assessment of amyloid deposition, Congo-red staining was performed using the Congo-red Staining Kit (04-210822A, Bio-Optica). Amyloid has been identified by apple-green birefringence when visualized under polarized light of Leica microscope. For histological and immunohistochemical evaluations, whole slide sections were scanned with Nanozoomer scanner from Hamamatsu and analyzed with Qu-Path v0.3.2 software using the positive cell detection tool for CD3+, B220+ and F4-80+ cell quantifications (expressed as % of positive cells) and pixel classifier tool for apple-green birefringence evaluation (expressed in pixel).

Label-free proteomic analysis

Following collection, snap-freezing on liquid nitrogen and storage at -80°C, spleens obtained from BAG3 β KO and control mice, were analyzed by label-free shotgun proteomics analysis according to the FASP (Filter Aided Sample Preparation) protocol. Briefly, as reported in our previous work (25), these spleens were lysed by mechanical homogenization using a turrax mixer (1-minute, highest speed) and ultrasonication (80% amplitude, Sonicator U200S control, IKA Labortechnik, Staufen, Germany) on ice in lysis buffer (urea 6 M in 100 mM Tris/HCl, p H = 7.5), followed by centrifugation (1300 rpm) to remove tissues debris. A total of 50 μ g

of proteins for each condition were digested using trypsin (0,5 µg/µl Trypsin, Sigma-Aldrich, St. Louis, MI, USA). For protein label-free identification and quantification, 5µl of tryptic peptides (corresponding to 0,01 µg/ml) from each condition were analyzed in triplicate with an EASY-spray Acclaim™ PepMap™ C18 (75 µm ID, 25 cm L, 2 µm PS, Thermo Fisher Scientific) nanoscale chromatographic column and a total run time of 125 min using a chromatographic gradient from 5 to 90% of 100% acetonitrile (ACN) and 0.1% formic acid (FA). Proteomics data were acquired according to Di Stefano et al., as previously mentioned.

Micro-proteomic analysis

Histology-grade solvents and Congo Red were purchased from DiaPath S.p.A. (Martinengo, Italy). SuperFrost Plus glass slides were purchased from VWR International, LLC (Radnor, PA, USA). LC-MS grade water, acetonitrile (ACN) and formic acid (FA) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Phosphoric acid was purchased from Merck (Darmstadt, Germany). All the other reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA). FFPE blocks of spleens from 3X Control and 3X BAG3 KO mice were sectioned (8 µm thickness) using a Leica RM2245 microtome (Leica, Wetzlar, Germany) and mounted on a charged glass slide using a warm bath (Leica HI 1210, Leica). Slides were heated at 60°C for one hour to melt paraffin and then washed twice for 15 minutes with xylene. Congo Red staining was performed according to the vendor's instructions. Slides were microdissected using a Zeiss Axio Observer Z1 microscope equipped with a PALM Microbeam laser and a RoboMover (both Zeiss, Oberkochen, Germany). Amyloid areas were detected in fluorescence mode1 (HPX 120C lamp, 43 DSRed filter, Zeiss) and confirmed as CR-stained under bright field using the PALMRobo software (v 4.9, Zeiss). The microdissection laser was operated in AutoLPC mode, with a distance between the spots of 10 µm and a distance from the region border of 5 µm. Fragments were collected in the cap of Adhesive cap-tubes and stored at -20°C until processing. Microdissected tissue fragments were transferred from the cap of the Adhesive Cap tube to a clean tube using thirty-five microliters of 10mM Tris/ 1mM EDTA/5% SDS lysis buffer. Heat-aided antigen retrieval was performed by heating the samples to 98°C for 90 minutes with occasional vortexing. Samples were lysed (15 cycles, 30 sec ON, 30 sec

OFF) using a Bioruptor Pico (Diagenode, Liège, Belgium). Samples were then heated to 95°C for 10 minutes. Protein quantification was performed using a modified microBCA protocol². In brief, sample aliquots were diluted 1:1 with water to lower the SDS content. Sample and BSA standards were incubated at 60°C for 1 hour with the working reagent. Absorbance was read using an Eppendorf BioSpectrometer (Eppendorf, Hamburg, Germany). The amount of protein extract subject to proteomics analysis from each microdissected tissue fragment was 0.35 µg. Proteins were reduced with dithiothreitol (DTT, final concentration 20 mM, 45°C for 30 min) and alkylated iodoacetamide (IAA, final concentration 40mM, RT for 30 minutes in the dark). Excess IAA was quenched by addition of another aliquot of DTT (final concentration 40mM). Proteins were digested using a S-TrapTM Micro (ProtiFI, Farmingdale, NY, USA). A ratio of 1:10 v/v of 27.5% phosphoric acid was added to each sample, followed by 400 µL of S-trap binding buffer (90% MeOH, final TEAB concentration of 100mM, pH 7.1). Samples were loaded onto the S-trap columns and centrifuged at 4000xg for 1 minute and the flow-through discarded. This process was repeated until all the sample was loaded on the column. Bound proteins were washed four times with 150 µL of S-trap binding buffer. 0.5 µg of trypsin/LysC (Promega, Madison, WI, USA) in 20 µL of digestion buffer (50 mM TEAB, pH 8) were added to the column and samples were incubated at 37°C for 18 hours. Peptides were eluted in three steps with 40 µL of 50 mM TEAB, 40 µL 0.2% FA and 35 µL of 50% ACN, each followed by a centrifugation at 4000xg for 1 minute. Peptides were vacuum-dried and resuspended in 10% formic acid. Peptides were separated on an EASY-nLC 1000 (Thermo Scientific) equipped with an Acclaim PepMap 100 pre-column (2 cm x 75 µm, C18, 3 µm, 100 Å; Thermo Scientific) and an EASY-Spray analytical column (ES803: 50 cm x 75 µm, C18, 2 µm, 100 Å; Thermo Scientific). Peptides were separated with a 60 minute gradient and analyzed using an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Diego, USA), using the Top Speed data dependent acquisition method, with a 3 seconds cycle time; MS1 scans were performed in the Orbitrap (m/z 375 to 1500 at 120K resolution, AGC Target 5x10⁵ and 100 ms maximum injection time) and MS2 scans were acquired in the ion trap using a 1.6 m/z isolation window, 30% HCD normalized Collision Energy and an AGC target of 5x10³.

Proteomics and micro-proteomics data processing

Proteomics raw data obtained by both label-free shotgun proteomics and micro-proteomics analyses, were processed using Thermo Proteome Discoverer (PD) version 2.4.0.305 (Thermo Fischer Scientific) to identify differentially expressed proteins (DEPs) between respectively BAG3 β KO versus control mice, BAG3 β KO Congo Red positive (Congo Red+) regions versus control mice, BAG3 β KO Congo Red negative (Congo Red-) regions versus control mice and BAG3 β KO Congo Red+ versus Congo Red- regions. As “Spectrum Selector”, MS1 precursor was used for the precursor selection with a mass-range about 375-1500 Da. Sequest HT was used to search sequence database “uniprotkb_mouse_AND_reviewed_true_2023.fasta” with maximum missed cleavage sites about 2 and precursor mass tolerance about 10 ppm. Carbamidomethylation of cysteines (C) was defined as fixed and quantification modification, while oxidation of methionines (M) and deamidation of asparagines (N) and glutamines (Q) were set as variable modifications. False discovery rate (FDR) was set at 1% of both for protein and for peptide levels. In the quantification process, as PD 2.4.0.305 parameters, we select “Unique + Razor” as peptides to use in the general quantification setting; the precursor abundance quantification was based on the intensity and the normalization was performed on the total peptide amount. Protein abundance was calculated by “Summed abundances”; protein ratios were calculated as “Protein Abundance Based” and the statistics hypothesis by ANOVA was based on individual proteins. The statistical analysis was performed to individuate DEPs between two experimental conditions using the “Protein Abundance Ratio” value and “p-value” for each quantified protein. In order to perform a robust quantification, we removed all contaminant proteins and set the parameters “High identification confidence” for each protein in at least two of three analytical replicates and number of “Unique peptides” greater than 1.

Functional gene ontology and enrichment analysis

Finally, DEPs lists were loaded into functional analysis tools such as FunRich and Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany). FunRich is a stand-alone software tool used mainly for functional enrichment and interaction network analysis of genes and proteins; so that, the analysis of biological processes was

performed with FunRich 3.1.3 analysis tool (28). Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany) is a powerful tool that allows to associate biological functions and diseases to experimental results, by leveraging the complex biological interactions that are stored in the Ingenuity Knowledge Base. IPA functional analysis was designed to organize biological information in a way that allows to gain a high-level overview of the general biology that is associated with “omics” data and then access the more specific and detailed information. So that, obtained lists of DEPs by each quantitative proteomics analysis, were uploaded to the IPA tool to perform a separate gene ontology and functional enrichment analysis and to map statistically each protein with its functional annotation. IPA uses the z-score algorithm to make predictions on the activation (z-scores ≥ 2.0) or inhibition (z-scores ≤ -2.0) of well-characterized metabolic and cell signaling pathways defined as “canonical pathways” analysis and some upstream transcriptional regulators that can explain the observed protein expression changes in the dataset, defined as “upstream regulators analysis”. Consistent with literature across most molecules, IPA predicts that molecules/diseases or functions will increase in the experimental sample; on the other hand, mostly inconsistent with the literature, IPA predicts that molecules/diseases or functions will decrease in the experimental sample; with no clear pattern related to the literature, IPA does not make a prediction on the modulation. “Canonical pathways analysis” depicts the proteins involved, their interactions, and the cellular and metabolic reactions in which the pathway is involved. IPA uses the p-value, calculated by the right-tailed Fisher’s Exact Test, to represent statistical overlap measurement between the protein dataset or function categories and to identify significant pathways; the significance is attributed to p-value < 0.05 and indicates the probability of association of molecules from proteins dataset with the canonical pathway by random chance alone.

Western blot analysis

Tissues were homogenized using ice-cold lysis buffer (TRIS-HCl 50 mM pH 8, NaCl 150 mM, TritonX-100 1%, NaF 100 mM, EDTA 1 mM, MgCl₂ 1 mM, Glycerol 10%) with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Proteins were quantified by using the Bradford method (Bio-Rad). The same quantity of

proteins was loaded on SDS-polyacrylamide gel electrophoresis gels (10%) and subsequently transferred to a nitrocellulose membrane (GE Healthcare Life Science, Piscataway, NJ, USA). The membranes were incubated overnight at 4 °C with primary antibodies: anti-SAP (Serum Amyloid P Component) used 1:1000 (Proteintech, Manchester, UK), anti-INSR (Insulin Receptor) and anti-GAPDH used 1:1000 supplied by Cell Signaling Technology (Danvers, MA, USA), anti- β -actin used 1:40000 (Sigma-Aldrich, Milan, Italy). Following that, the membranes were washed and incubated for 1h at RT with the secondary antibody, diluted 1:20,000 (Bio-Rad, Milan, Italy). The enhanced chemiluminescent (ECL) method (PerkinElmer Italia, Milan, Italy) was employed to detect bound antibodies.

Plasma SAP measurement

Plasma SAP concentration was quantified using enzyme-linked immunosorbent (ELISA) assay following the manufacturer's instructions. The kit was purchased from Crystal Chem (Crystal Chem Inc., Downers Grove, IL, USA).

Statistical analysis

Experimental results were expressed as means $\pm$ standard error (SEM). GraphPad Prism v.9 (GraphPad Software, Inc.) was used to perform statistical analyses. Student's t-test or Mann-Whitney U test were used. Unless specified otherwise, asterisks in the figures indicate statistical significance *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

References

1. Damiani V, Lamolinara A, Cicalini I, Cufaro MC, Del Pizzo F, Di Marco F, et al. Pancreatic beta-cell specific BAG3 knockout results in chronic hyperinsulinemia inducing insulin resistance. *Mol Metab.* agosto 2023;74:101752.
2. Brenner CM, Choudhary M, McCormick MG, Cheung D, Landesberg GP, Wang JF, et al. BAG3: Nature's Quintessential Multi-Functional Protein Functions as a Ubiquitous Intra-Cellular Glue. *Cells.* 19 marzo 2023;12(6):937.
3. Marzullo L, Turco MC, De Marco M. The multiple activities of BAG3 protein: Mechanisms. *Biochim Biophys Acta Gen Subj.* agosto 2020;1864(8):129628.
4. Fujimoto WY. The importance of insulin resistance in the pathogenesis of type 2 diabetes mellitus. *Am J Med.* 17 aprile 2000;108 Suppl 6a:9S-14S.
5. Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, et al. Pathophysiology of Type 2 Diabetes Mellitus. *Int J Mol Sci.* 30 agosto 2020;21(17):6275.
6. Groop L. Pathogenesis of type 2 diabetes: the relative contribution of insulin resistance and impaired insulin secretion. *Int J Clin Pract Suppl.* ottobre 2000;(113):3–13.
7. Roden M, Shulman GI. The integrative biology of type 2 diabetes. *Nature.* dicembre 2019;576(7785):51–60.
8. Yan T, Huang Y, Wu JHY, Zhuang XD, Pan XF. Editorial: Insulin Resistance, Metabolic Syndrome, and Cardiovascular Disease. *Front Cardiovasc Med.* 2022;9:959680.
9. Perdomo CM, Garcia-Fernandez N, Escalada J. Diabetic Kidney Disease, Cardiovascular Disease and Non-Alcoholic Fatty Liver Disease: A New Triumvirate? *J Clin Med.* 10 maggio 2021;10(9):2040.

10. Seo DH, Suh YJ, Cho Y, Ahn SH, Seo S, Hong S, et al. Advanced Liver Fibrosis Is Associated with Chronic Kidney Disease in Patients with Type 2 Diabetes Mellitus and Nonalcoholic Fatty Liver Disease. *Diabetes Metab J.* luglio 2022;46(4):630–9.
11. Iorio V, Festa M, Rosati A, Hahne M, Tiberti C, Capunzo M, et al. BAG3 regulates formation of the SNARE complex and insulin secretion. *Cell Death Dis.* 12 marzo 2015;6(3):e1684.
12. Youn DY, Lee DH, Lim MH, Yoon JS, Lim JH, Jung SE, et al. Bis deficiency results in early lethality with metabolic deterioration and involution of spleen and thymus. *Am J Physiol Endocrinol Metab.* dicembre 2008;295(6):E1349-1357.
13. Hawkins PN. Amyloidosis. *Blood Reviews.* dicembre 1988;2(4):270–80.
14. Khalighi MA, Dean Wallace W, Palma-Diaz MF. Amyloid nephropathy. *Clin Kidney J.* aprile 2014;7(2):97–106.
15. Cuddy SAM, Falk RH. Amyloidosis as a Systemic Disease in Context. *Canadian Journal of Cardiology.* marzo 2020;36(3):396–407.
16. Palladini G, Milani P. Diagnosis and Treatment of AL Amyloidosis. *Drugs.* febbraio 2023;83(3):203–16.
17. Goldis R, Kaplan B, Kukuy OL, Arad M, Magen H, Shavit-Stein E, et al. Diagnostic Challenges and Solutions in Systemic Amyloidosis. *Int J Mol Sci.* 28 febbraio 2023;24(5):4655.
18. Ryšavá R. AL amyloidosis: advances in diagnostics and treatment. *Nephrol Dial Transplant.* 1 settembre 2019;34(9):1460–6.
19. Muchtar E, Dispenzieri A, Magen H, Grogan M, Mauermann M, McPhail ED, et al. Systemic amyloidosis from A (AA) to T (ATTR): a review. *J Intern Med.* marzo 2021;289(3):268–92.
20. Mirioglu S, Uludag O, Hurdogan O, Kumru G, Berke I, Doumas SA, et al. AA Amyloidosis: A Contemporary View. *Curr Rheumatol Rep.* luglio 2024;26(7):248–59.
21. Dogan A. Amyloidosis: Insights from Proteomics. *Annu Rev Pathol.* 24 gennaio 2017;12:277–304.

22. Ni R. Magnetic Resonance Imaging in Animal Models of Alzheimer's Disease Amyloidosis. *Int J Mol Sci*. 25 novembre 2021;22(23):12768.
23. Salahuddin P, Fatima MT, Uversky VN, Khan RH, Islam Z, Furkan M. The role of amyloids in Alzheimer's and Parkinson's diseases. *International Journal of Biological Macromolecules*. novembre 2021;190:44–55.
24. Wechalekar AD, Gillmore JD, Hawkins PN. Systemic amyloidosis. *The Lancet*. giugno 2016;387(10038):2641–54.
25. Di Stefano J, Garcia-Pupo L, Di Marco F, Motaln H, Govaerts J, Van Breedam E, et al. Transcriptomic and proteomic profiling of bi-partite and tri-partite murine iPSC-derived neurospheroids under steady-state and inflammatory condition. *Brain Behav Immun*. ottobre 2024;121:1–12.
26. Vrana JA, Gamez JD, Madden BJ, Theis JD, Bergen HR, Dogan A. Classification of amyloidosis by laser microdissection and mass spectrometry-based proteomic analysis in clinical biopsy specimens. *Blood*. 2009;114(24):4957–9.
27. De Graaf EL, Pellegrini D, McDonnell LA. Set of Novel Automated Quantitative Microproteomics Protocols for Small Sample Amounts and Its Application to Kidney Tissue Substructures. *Journal of Proteome Research*. 2016;15(12):4722–30.
28. Pathan M, Keerthikumar S, Ang CS, Gangoda L, Quek CYJ, Williamson NA, et al. FunRich: An open access standalone functional enrichment and interaction network analysis tool. *Proteomics*. agosto 2015;15(15):2597–601.
29. Lenti MV, Luu S, Carsetti R, Osier F, Ogowang R, Nnodu OE, et al. Asplenia and spleen hypofunction. *Nat Rev Dis Primers*. 3 novembre 2022;8(1):71.
30. Lundmark K, Vahdat Shariatpanahi A, Westermarck GT. Depletion of spleen macrophages delays AA amyloid development: a study performed in the rapid mouse model of AA amyloidosis. *PLoS One*. 2013;8(11):e79104.

CHAPTER 4

STUDY 3

From BAG3albKO mouse model generation to proteomic analysis

Liver-specific BAG3 knockout delays chemically induced hepatocellular carcinoma development

This research manuscript has been submitted as:

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BAG3 in Hepatocellular carcinoma: insights into tumor development, progression, and signaling pathways from hepatocyte-specific knockout model

Bcl-2-associated athanogene 3 (BAG3) is an evolutionary conserved, multifunctional protein that plays critical roles in a variety of cellular processes (1), (2). Functionally, BAG3 acts as a co-chaperone, assisting in the refolding of misfolded proteins and directing them toward degradation via the ubiquitin-proteasome system or autophagy (3), (4). In addition to its chaperone activities, BAG3 regulates apoptosis, cell adhesion, and cytoskeletal dynamics, thereby contributing to cellular homeostasis and the cellular response to stress (5), (6), (7), (8), (9). Consequently, dysregulation of BAG3 has been implicated in numerous pathological conditions, including cancer, cardiovascular diseases, and impaired glucose metabolism (2), (10), (11), (12), (13), (14). BAG3 is frequently reported to be overexpressed in various cancer types, where its expression is predominantly linked to aggressive phenotypes. Through its anti-apoptotic properties, BAG3 promotes tumor cell survival, enabling these cells to evade programmed cell death mechanisms and thrive under adverse conditions (15), (16), (17). Hepatocellular carcinoma (HCC), the most common primary liver cancer in humans, ranks among the most aggressive malignancies globally. It represents a significant public health challenge due to its rising incidence and mortality rates (Globocan 2020) (18). The primary etiological factors for HCC include genetic mutations, chronic hepatitis, and infections with hepatitis B or C viruses (HBV, HCV, or both). In Western countries, alcohol-induced liver damage remains the leading cause of liver cirrhosis and the most significant risk factor for HCC (19), (20), (21). Additional risk factors include exposure to aflatoxins, iron overload (e.g., hemochromatosis), prolonged use of oral contraceptives, and excessive anabolic steroid consumption (22), (23), (24). HCC progression is a complex, multistage process. The early phases are characterized by hepatocyte exposure to damaging agents that initiate molecular and cellular changes within the liver microenvironment, ultimately disrupting critical signaling pathways (25), (26), (27), (28), (29), (30). Elevated BAG3 expression has been associated with aggressive tumor phenotypes and poor prognosis in HCC. However, the role of BAG3 in HCC is nuanced and sometimes contradictory (21), (31). For instance, a study by Xiao et al. demonstrated that BAG3 promotes epithelial-mesenchymal transition (EMT), tumor growth,

invasiveness, and angiogenesis in HCC (32). Furthermore, the study identified BAG3 and hypoxia-inducible factor 1-alpha (HIF-1 α) as reliable prognostic markers in HCC patients post-liver transplantation (33). Conversely, research by Kong et al. suggested that BAG3 may exert tumor-suppressive effects. According to this study, BAG3 directly interacts with glucose-6-phosphate dehydrogenase (G6PD), suppressing the pentose phosphate pathway (PPP), de novo DNA synthesis, and cell proliferation in HCC (34). These conflicting findings highlight the ambiguous role of BAG3 in HCC pathogenesis and underscore the necessity for further investigation to delineate its exact functions in liver cancer progression. To address this knowledge gap, the present study explores the role of BAG3 in tumor initiation and HCC development using a hepatocyte-specific BAG3 knockout mouse model (BAG3albKO). The study employed the diethyl-nitrosamine (DEN)-induced hepatocarcinogenesis model, a widely recognized and versatile tool that allows researchers to investigate various aspects of HCC progression, spanning from its early stages to advanced malignancy.

Results

Hepatocytes-specific BAG3 knockout liver development

To elucidate the liver-specific function of BAG3 during the pathogenesis of hepatocellular carcinoma (HCC), we generated hepatocyte-specific BAG3 knockout mice (BAG3albKO). These mice were developed by crossing BAG3 flox/flox mice with mice expressing Cre recombinase under the control of the albumin promoter. Cre recombinase expression facilitated the deletion of BAG3 floxed exons 2 and 3 (Figure 1A), resulting in the complete absence of BAG3 mRNA and protein expression. This was confirmed through qPCR, Western blot, and immunohistochemical analyses (Figures 1B–1D). To ensure recombination specificity, we evaluated BAG3 protein expression across various tissues and observed no evidence of off-target recombination (Figure 1E). Consistent with prior findings in a similar mouse model (35), BAG3albKO mice exhibited normal fertility, litter sizes, and longevity, without any apparent anatomical or phenotypic abnormalities. We specifically assessed potential liver abnormalities by analyzing hematoxylin and eosin (H&E)-stained liver sections from both control and BAG3albKO mice. Up to 12 weeks of age, no discernible differences were observed between

the groups. However, while the control mice showed no abnormalities up to 28 weeks of age, BAG3albKO mice began to display small clusters of altered cells at 16 and 20 weeks of age. At 28 weeks, BAG3albKO mice demonstrated the presence of hepatic bipotential progenitor cells (oval cells) forming foci of biliary duct hyperplasia, as confirmed by cytokeratin 19 immunohistochemical staining. Between 32 and 48 weeks of age, both BAG3albKO and control mice exhibited focal cellular changes and areas of extramedullary hematopoiesis, though these findings were not considered pathologically significant (Figures 1F and 1G).

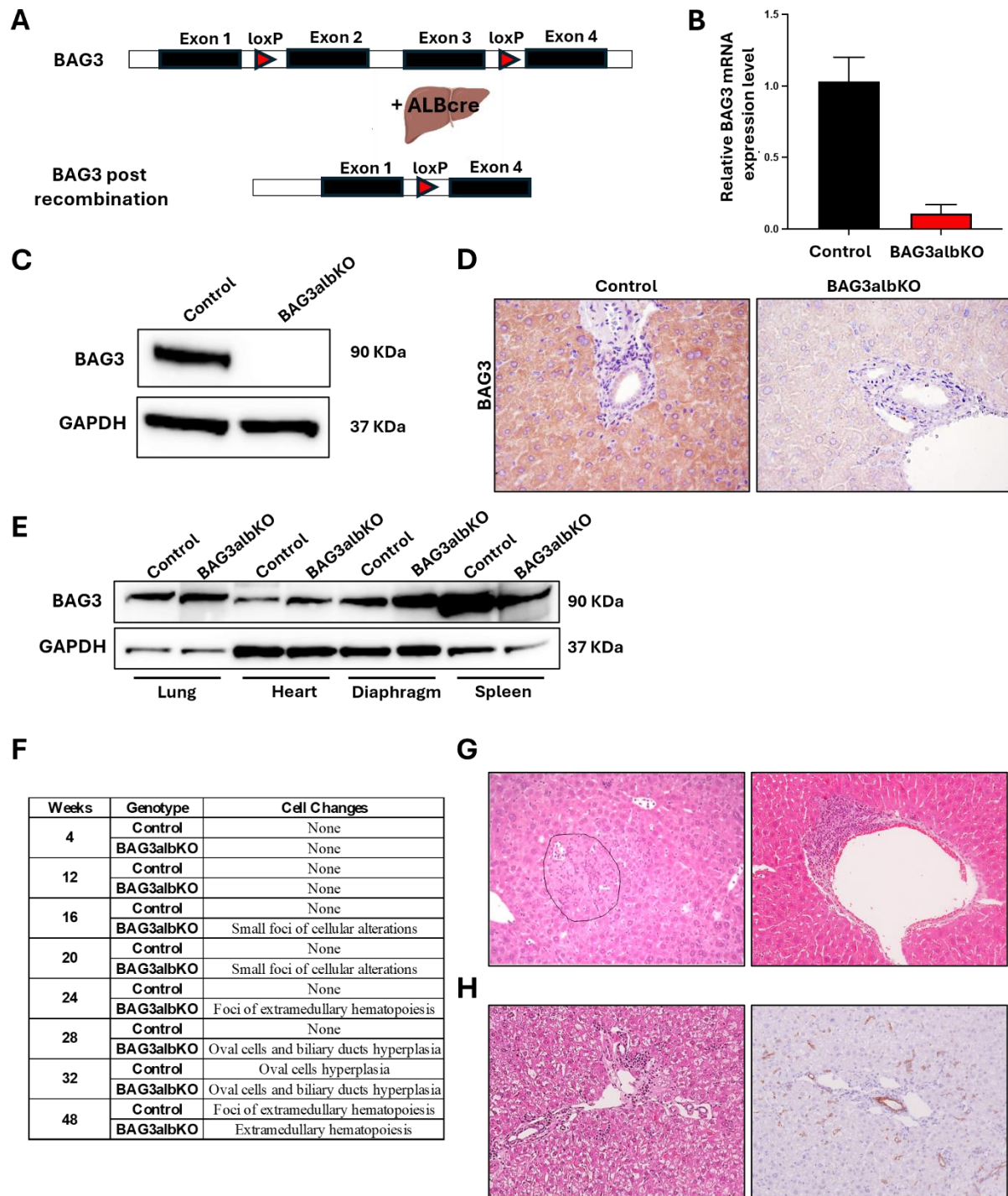


Figure 1: Establishment of hepatocytes-specific BAG3 knockout mice. (A) Simplified allele scheme showing hepatocytes specific BAG3 knockout strategy. (B) Relative BAG3 mRNA expression level in liver tissue detected by qRT-PCR. Data are presented as mean $\pm$ SEM (n=4 for each group). (C) Western Blot and (D) IHC analyses demonstrating BAG3 loss in the liver of BAG3albKO mice compared to Control. (E) Western Blot analysis confirms no differences in BAG3 expressions in lung, heart, diaphragm, and spleen between BAG3albKO and Control mice. (F) Table of major Pathological findings observed overtime, from 1 to 12 months of age, between BAG3albKO and Control mice (n=3-4 for each group). (G)

Representative H&E images of small foci of altered cells, encircled (left), of foci of extramedullary hematopoiesis (left) and (H) biliary ducts hyperplasia (left) confirmed by cytokeratin 19 immunostaining (right).

BAG3albKO delays early stages of DEN-induced hepatocarcinogenesis

To explore the potential role of BAG3 in tumorigenesis, hepatocellular carcinoma (HCC) was induced in mice using the genotoxic agent diethylnitrosamine (DEN). A single intraperitoneal (IP) injection of DEN at a dose of 25 mg/kg body weight, administered to 2-week-old male mice, is sufficient to induce liver tumor formation within 26–40 weeks (36), (37). At this developmental stage, DEN effectively promotes tumor initiation and progression (38). In this study, 2-week-old male mice received a single dose of DEN and were sacrificed at three time points: 22, 30, and 38 weeks of age (Figure 2A). Analysis of body weight and liver-to-body weight ratios revealed no significant differences between control and BAG3albKO mice at any of the time points evaluated (Figure 2C). Microscopic examination of liver lesions, however, uncovered a notable finding in BAG3albKO mice. At 22 and 30 weeks following DEN administration, BAG3albKO livers exhibited a reduced incidence of focal lesions, adenomas, and carcinomas compared to those of control mice (Figures 2B, 2D–E). Specifically, 62% and 19% of BAG3albKO mice were free of liver lesions at 22 and 30 weeks, respectively, while only 14% and 0% of control mice displayed no liver lesions at the corresponding time points. By 38 weeks after HCC induction, the differences in histopathological alterations between BAG3albKO and control mice were no longer evident. These findings suggest that the loss of BAG3 delays, but does not completely prevent DEN-induced hepatocarcinogenesis.

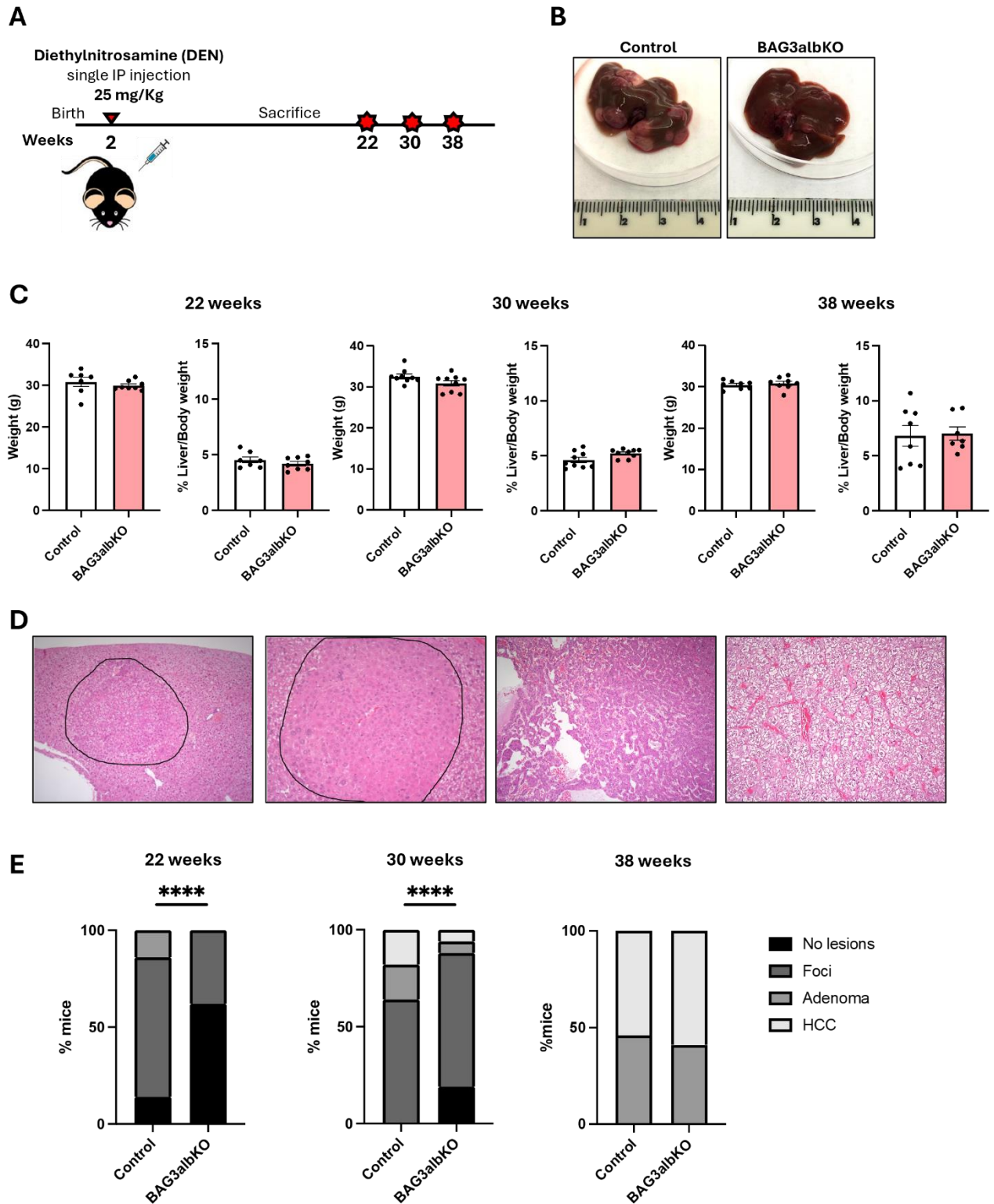


Figure 2: BAG3albKO delayed the development of pathological lesions in the liver in a DEN-induced HCC model. (A) Scheme of experimental procedure for IP injection of DEN (25 mg/kg of body weight) 2 weeks after birth into Control and BAG3albKO mice. (B) Representative livers of 30-weeks-old DEN-treated mice. (C) Comparison of liver weights and liver/body weight ratio between Control and BAG3albKO mice at different times. Data are presented as mean $\pm$ SEM (n=7-9 for each group). (D) Representative images of H&E staining of liver sections showing pathological lesions: focal lesions,

adenoma and HCC (starting from left). (E) Percentage of mice presenting different liver lesions in each group evaluated at 22, 30 and 38 weeks of age. Data are presented as percentage of mice harbouring specific liver lesions (n=7-17 for each group). Data were analysed by Fisher's exact test (***p<0.0001).

BAG3 regulates EMT in mouse derived HCC cell lines

To further investigate the role of BAG3 in liver carcinogenesis, two hepatocellular carcinoma (HCC) cell lines were established from liver lesions of control and BAG3albKO mice. These cell lines exhibited distinct morphological characteristics *in vitro*: the control-derived line, which expressed BAG3, displayed larger cell size with abundant cytoplasm, whereas the BAG3albKO-derived line was smaller with reduced cytoplasm (Figure 3A). Despite these morphological differences, the cell cycle appeared similar between the two lines, as indicated by comparable growth rates and population doubling times of 0.79 and 21.2 hours for the control-derived line and 0.70 and 23.7 hours for the BAG3albKO-derived line, respectively (Figures 3B and 3C). Additionally, cell viability assessed over a 72-hour period showed no significant differences between the two lines (Figure 3D). However, a wound healing assay revealed a significantly reduced migratory capacity in the BAG3-deficient cells compared to the control cell line (Figure 3C). Further investigation of epithelial and mesenchymal markers demonstrated downregulation of vimentin and alpha-SMA, alongside a marked upregulation of E-cadherin in the BAG3albKO-derived HCC cells (Figure 3E). These findings indicate that the BAG3albKO-derived HCC line exhibits a more epithelial-like phenotype than its BAG3-expressing counterpart. Collectively, these results suggest that BAG3 plays a crucial role in regulating the epithelial-to-mesenchymal transition (EMT) in HCC cells.

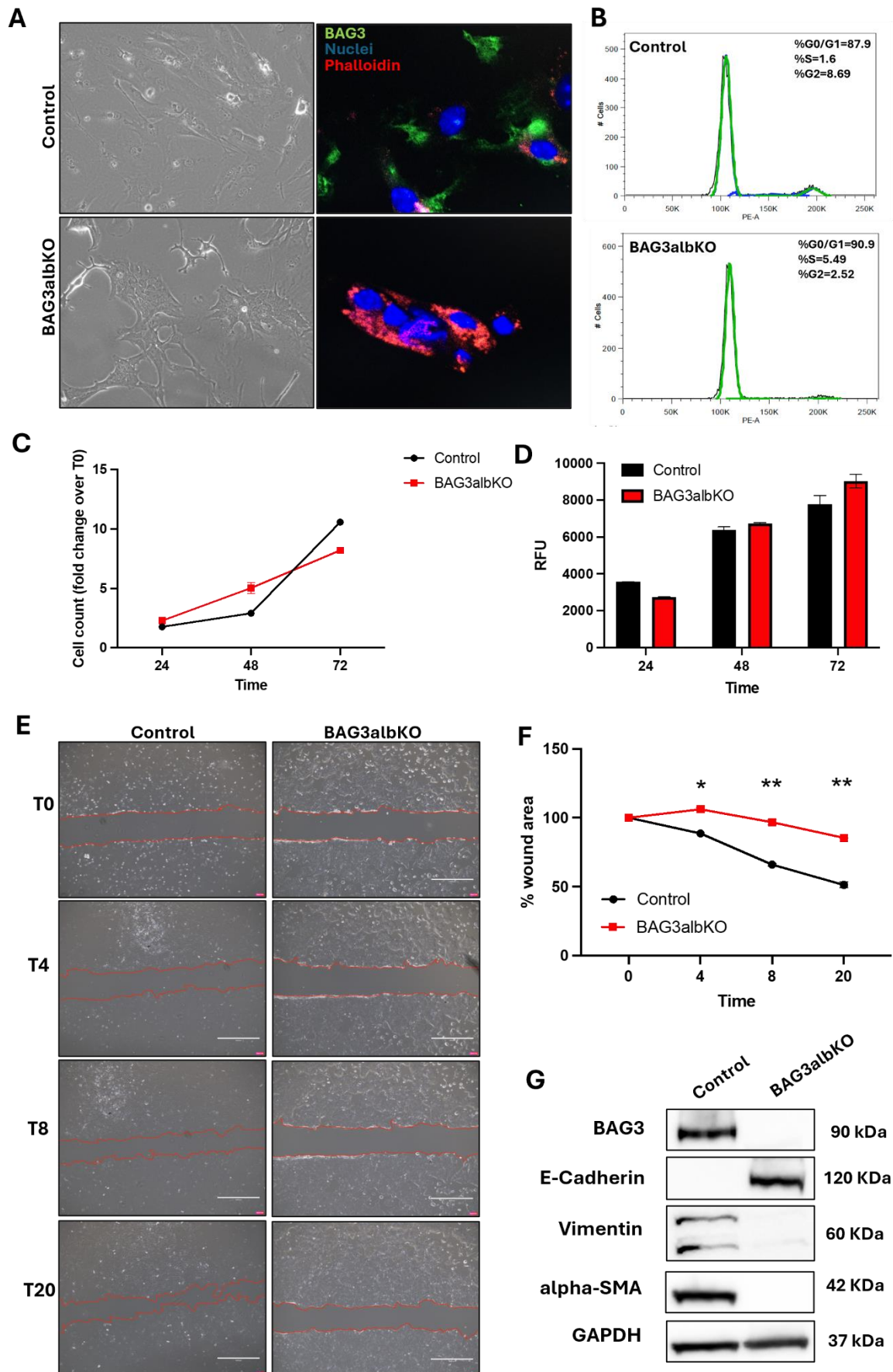


Figure 3: Establishment of HCC cell lines derived from control and BAG3albKO liver lesions. (A) Representative images, brightfield microscopy (left) and immunofluorescence staining (right) of control- and BAG3albKO-derived HCC cell lines; (B) Flow cytometry cell-cycle analysis; (C) Percentage of fold over control cell proliferation assay of control- and BAG3albKO-derived HCC cell lines at the indicated time points (hours). Data are shown as means $\pm$ SD of two different experiments, each performed in duplicate; (D) Viability assay of control- and BAG3albKO-derived HCC cell lines at the indicated time points (hours). Data are shown as means $\pm$ SD of two different experiments, each performed in triplicate; (E) Images of wound healing assay and (F) graph showing the percentage of wound area closure after 4, 8 and 20 hours. Data are shown as means $\pm$ SD of two independent experiments (* p <0.05; ** p <0.01); (G) Western blot analysis of EMT markers.

Evaluation of BAG3-dependent cell functions and pathways involved in response to DEN-induced acute liver injury

To explore the early pathways activated by inflammation and their potential contribution to tumorigenesis in the DEN model, we utilized an acute model of diethyl-nitrosamine (DEN)-induced liver injury combined with proteomic analysis. Eight-week-old male mice received a single intraperitoneal injection of DEN (100 mg/kg), and liver samples were collected after 24 hours (Figure 4A). To identify potential alterations in signaling pathways, pooled liver homogenates from four control and four BAG3albKO mice treated with DEN were analyzed using nanoLC-Orbitrap-MS/MS (Figure 4B). A total of 2,205 proteins were identified, with proper clustering verified in triplicate experiments (Figure 4C). Among these, 2,190 proteins were common between DEN-treated controls and BAG3albKO mice, as shown by Venn diagram analysis (Figure 4D). Statistical analysis revealed 74 differentially expressed proteins (DEPs) between the two groups: 35 were upregulated and 39 were downregulated in BAG3albKO mice (Figure 4D). The functional analysis by Ingenuity Pathway Analysis (IPA) tool revealed distinct differences in pathways activated or inhibited in DEN-treated BAG3albKO mice compared to controls (Figure 4E). BAG3albKO mice exhibited activation of metabolic signaling pathways, while pathways related to cellular stress and injury (e.g., NRF2-mediated oxidative stress response, wound healing signaling), organismal growth and development (e.g., actin cytoskeleton signaling, autophagy), and proliferation and development (e.g., eIF2 signaling, integrin signaling, remodeling of epithelial adherens junctions) were predominantly inhibited. Furthermore, the NAD salvage pathway and p53 signaling were also predicted to be inhibited in BAG3albKO mice relative to controls.

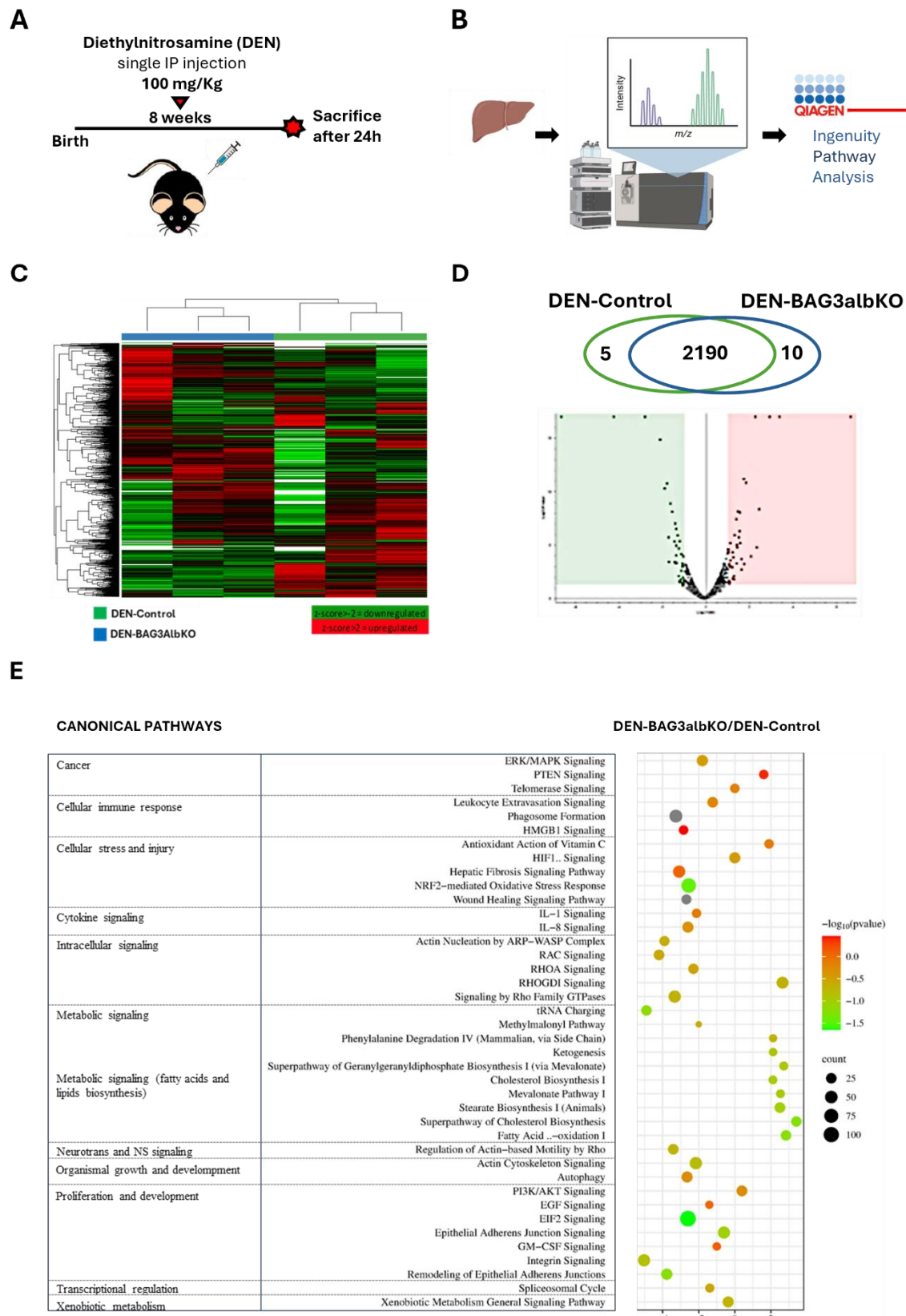
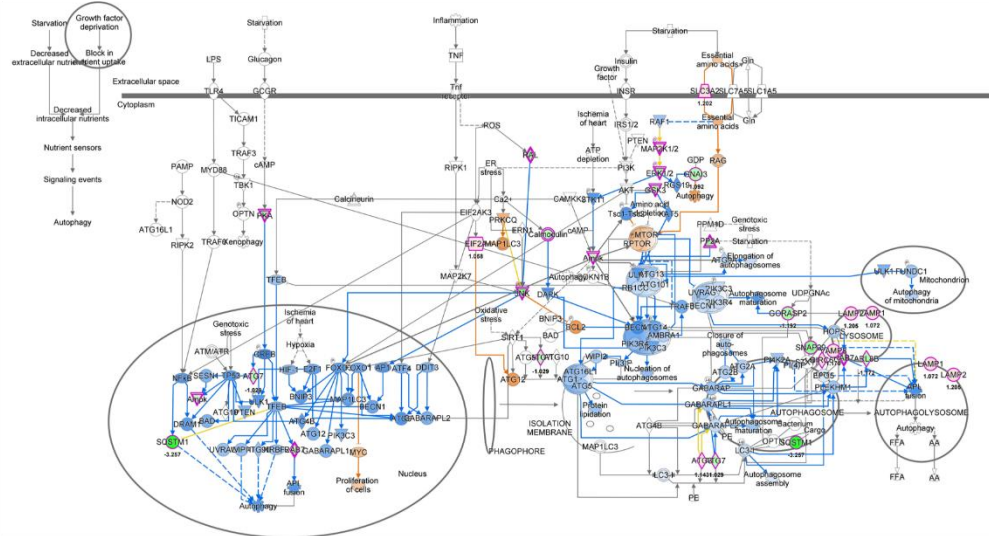


Figure 4: Liver proteomics profile of DEN-induced acute liver damage in BAG3albKO mice. (A) Scheme of experimental procedure for DEN-induced acute liver injury, with one single IP injection of DEN (100 mg/kg of body weight) at 8 weeks

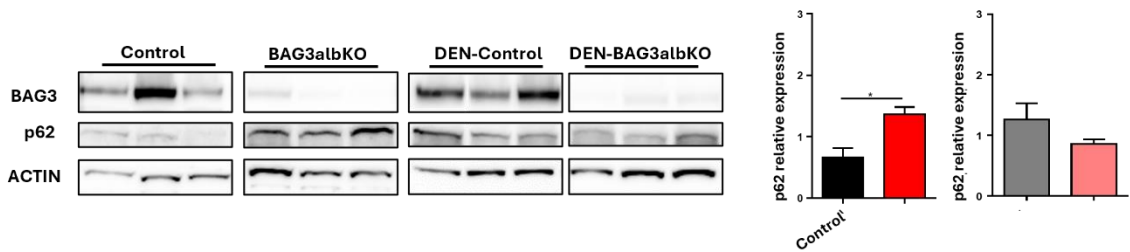
of age. (B) Schematic workflow of proteomic analysis with nanoLC-Orbitrap-MS/MS followed by IPA analysis. (C) Heatmap of differentially expressed proteins. (D) Venn diagram and volcano plot of quantified proteins in DEN-treated mice. (E) Bubble plot of IPA Canonical pathways of DEN-treated BAG3albKO vs DEN-treated control groups.

To validate the proteomic findings, we focused on specific pathways predicted to be differentially modulated. First, we examined the autophagic pathway (Figure 5A), as BAG3 is known to play a critical role in maintaining cellular homeostasis through autophagy regulation (39). Under acute stress and during cellular aging, BAG3 collaborates with the ubiquitin receptor p62/SQSTM1 (hereafter referred to as p62) to target aggregation-prone proteins for autophagic degradation (40). Consistent with IPA predictions, we observed decreased p62 protein levels in DEN-treated BAG3albKO mice compared to controls. However, in the absence of DEN, p62 levels were significantly elevated in BAG3albKO mice, corroborating previous findings by Jung et al. (35). Next, we examined the eIF2 signaling pathway, which was predicted to be significantly inhibited in DEN-treated BAG3albKO mice (Figure 5C) (41), (42). Activation of eIF2 α is a key component of the integrated stress response and is associated with autophagy regulation. In agreement with IPA predictions, phosphorylation of eIF2 α was significantly reduced in DEN-treated BAG3albKO mice compared to controls. Conversely, in untreated conditions, BAG3albKO mice exhibited significantly increased eIF2 α phosphorylation (Figure 5D).

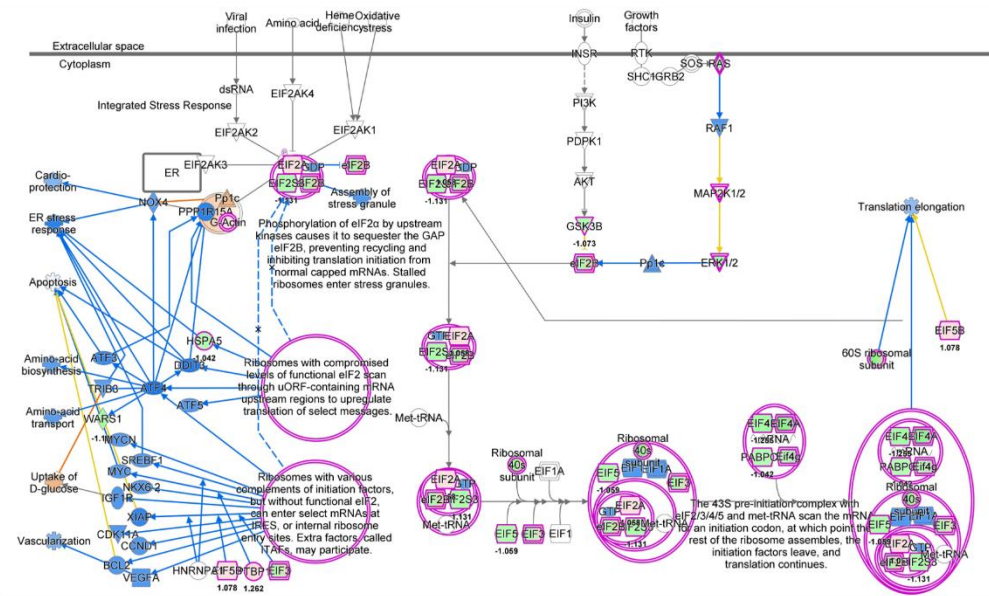
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B



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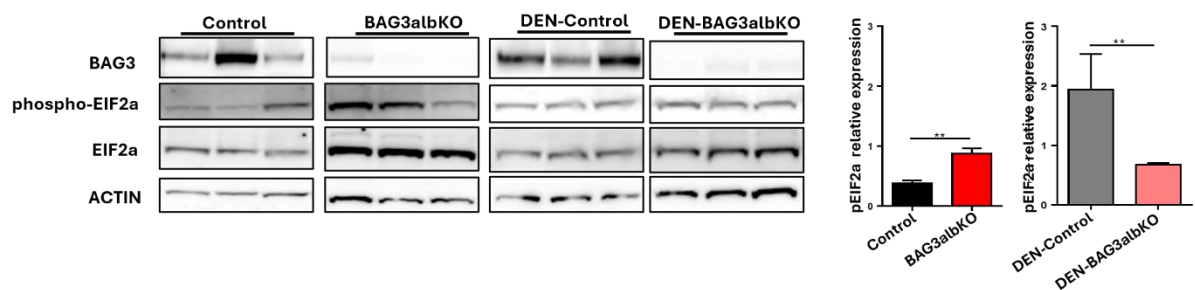
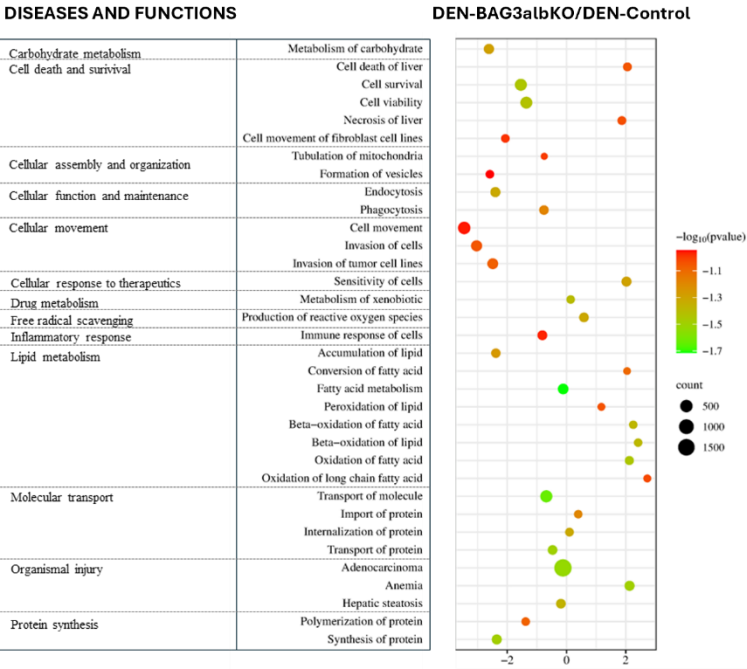


Figure 5: Acute DEN liver injury alters several proteins involved in organismal growth and proliferation. (A) IPA autophagy pathway. (B) Western blot experiment showing the expression of BAG3 and p62 proteins in control and BAG3albKO mice without or after DEN treatment and the relative protein expression analysis. Data are presented as mean values $\pm$ SEM; n=4 animals for each group; *p<0.01. (C) IPA eIF2 signalling pathway. (D) Western blot analysis of BAG3, phospho-eIF2a and eIF2a protein levels in control and BAG3albKO mice without or after DEN treatment and relative protein expression analysis. Data are presented as mean values $\pm$ SEM, n=4 animals for each group; **p<0.01.

Finally, analysis of IPA-predicted downstream diseases and functions (Figure 6A) suggested increased liver cell death and necrosis, along with enhanced lipid oxidation and inhibition of cell movement, vesicle formation, and protein synthesis in DEN-treated BAG3albKO mice compared to controls. These predictions were confirmed by histological analysis of H&E-stained liver sections, which revealed increased necrosis characterized by small foci of necrotic cells with neutrophilic infiltrates in BAG3albKO mice (Figure 6C). Plasma analyses further validated these findings, showing elevated alanine transaminase (ALT) and alkaline phosphatase (ALP) levels in DEN-treated BAG3albKO mice compared to controls (Figure 6D).

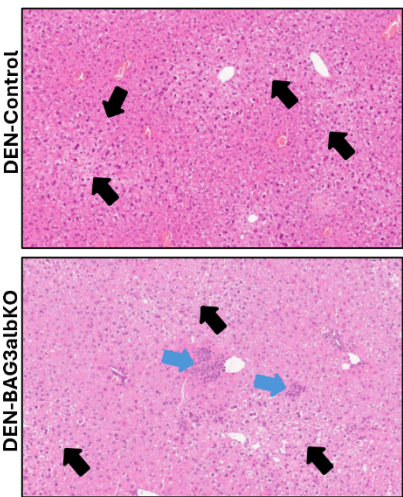
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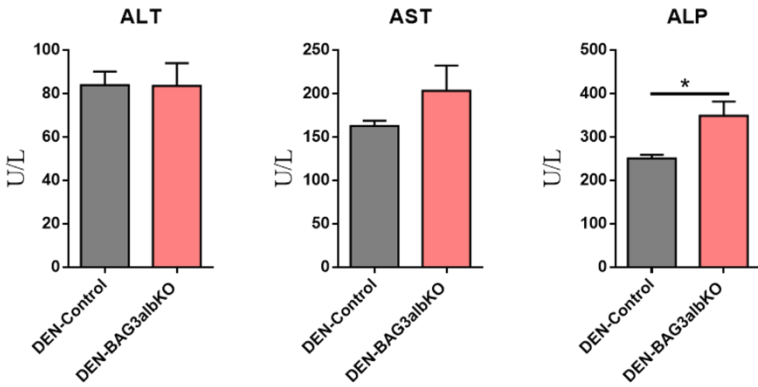


Figure 6: Downstream analyses of DEN-induced acute liver damage in BAG3albKO mice. (A) Bubble plot of IPA Diseases and Functions of DEN-treated BAG3albKO vs DEN- treated control groups. (B) IPA downstream liver necrosis. (C) Representative images of H&E liver sections after DEN treatment. Blue arrows indicate the presence of small focal necrotic areas with neutrophilic infiltrate, while black arrows show glycogenated hepatocytes. (D) Blood biochemical profile of mice (levels of alanine aminotransferase ALT, aspartate aminotransferase AST, and alkaline phosphatase ALP). Data are presented as mean values $\pm$ SEM, n=4 animals for each group; *p < 0.05.

These results collectively highlight the critical role of BAG3 in modulating early inflammatory responses and maintaining cellular homeostasis during DEN-induced acute liver injury, ultimately influencing tumorigenesis pathways.

Discussion and conclusions

Hepatocellular carcinoma (HCC) is a prevalent malignancy with a high mortality rate. Hepatocarcinogenesis is a multifaceted process characterized by chronic liver injury, inflammation, and the progression from precancerous lesions (e.g., dysplastic foci and nodules) to overt HCC. A deeper understanding of the molecular pathways driving HCC pathogenesis is crucial for the development of effective therapeutic strategies. In this study, we established a hepatocyte-specific BAG3 knockout (BAG3albKO) mouse model through Albumin-Cre recombinase-mediated deletion of BAG3 floxed exons. Comprehensive analyses validated the efficacy and specificity of this knockout approach, with the complete absence of BAG3 mRNA and protein expression in hepatocytes. Importantly, BAG3albKO mice exhibited normal physiological and anatomical characteristics, aligning with prior studies (35). To investigate the role of BAG3 in HCC development, we utilized diethyl-nitrosamine (DEN), a genotoxic alkylating agent commonly used to induce acute or chronic liver injury and hepatocarcinogenesis in murine models (37). DEN causes hepatocyte and endothelial cell damage, necrosis, and apoptosis mediated by oxidative stress and inflammation (36), (38). Body and liver weight are key initial indicators of liver pathology. Following a single administration of DEN to 2-week-old mice, BAG3albKO mice showed no significant changes in these parameters but exhibited a

marked reduction in liver lesions, including focal lesions, adenomas, and carcinomas, at early time points compared to control mice. These findings suggest that BAG3 facilitates the development of liver lesions in response to chemical carcinogenesis, delaying cancer onset and reducing tumor burden. The pro-survival role of BAG3 in tumors is well-documented (2). BAG3 enhances cell survival under stressful tumor microenvironmental conditions, such as nutrient deprivation, hypoxia, and chemotherapy, through its interactions with heat shock proteins like Hsp70. This interaction prevents apoptosis and promotes protein folding, enabling tumor cells to evade cell death (43), (44). BAG3 also supports cancer cell migration, invasion, and metastasis by modulating cytoskeletal dynamics and adhesion molecules (39). Supporting this, primary HCC cell lines derived from control and BAG3albKO liver lesions exhibited distinct morphological and migratory characteristics. BAG3 deficiency was associated with reduced migration and a downregulation of epithelial-to-mesenchymal transition (EMT) markers, indicating that BAG3 promotes HCC progression and metastasis, consistent with findings by Xiao et al. (32). Proteomic analyses of DEN-treated BAG3albKO liver tissues following acute liver injury revealed significant alterations in protein expression profiles compared to DEN-treated controls. The findings highlighted inhibited canonical pathways associated with cellular adaptation and survival, including autophagy, eIF2 signaling, the NAD salvage pathway, and p53 signaling. Conversely, hepatocellular cell death and necrosis were markedly increased in BAG3albKO mice, as evidenced by elevated levels of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP), as well as histological confirmation of necrotic foci and neutrophilic infiltrates. BAG3 plays a critical role in maintaining cellular homeostasis during stress, largely through its regulation of autophagy and eIF2 signaling. Autophagy is essential for the clearance of damaged cellular components and the maintenance of NAD levels necessary for cell survival. In DEN-treated BAG3albKO mice, autophagic impairment contributed to uncontrolled NAD depletion and subsequent cell death (2). In particular, our findings show that the NAD salvage pathway is inhibited in DEN-treated BAG3albKO mice. Autophagy plays an evolutionarily conserved role in maintaining NAD levels, which are essential for cellular survival (45). A loss of NAD leads to uncontrolled depletion, ultimately contributing to cell death (46), which is consistent with the increased necrosis observed in our proteomic analysis. Additionally, DEN-induced alkylating DNA damage triggers a

regulated form of necrotic cell death that does not rely on the central apoptotic mediator, p53, whose signaling is already suppressed in BAG3^{alb}KO mice. Instead, this form of cell death involves the activation of poly (ADP-ribose) polymerase (PARP), a DNA repair protein. However, PARP activation alone is insufficient to determine cell fate; cell death is ultimately influenced by the effect of PARP-mediated NAD consumption on cellular metabolism (47), which aligns with our findings. Similarly, eIF2 signaling, crucial for the cellular stress response (41)—particularly in modulating global protein synthesis and activating stress-responsive genes—was found to be impaired in the absence of BAG3. This impairment is particularly significant in liver pathology, where the cell's ability to withstand and recover from injury is critical in preventing the progression to more severe disease states. The increased propensity for cell death and necrosis observed in DEN-treated BAG3^{alb}KO mice provides compelling evidence for the role of BAG3 in mechanisms that mitigate excessive cell damage and liver pathology. However, compensatory proliferation of surviving hepatocytes to repair the damaged parenchyma may eventually lead to neoplasia (48), (49). Cancer often arises in epithelial tissues subjected to repeated cycles of injury and repair (50), which could explain the development of adenomas and carcinomas in the liver of BAG3^{alb}KO mice at 38 weeks of age in the HCC model. The relationship between liver regeneration and the onset of HCC, however, remains poorly understood, and further studies are needed to elucidate this mechanism.

In conclusion, our findings underscore the critical role of BAG3 in supporting hepatocyte survival under stress, preventing excessive liver damage, and facilitating HCC progression. By modulating autophagy, stress signaling, and EMT pathways, BAG3 emerges as a key player in liver tumorigenesis. Targeting BAG3 may represent a promising therapeutic or prophylactic strategy for liver cancer, offering novel avenues for intervention. Future studies should further explore the mechanisms linking liver regeneration and HCC onset to refine therapeutic approaches.

Materials and methods

Animals and maintenance

To generate hepatocyte-specific BAG3 knockout mice, Cre-loxP recombination technology was employed. The Bag3^{tm1a(EUCOMM)Hmgu} strain, obtained from the EUCOMM consortium, was crossed with transgenic mice expressing Flpo recombinase to produce BAG3 floxed mice (Bag3^{fl/fl}) (51). These Bag3^{fl/fl} mice were then crossed with homozygous mice expressing Cre recombinase under the control of the mouse Albumin promoter (Alb-Cre^{+/+}), sourced from Charles River Italia SPA. For experimental purposes, control mice carried two floxed BAG3 alleles, while hepatocyte-specific BAG3 knockout mice possessed two floxed alleles and Alb-Cre^{+/+} simultaneously (BAG3albKO). For hepatocellular carcinoma (HCC) induction, 14-day-old male mice were administered a single intraperitoneal (i.p.) injection of 25 mg/kg of diethyl-nitrosamine (DEN), with animals being euthanized at 22, 30, and 38 weeks of age. For liver injury models, 8-week-old mice received a higher intraperitoneal dose of DEN (100 mg/kg) and were euthanized 24 hours after injection. All mice were housed in a pathogen-free environment (22 °C, 12-hour light/dark cycle) with ad libitum access to food and water. Experimental protocols were approved by the Institutional Animal Healthcare of the University of Chieti and adhered to the guidelines set by the Italian Ministry of Health (Protocol N° 219/2019PR). Animal care and experimental procedures complied with the NIH guidelines.

Genotyping

DNA was extracted from ear punches and subsequently analyzed by PCR (Polymerase Chain Reaction) using the Xpert Direct Extract PCR Kit. BAG3 genotyping was performed using the following primers: BAG3-471-FW (ACTATCTGCTGTCTACACTGC) and BAG3-459-RV (TGAGCGGCACTTTAAGGTTT). For Albumin Cre recombinase genotyping, the protocol was followed as detailed on The Jackson Laboratory's website (<http://jaxmice.jax.org/>). PCR amplification was carried out using the MJ Mini 48-Well Personal Thermal Cycler with the following cycling conditions: 95 °C for 3 minutes (1 cycle); 95 °C for 15 seconds, 55 °C for 15 seconds,

and 72 °C for 2 seconds (40 cycles); followed by 72 °C for 3 minutes (1 cycle) and a final hold at 4 °C. PCR products were resolved on a 2% agarose gel.

RNA extraction and qPCR

Total RNA was extracted from liver tissue using the RNeasy Kit. RNA concentration was measured by absorbance using a NanoDrop spectrophotometer. RNA was then reverse transcribed into cDNA using TaqMan™ Reverse Transcription Reagents according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using SsoAdvanced Universal SYBR Green Supermix on a Bio-Rad CFX96 Touch System. Transcript levels were normalized to actin. The following primers were used: FW-BAG3 (CCAACTGCTCATGGACCTG), RV-BAG3 (GCCGAGGAGGAAGAGGAT), FW-ACTIN (CTAAGGCCAACCGTGAAAAG), and RV-ACTIN (ACCAGAGGCATACAGGGACA).

Western blot analysis

Tissues were homogenized in ice-cold lysis buffer (She-Lysis buffer, PhosphoStop 10X, and Protease Inhibitor 100X). Protein concentrations were quantified using the Bradford method. For Western blot analysis, the Invitrogen Mini Gel System and Semi-Dry Transfer were used. Protein samples (30 µg) were prepared with 4X Bolt™ LDS Sample Buffer and 10X Bolt™ Sample Reducing Agent. The samples were heated at 70°C for 10 minutes before being loaded onto a pre-cast polyacrylamide gel (4-12%) with 20X Bolt™ MOPS SDS Running Buffer and Bolt™ Antioxidant, which prevents reoxidation and maintains proteins in a reduced state during electrophoresis and transfer. The gel was run at 200 V for 32 minutes using the Thermo Fisher Mini Gel Tank and Power Supply. After electrophoresis, proteins were transferred to a nitrocellulose membrane using the Thermo Fisher Power Blotter XL System, which enabled a rapid semi-dry transfer in 7 minutes. Membranes were incubated overnight at 4°C with primary antibodies: anti-BAG3 (1:2000, Proteintech, Manchester, UK), anti-phospho-eIF2α (1:1000, #3597), anti-Vimentin (1:1000, #5741), anti-GAPDH (1:2000, #5174), anti-p62/SQSTM1 (1:1000, BD Biosciences, San Jose, CA, USA), anti-E-Cadherin (1:1000, BD Biosciences), anti-eIF2α

(1:500, Santa Cruz Biotechnology, Dallas, TX, USA), anti- α SMA (1:500, Sigma-Aldrich, St. Louis, MO, USA), and anti- β -actin (1:40,000, Sigma-Aldrich). Following incubation, the membranes were washed and incubated with a secondary antibody (1:20,000, Bio-Rad) at room temperature for 1 hour. Protein bands were detected using the enhanced chemiluminescent (ECL) method.

Histological and immunohistochemical analysis

Necropsy was performed, and the livers were harvested and fixed in 10% neutral buffered formalin for 24 hours. The tissues were then processed overnight in a tissue processor, followed by paraffin embedding. The embedded tissue was sectioned at 5 μ m thickness using a manual microtome and mounted on slides. After deparaffinization, liver tissue sections were stained with Hematoxylin and Eosin for histological analysis. Slides were independently evaluated by two pathologists. For immunohistochemistry, slides were deparaffinized and serially rehydrated. Antigen retrieval was performed using a microwave citrate buffer (pH 6.0) for 10 or 20 minutes. The slides were then incubated overnight with the following primary antibodies: rabbit anti-BAG3 antibody (NBP2-27398, Bio-Techne, Milan, Italy) and rabbit anti-cytokeratin 19 antibody (rb-9021-r7, Thermo Fisher). Biotin-SP-conjugated anti-rabbit IgG (111-065-144, Jackson ImmunoResearch, Cambridge, UK) and Streptavidin HRP (ab64269, Abcam, Cambridge, UK) or Dako EnVision System-HRP-labelled polymer anti-rabbit (K4001, Agilent, Santa Clara, CA, USA) were used as secondary antibodies for 30 minutes, followed by incubation with 3,3'-diaminobenzidine (DAB) (K3468, Agilent) as the chromogenic agent. After chromogen incubation, slides were counterstained with Hematoxylin, and images were acquired using a Leica DMRD optical microscope.

Murine liver tumour cell lines establishment

DEN-treated control and BAG3albKO mice were sacrificed at 38 weeks of age. Liver tissue was aseptically collected and washed in phosphate-buffered saline (PBS) containing antibiotics (penicillin, 100 U/mL; streptomycin, 100 μ g/mL). After washing, the liver was transferred to a petri dish with PBS containing

antibiotics and minced using surgical scissors and a scalpel. The minced tissue was then incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) and enzymatically dissociated using a mix of collagenase and hyaluronidase for 30 minutes at 37°C. The enzymatic digestion was stopped by adding complete medium (DMEM supplemented with 10% fetal bovine serum [FBS] and antibiotics), followed by centrifugation at 1500 rpm for 5 minutes. The dissociated tissue was filtered through a 0.70 µm cell strainer to remove any undigested fragments. The isolated cells were cultured in complete medium and incubated at 37°C in a 5% CO₂ incubator. Once cell confluence reached 90–100%, subculturing was performed at a 1:2 split using the standard trypsinization method. Cells were considered stable and ready for subsequent experiments once they reached passage 20.

Cell cycle analysis, cell proliferation and cell viability assay

Cell cycle analysis was performed using flow cytometry. Approximately 1×10^6 cells per experimental condition were harvested, fixed in 70% cold ethanol, and stored at 4°C overnight. The cells were then pelleted, resuspended in 100 µg/mL propidium iodide and 200 µg/mL RNase, and incubated for at least 30 minutes, as previously described (52). 1×10^4 cells were acquired using a FACSCanto II flow cytometer. Data were analyzed using FlowJo software. Cell proliferation was monitored by counting viable cells using Trypan Blue reagent after seeding cell lines into 12-well plates at the same confluency and at the indicated time points. Additionally, cell viability was assessed using the CellTiter-Blue Cell Viability Assay, according to the manufacturer's instructions, after seeding cell lines into 96-well plates at the same confluency and at the indicated time points.

Wound healing assay

Cells were seeded into a 6-well culture plate and incubated for 24 hours. A sterile 200 µL pipette tip was used to create a scratch wound across the cell monolayer. After rinsing with PBS, the cells were cultured in complete

DMEM. Images of the scratch were captured using an EVOS M5000 microscope at time 0 and after 4, 8, and 20 hours. The cell migration capacity was assessed by measuring the relative distance of cell migration from the initial time point, as previously described (53).

Shotgun label-free proteomic analysis

Liver tissues from BAG3albKO and control mice were processed using the FASP (Filter Aided Sample Preparation) method. First, the tissues were mechanically homogenized with a Turrax mixer (1-minute, highest speed) and ultrasonicated on ice at 80% amplitude in a lysis buffer (6 M urea in 100 mM Tris/HCl, pH 7.5). The homogenates were then centrifuged to remove tissue debris. A total of 50 µg of protein from each sample, quantified by the Bradford Protein Assay using Bovine Serum Albumin as a standard, were digested by trypsin. For protein identification and quantification, the tryptic digests from each liver sample were analyzed in triplicate using LC-MS/MS with an UltiMate™ 3000 UPLC system (Thermo Fischer Scientific) coupled to an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fischer Scientific). Proteomics data were processed as previously described by Di Stefano et al. (54), using Thermo Proteome Discoverer (PD) version 2.4.0.305 to identify differentially expressed proteins (DEPs) between the livers of BAG3albKO and control mice (54). Lists of DEPs were uploaded to the Ingenuity Pathway Analysis (IPA) tool (Qiagen) for gene ontology, functional enrichment analysis, and mapping of each protein's functional annotation. IPA Functional Analysis connects experimental results to biological functions and diseases by utilizing complex biological interactions stored in the Ingenuity Knowledge Base. IPA uses a z-score algorithm to predict the activation (z-scores ≥ 2.0) or inhibition (z-scores ≤ -2.0) of well-characterized metabolic and cell signaling pathways through "Canonical Pathways Analysis" and to assess downstream biological effects through "Disease and Biofunctions Analysis." IPA also employs the right-tailed Fisher's Exact Test p-value of overlap to identify significant pathways, which reflects the probability that molecules in the dataset are associated with a canonical pathway by random chance. The Downstream Effects Analysis predicts the impact of molecular changes on biological processes and diseases. This analysis identifies diseases and functions expected to increase or decrease based

on causal effects derived from the literature compiled in the QIAGEN Knowledge Base. It compares the direction of change in molecules with expectations from the literature, making predictions for each disease and function based on the observed molecular alterations.

Biochemical analysis

Blood samples were collected via cardiac puncture from euthanized mice after an overnight fasting period. The samples were collected in Microvette® 200 Lithium Heparin tubes. Plasma was separated by centrifuging the blood samples at 4 °C for 10 minutes. The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured using a VChemys Automatic Biochemical Analyzer, following the manufacturer's instructions.

Statistical analysis

Experimental results are presented as means $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism v.9. For normally distributed data, Student's t-test was applied, whereas the Mann–Whitney U test was used for non-normally distributed data, as determined by the Shapiro–Wilk normality test. Fisher's exact test was employed to analyze differences between Control and BAG3albKO groups following DEN treatment. Statistical significance is indicated by asterisks in the figures: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

References

1. Rosati A, Graziano V, De Laurenzi V, Pascale M, Turco MC. BAG3: a multifaceted protein that regulates major cell pathways. *Cell Death Dis.* 7 aprile 2011;2(4):e141–e141.
2. Kögel D, Linder B, Brunschweiler A, Chines S, Behl C. At the Crossroads of Apoptosis and Autophagy: Multiple Roles of the Co-Chaperone BAG3 in Stress and Therapy Resistance of Cancer. *Cells.* 28 febbraio 2020;9(3):574.
3. Gamerding M, Kaya AM, Wolfrum U, Clement AM, Behl C. BAG3 mediates chaperone-based aggresome-targeting and selective autophagy of misfolded proteins. *EMBO Reports.* febbraio 2011;12(2):149–56.
4. Carra S, Seguin SJ, Landry J. HspB8 and Bag3: A new chaperone complex targeting misfolded proteins to macroautophagy. *Autophagy.* 16 febbraio 2008;4(2):237–9.
5. Rosati A, Ammirante M, Gentilella A, Basile A, Festa M, Pascale M, et al. Apoptosis inhibition in cancer cells: A novel molecular pathway that involves BAG3 protein. *The International Journal of Biochemistry & Cell Biology.* luglio 2007;39(7–8):1337–42.
6. Iwasaki M, Tanaka R, Hishiya A, Homma S, Reed JC, Takayama S. BAG3 directly associates with guanine nucleotide exchange factor of Rap1, PDZGEF2, and regulates cell adhesion. *Biochemical and Biophysical Research Communications.* settembre 2010;400(3):413–8.
7. Fuchs M, Luthold C, Guilbert SM, Varlet AA, Lambert H, Jetté A, et al. A Role for the Chaperone Complex BAG3-HSPB8 in Actin Dynamics, Spindle Orientation and Proper Chromosome Segregation during Mitosis. Serio TR, curatore. *PLoS Genet.* 23 ottobre 2015;11(10):e1005582.

8. Chakraborty D, Felzen V, Hiebel C, Stürner E, Perumal N, Manicam C, et al. Enhanced autophagic-lysosomal activity and increased BAG3-mediated selective macroautophagy as adaptive response of neuronal cells to chronic oxidative stress. *Redox Biology*. giugno 2019;24:101181.
9. Hiebel C, Stürner E, Hoffmeister M, Tascher G, Schwarz M, Nagel H, et al. BAG3 Proteomic Signature under Proteostasis Stress. *Cells*. 4 novembre 2020;9(11):2416.
10. De Marco M, Basile A, Iorio V, Festa M, Falco A, Ranieri B, et al. Role of BAG3 in cancer progression: A therapeutic opportunity. *Seminars in Cell & Developmental Biology*. giugno 2018;78:85–92.
11. Knezevic T, Myers VD, Gordon J, Tilley DG, Sharp TE, Wang J, et al. BAG3: a new player in the heart failure paradigm. *Heart Fail Rev*. luglio 2015;20(4):423–34.
12. Kirk JA, Cheung JY, Feldman AM. Therapeutic targeting of BAG3: considering its complexity in cancer and heart disease. *Journal of Clinical Investigation*. 16 agosto 2021;131(16):e149415.
13. Iorio V, Festa M, Rosati A, Hahne M, Tiberti C, Capunzo M, et al. BAG3 regulates formation of the SNARE complex and insulin secretion. *Cell Death Dis*. 12 marzo 2015;6(3):e1684–e1684.
14. Damiani V, Lamolinara A, Cicalini I, Cufaro MC, Del Pizzo F, Di Marco F, et al. Pancreatic beta-cell specific BAG3 knockout results in chronic hyperinsulinemia inducing insulin resistance. *Molecular Metabolism*. agosto 2023;74:101752.
15. Furusawa Y, Yunoki T, Hirano T, Minagawa S, Izumi H, Mori H, et al. Identification of genes and genetic networks associated with BAG3-dependent cell proliferation and cell survival in human cervical cancer HeLa cells. *Mol Med Report* [Internet]. 10 agosto 2018 [citato 2 dicembre 2024]; Disponibile su: <http://www.spandidos-publications.com/10.3892/mmr.2018.9383>
16. Iwasaki M, Homma S, Hishiya A, Dolezal SJ, Reed JC, Takayama S. BAG3 Regulates Motility and Adhesion of Epithelial Cancer Cells. *Cancer Research*. 1 novembre 2007;67(21):10252–9.

17. An MX, Li S, Yao HB, Li C, Wang JM, Sun J, et al. BAG3 directly stabilizes Hexokinase 2 mRNA and promotes aerobic glycolysis in pancreatic cancer cells. *Journal of Cell Biology*. 4 dicembre 2017;216(12):4091–105.
18. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA A Cancer J Clinicians*. maggio 2021;71(3):209–49.
19. D'souza S, Lau KC, Coffin CS, Patel TR. Molecular mechanisms of viral hepatitis induced hepatocellular carcinoma. *WJG*. 14 ottobre 2020;26(38):5759–83.
20. Shlomai A, De Jong YP, Rice CM. Virus associated malignancies: The role of viral hepatitis in hepatocellular carcinoma. *Seminars in Cancer Biology*. giugno 2014;26:78–88.
21. Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, et al. Hepatocellular carcinoma. *Nat Rev Dis Primers*. 21 gennaio 2021;7(1):6.
22. Ganne-Carrié N, Chaffaut C, Bourcier V, Archambeaud I, Perarnau JM, Oberti F, et al. Estimate of hepatocellular carcinoma incidence in patients with alcoholic cirrhosis. *Journal of Hepatology*. dicembre 2018;69(6):1274–83.
23. Jacob R, Prince DS, Kench C, Liu K. Alcohol and its associated liver carcinogenesis. *J of Gastro and Hepatol*. agosto 2023;38(8):1211–7.
24. Ganne-Carrié N, Nahon P. Hepatocellular carcinoma in the setting of alcohol-related liver disease. *Journal of Hepatology*. febbraio 2019;70(2):284–93.
25. Giannitrapani L, Soresi M, La Spada E, Cervello M, D'Alessandro N, Montalto G. Sex Hormones and Risk of Liver Tumor. *Annals of the New York Academy of Sciences*. novembre 2006;1089(1):228–36.
26. Hardt A, Stippel D, Odenthal M, Hölscher AH, Dienes HP, Drebber U. Development of Hepatocellular Carcinoma Associated with Anabolic Androgenic Steroid Abuse in a Young Bodybuilder: A Case Report. *Case Reports in Pathology*. 2012;2012:1–5.

27. Paganoni R, Lechel A, Vujic Spasic M. Iron at the Interface of Hepatocellular Carcinoma. *IJMS*. 15 aprile 2021;22(8):4097.
28. Melaram R. Environmental Risk Factors Implicated in Liver Disease: A Mini-Review. *Front Public Health*. 24 giugno 2021;9:683719.
29. Kew MC. Aflatoxins as a cause of hepatocellular carcinoma. *J Gastrointestin Liver Dis*. settembre 2013;22(3):305–10.
30. Magnussen A. Aflatoxins, hepatocellular carcinoma and public health. *WJG*. 2013;19(10):1508.
31. Llovet JM, Pinyol R, Kelley RK, El-Khoueiry A, Reeves HL, Wang XW, et al. Molecular pathogenesis and systemic therapies for hepatocellular carcinoma. *Nat Cancer*. 28 aprile 2022;3(4):386–401.
32. Xiao H, Cheng S, Tong R, Lv Z, Ding C, Du C, et al. BAG3 regulates epithelial–mesenchymal transition and angiogenesis in human hepatocellular carcinoma. *Laboratory Investigation*. marzo 2014;94(3):252–61.
33. Xiao H, Tong R, Cheng S, Lv Z, Ding C, Du C, et al. BAG3 and HIF-1 α Coexpression Detected by Immunohistochemistry Correlated with Prognosis in Hepatocellular Carcinoma after Liver Transplantation. *BioMed Research International*. 2014;2014:1–9.
34. Kong DH, Li S, Du ZX, Liu C, Liu BQ, Li C, et al. BAG3 elevation inhibits cell proliferation via direct interaction with G6PD in hepatocellular carcinomas. *Oncotarget*. 5 gennaio 2016;7(1):700–11.
35. Jung SY, Yun HH, Lim JH, Lee DH, Seo SB, Baek JY, et al. Hepatocyte-specific deletion of Bis causes senescence in the liver without deteriorating hepatic function. *Biochemical and Biophysical Research Communications*. settembre 2022;619:42–8.
36. Tolba R, Kraus T, Liedtke C, Schwarz M, Weiskirchen R. Diethylnitrosamine (DEN)-induced carcinogenic liver injury in mice. *Lab Anim*. aprile 2015;49(1_suppl):59–69.
37. Sotty J, Bablon P, Weiss PH, Soussan P. Diethylnitrosamine Induction of Hepatocarcinogenesis in Mice. In: Kroemer G, Pol J, Martins I, curatori. *Liver Carcinogenesis* [Internet]. New York, NY: Springer US;

2024 [citato 2 dicembre 2024]. p. 15–25. (Methods in Molecular Biology; vol. 2769). Disponibile su: https://link.springer.com/10.1007/978-1-0716-3694-7_2

38. Schulien I, Hasselblatt P. Diethylnitrosamine-induced liver tumorigenesis in mice. In: Methods in Cell Biology [Internet]. Elsevier; 2021 [citato 2 dicembre 2024]. p. 137–52. Disponibile su: <https://linkinghub.elsevier.com/retrieve/pii/S0091679X20301527>

39. Brenner CM, Choudhary M, McCormick MG, Cheung D, Landesberg GP, Wang JF, et al. BAG3: Nature's Quintessential Multi-Functional Protein Functions as a Ubiquitous Intra-Cellular Glue. Cells. 19 marzo 2023;12(6):937.

40. Gamerdinger M, Hajieva P, Kaya AM, Wolfrum U, Hartl FU, Behl C. Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. EMBO J. 8 aprile 2009;28(7):889–901.

41. B'chir W, Maurin AC, Carraro V, Averous J, Jousse C, Muranishi Y, et al. The eIF2 α /ATF4 pathway is essential for stress-induced autophagy gene expression. Nucleic Acids Research. settembre 2013;41(16):7683–99.

42. Humeau J, Leduc M, Cerrato G, Loos F, Kepp O, Kroemer G. Phosphorylation of eukaryotic initiation factor-2 α (eIF2 α) in autophagy. Cell Death Dis. 8 giugno 2020;11(6):433.

43. Antonietti P, Linder B, Hehlhans S, Mildenerberger IC, Burger MC, Fulda S, et al. Interference with the HSF1/HSP70/BAG3 Pathway Primes Glioma Cells to Matrix Detachment and BH3 Mimetic-Induced Apoptosis. Molecular Cancer Therapeutics. 1 gennaio 2017;16(1):156–68.

44. Colvin TA, Gabai VL, Gong J, Calderwood SK, Li H, Gummuluru S, et al. Hsp70–Bag3 Interactions Regulate Cancer-Related Signaling Networks. Cancer Research. 1 settembre 2014;74(17):4731–40.

45. Kataura T, Sedlackova L, Otten EG, Kumari R, Shapira D, Scialo F, et al. Autophagy promotes cell survival by maintaining NAD levels. Developmental Cell. novembre 2022;57(22):2584–2598.e11.

46. Schultz MD, Dadali T, Jacques SA, Muller-Steffner H, Foote JB, Sorci L, et al. Inhibition of the NAD salvage pathway in schistosomes impairs metabolism, reproduction, and parasite survival. Collins JJ, curatore. *PLoS Pathog.* 27 maggio 2020;16(5):e1008539.
47. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev.* 1 giugno 2004;18(11):1272–82.
48. Rigual MDM, Sánchez Sánchez P, Djouder N. Is liver regeneration key in hepatocellular carcinoma development? *Trends in Cancer.* febbraio 2023;9(2):140–57.
49. Michalopoulos GK, Bhushan B. Liver regeneration: biological and pathological mechanisms and implications. *Nat Rev Gastroenterol Hepatol.* gennaio 2021;18(1):40–55.
50. Guest RV, Boulter L, Dwyer BJ, Forbes SJ. Understanding liver regeneration to bring new insights to the mechanisms driving cholangiocarcinoma. *npj Regen Med.* 22 maggio 2017;2(1):13.
51. Kranz A, Fu J, Duerschke K, Weidlich S, Naumann R, Stewart AF, et al. An improved Flp deleter mouse in C57Bl/6 based on Flpo recombinase. *Genesis.* agosto 2010;48(8):512–20.
52. Veschi S, De Lellis L, Florio R, Lanuti P, Massucci A, Tinari N, et al. Effects of repurposed drug candidates nitroxoline and nelfinavir as single agents or in combination with erlotinib in pancreatic cancer cells. *J Exp Clin Cancer Res.* dicembre 2018;37(1):236.
53. Damiani V, Cufaro MC, Fucito M, Dufrusine B, Rossi C, Del Boccio P, et al. Proteomics Approach Highlights Early Changes in Human Fibroblasts-Pancreatic Ductal Adenocarcinoma Cells Crosstalk. *Cells.* 29 marzo 2022;11(7):1160.
54. Di Stefano J, Garcia-Pupo L, Di Marco F, Motaln H, Govaerts J, Van Breedam E, et al. Transcriptomic and proteomic profiling of bi-partite and tri-partite murine iPSC-derived neurospheroids under steady-state and inflammatory condition. *Brain, Behavior, and Immunity.* ottobre 2024;121:1–12.

CHAPTER 5

Technological and scientific achievements during the research stay abroad

Introduction

As part of my PhD program performed at CAST (University “G. d’Annunzio” of Chieti-Pescara), this chapter outlines the most significant technological and scientific achievements obtained during my research visit abroad. This international exposure has provided me with an invaluable opportunity - in a truly collaborative nature - to consolidate my knowledge in proteomic analyses within the course of ongoing research projects in the *Laboratory of Experimental Hematology (LEH)* from the *Vaccine and Infectious Disease Institute (Vaxinfectio)* at the *University of Antwerp (UA)*, under the supervision of Prof. Peter Ponsaerts. Here I gained unique insights and enhanced my technical skills in multi-omics data analysis, engaged with researchers from different fields, fostered collaboration and knowledge exchange. During this period, I also participated in advanced cell culture training, attended international conferences and seminars, but most importantly contributed to multiple UA-CAST joint research projects.

The section below will highlight the objectives of my research activities, as well as the resulting outcomes during and beyond my research period abroad, underscoring the importance of this experience in shaping my academic, professional, and personal development. I spent three months at the *University of Antwerp*, from October 2023 to December 2023 and during this period, I primarily focused on the processing and integration of proteomic analyses that had been previously conducted at the CAST laboratories with transcriptomic analyses on the same specimens that had been performed previously at LEH (UA). My work was therefore mainly focused on gene ontology and functional enrichment analysis using the IPA tool across various projects, whereby my major achievement was setting the basis for current and future transcriptome-proteome integration analyses performed both at UA and at CAST. Furthermore, I was trained in the initiation and maintenance of hiPSC-derived human brain organoids, for which I – after my research period abroad – further contributed to the optimized advanced multi-omics analyses by active knowledge transfer to multiple members of LEH at UA.

General concepts of multi-omics data analysis

To achieve a comprehensive understanding of human health and disease, it is essential to interpret molecular complexity and variability across multiple biological levels, including the genome, epigenome, transcriptome, proteome, and metabolome (1). The advancement of “omics” technologies has markedly increased biology’s reliance on data generated at these levels, collectively referred to as “multi-omics” data. The availability of multi-omics data has transformed the fields of medicine and biology by facilitating integrated, systems-level understandings (2). The analysis of multi-omics data, when combined with clinical information, either from animal experiments or human patients, has become central to extracting meaningful insights into cellular processes. Integrating multi-omics data, which encompasses information on biomolecules across different biological layers and/or specimens, offers a promising approach for a systematic and holistic understanding of complex biological systems (3). Integrated methodologies merge individual omics datasets, either sequentially or concurrently, to elucidate molecular interactions. These approaches enable the assessment of information flow across omics layers, thereby bridging the gap from genotype to phenotype. Due to their holistic perspective, integrative approaches hold the potential to enhance prognostic and predictive precision for disease phenotypes, ultimately supporting more effective treatment and prevention strategies (4). The scope of “omics” can be further expanded to include additional biological data types, such as the lipidome, phosphoproteome, and glycoproteome. When multi-omics data are generated for the same set of samples, they offer valuable insights into the flow of biological information across different levels, facilitating a deeper understanding of the underlying mechanisms of the biological condition under investigation (1). The integration of multi-omics datasets to achieve a comprehensive understanding of biological processes and diseases presents several challenges; these include the inherent heterogeneity across individual omics data, the large size of datasets that require computationally intensive analyses, and the absence of standardized approaches for prioritizing the diverse set of tools available for multi-omics integration. Given that multi-omics data are generated using a wide range of platforms, the storage and formatting of these datasets can vary significantly. Most integrative analysis tools for multi-omics data require the data to be in specific formats, typically represented as a Feature X Sample matrix, necessitating preprocessing of the individual omics

datasets. Preprocessing steps generally include data filtering, systematic normalization, removal of batch effects, and quality control checks. These steps must be carefully taken, as they have a significant impact on the success of integrative analyses. For example, the data filtering step is crucial for reducing noise and minimizing the number of features used in integrative models, as many integrative methods are computationally demanding. Thus, it is essential to reduce the size of the input datasets beforehand. However, determining appropriate filtering criteria remains a challenge due to the lack of universally accepted standards; in this context, the development of new integrative methods and tools must prioritize efficient handling of large datasets. The integrative approach using multi-omics data is a powerful strategy for unraveling the mechanistic details of information flow within a cell. Currently, a wide range of tools and methods are available in the public domain for integrating multi-omics datasets to derive meaningful insights (5), (6), (7). Given that most tools and methods remain largely fragmented, there is a pressing need for a standardized framework capable of processing and analyzing multi-omics data in a comprehensive, end-to-end manner. This framework should also facilitate user-friendly visualization and interpretation, making it more accessible to biologists (1).

The multi-omics approach has emerged as a promising and powerful tool for conducting comprehensive studies of human diseases at a systems level, spanning multiple functional layers and overtime. To fully elucidate the dynamic molecular system and gain a deeper understanding of the biological processes involved in disease development, it is essential to not only examine the appropriate “omic” layer at the right time but also to capture multiple dimensions of biological information flow simultaneously. The exploration of complementary omics beyond a single “omic” layer has been undertaken for various diseases and model systems, demonstrating the utility and feasibility of multi-omics research (8).

In this dissertation, I took the first steps at CAST and UA in performing multi-omics data integration analyses based on both proteomic and transcriptomic data, thereby contributing to the study of complex biological processes in murine and human brain organoids, as well as brain tissue from mice.

Experimental choices for multi-omics data analysis

Throughout this work, Qiagen IPA software was applied to perform multi-omics data integration analyses. IPA software operates with a single, comprehensive matrix that includes the ID codes of proteins or/and genes, along with their respective “abundance ratio values” or “fold changes” and associated “p-values” indicating statistical significance. IPA tool returns the results by assigning to each pathway (“canonical pathways”, “upstream”, and “downstream”) a value of $-\log_{10}$ (p-value) and a z-score value. The $-\log_{10}$ (p-value) measures the statistical overlap between the protein dataset and the function categories; the significance is attributed to $-\log_{10}$ (p-value) greater than 1.3. Instead, the predicted regulation of each “canonical pathways”, “upstream regulators” and “downstreams” network effects is inferred by the z-score generated by IPA system (z-scores ≥ 2.0 means that a molecule or pathway is activated, whereas z-scores ≤ -2.0 means the inhibition of target molecules or pathways). IPA provides critical insights into the pathways in which the identified proteins or/and genes are involved, as well as how these pathways may be modulated through upregulation or downregulation. Moreover, the software is user-friendly, making it accessible even to biologists or general users who may not have expertise in bioinformatics. Finally, IPA enables the integration of multiple “omics” analysis layers, allowing for the combination of datasets to derive deeper functional insights.

A more detailed workflow is provided below in the summary of 4 research manuscripts and 1 review manuscript I contributed to, with one recently been published, one accepted for publication, one under review and two in preparation.

1. Transcriptomic and proteomic profiling of bi-partite and tri-partite murine iPSC-derived neurospheroids under steady-state and inflammatory condition

This research manuscript has been published as:

Julia Di Stefano, Laura Garcia-Pupo, **Federica Di Marco**, Helena Motaln, Jonas Govaerts, Elise Van Breedam, Ligia Monica Mateiu, Siebe Van Calster, Leonardo Ricciardi, Alessandra Quarta, Peter Verstraelen, Winnok H De Vos, Boris Rogelj, Ilaria Cicalini, Vincenzo De Laurenzi, Piero Del Boccio, Una FitzGerald, Wim Vanden Berghe, Marleen Verhoye, Damiana Pieragostino and Peter Ponsaerts – *Brain Behavior and Immunity*, 2024 [doi: 10.1016/j.bbi.2024.07.008]

2. Generation, interrogation, and future applications of microglia-containing brain organoids

This review manuscript has been published as:

Julia Di Stefano, **Federica Di Marco**, Ilaria Cicalini, Una FitzGerald, Damiana Pieragostino, Marleen Verhoye, Peter Ponsaerts, and Elise Van Breedam - *Neural regeneration research*, 2025 [not yet peer-reviewed in the journal]

3. The immune-modulating properties of conditioned medium derived from human amniotic membrane-derived mesenchymal stromal cells confer neuroprotection to miPSC-derived neurospheroids under pro-inflammatory stimulation

This research manuscript is in preparation as:

Di Stefano J., **Di Marco E.**, Bufi A., Cicalini I., Papait A., Motaln H., Mateiu L., Garcia-Pupo L., Van Breedam E., Govaerts J., Van Calster S., Rogelj B., Vanden Berghe W., De Laurenzi V., Del Boccio P., FitzGerald U., Parolini O., Verhoye M., Pieragostino D., Silini A. and Ponsaerts P. - 2024.

4. Magnetic resonance imaging and transcriptome-proteome integration analysis demonstrate improved disease-outcome in the cuprizone mouse model following AAV-mediated delivery of A Proliferation Inducing Ligand

This research manuscript has been submitted as:

Leonardo Ricciardi, Federica Di Marco, Marion Decrop, Julia Di Stefano, Tejaswini Kakunuri, Joëlle van Rijswijk, Nicholas Vidas-Guscic, Alexandro A. Bui, Ignace Van Spilbeeck, Johan Van Audekerke, Carole Faghel, Esther Bartholomeus, Eva Lion, Steven Van Laere, Ilaria Cicalini, Vincenzo De Laurenzi, Una FitzGerald, Damiana Pieragostino, Bertrand Huard, Piero Del Boccio, Peter Ponsaerts and Marleen Verhoye – 2024.

5. Multi-omics analysis reveals stroke-like features in matured hiPSC-derived neurospheroids after oxygen-glucose deprivation/reoxygenation

This research manuscript is in preparation as:

Siebe Van Calster, Federica Di Marco, Jonas Govaerts, Julia Di Stefano, Carole Faghel, Esther Bartholomeus, Eva Lion, Winnok H De Vos, Damiana Pieragostino, Piero Del Boccio, Peter Ponsaerts and Elise Van Breedam – 2024.

Development, characterization and application of murine iPSC-derived bi-partite and tri-partite neurospheroids

In collaboration with Julia Di Stefano, PhD student at LEH (UA)

Introduction

Compared to conventional 2D cell cultures, neurospheroids offer several advantages, the most notable of which is their closer resemblance to brain tissue and the more accurate representation of physiological responses. In neurospheroids, cells maintain their physiological morphology and are able to proliferate and differentiate in multiple directions, forming complex, multilayered structures. Cell-cell interactions in neurospheroids become more intricate, occurring in three dimensions and involving multiple cell types. Furthermore, the interaction between cells and the extracellular environment is enhanced in floating neurospheroid cultures, as they are not confined to rigid 2D plastic substrates, which can affect cell viability, differentiation, and gene expression (9). Even more significantly, 2D cultures exhibit limited metabolic activity, low resistance, and are more prone to apoptosis, which can distort the perceived efficacy of drugs, making them appear effective even at low concentrations. In contrast, neurospheroids exhibit a more nuanced response to mechanical and chemical stimuli, display physiologically relevant metabolic activation, demonstrate increased resistance, and have a higher threshold for apoptosis, thereby more accurately reflecting the response of an organ or organism (10), (11), (12). These features make neurospheroids better suited for studying drug administration and testing.

Summary of Materials and Methods

In this study, we worked with CX₃CR1-eGFP^{+/+}-CCR2-RFP^{+/+} murine (m)iPSC-derived bi-partite (neurons + astrocytes) and tri-partite (neurons + astrocytes + microglia) NSPH models that can be subjected to cellular activation following pro-inflammatory stimulation in both bi-partite and tri-partite NSPHs with, respectively,

TNF α + IL1 β + IFN γ and LPS + IFN γ . Bi-partite and tri-partite neurospheroids (NSPHs) were generated from a previously established and validated C57BL/6 CX₃CR1-eGFP^{+/+}-CCR2-RFP^{+/+} murine iPSC line, as described in detail in previous studies (13), (14), (15).

Both bi- and tri-partite NSPHs were utilized for downstream experiments at 4.5 weeks of age. At this stage, they were individually cultured in a 96-well format, with 200 μ L of neural differentiation (ND) medium, which consisted of a 1:1 ratio of DMEM/F12 and Neurobasal-A medium, supplemented with 0.5% N2 supplement (Gibco), 1% B27 supplement (Gibco), and 100 U/mL–100 μ g/mL penicillin-streptomycin (Gibco). Next, in order to further investigate the observed signs of inflammatory response and cellular stress, we performed an untargeted transcriptomic and proteomic analysis of bi- and tri-partite NSPHs under steady-state and inflammatory condition. Here, using the combined differential gene and protein expression profiles between unstimulated and stimulated NSPHs, Ingenuity Pathway Analysis (IPA) confirms the activation of canonical pathways associated with inflammation and cellular stress in both bi-partite and tri-partite NSPHs (15).

Lastly, using an untargeted proteomic analysis we tested the immune-modulating potential of (i) the secretome of hAMSC (Human Amniotic Mesenchymal Stromal Cells) (16), (17), (18), (19), (20), and (ii) the APRIL protein (a member of the TNF superfamily) on bi-partite and/or tri-partite miPSC-derived neurospheroids. Experiments related to the application of hAMSC conditioned medium are described within this section. Experiments related to the application of the APRIL protein are described below.

Summary of Results

A. Generation, characterization and stimulation of miPSC-derived bi-partite and tri-partite NSPHs

Following previously established protocols (15), CX₃CR1-eGFP^{+/+}-CCR2-RFP^{+/+} C57BL/6 miPSCs were differentiated over a 5-week cell culture period into bi-partite NSPHs, approximately 1 mm in diameter, containing β 3-tubulin⁺ neurons and GFAP⁺ astrocytes (Figure 1A), and tri-partite NSPHs, containing β 3-tubulin⁺ neurons, GFAP⁺ astrocytes, and CX₃CR1-eGFP⁺ microglia (Figure 1B). These neurospheroids, which

recapitulate immune responsiveness, exhibited the ability to readily secrete the pro-inflammatory cytokines IL6 and CXCL10. In bi-partite NSPHs, this secretion was triggered by stimulation with $\text{TNF}\alpha + \text{IL1}\beta + \text{IFN}\gamma$, while in tri-partite NSPHs, it was induced by LPS + $\text{IFN}\gamma$ stimulation (Figure 1C and 1D).

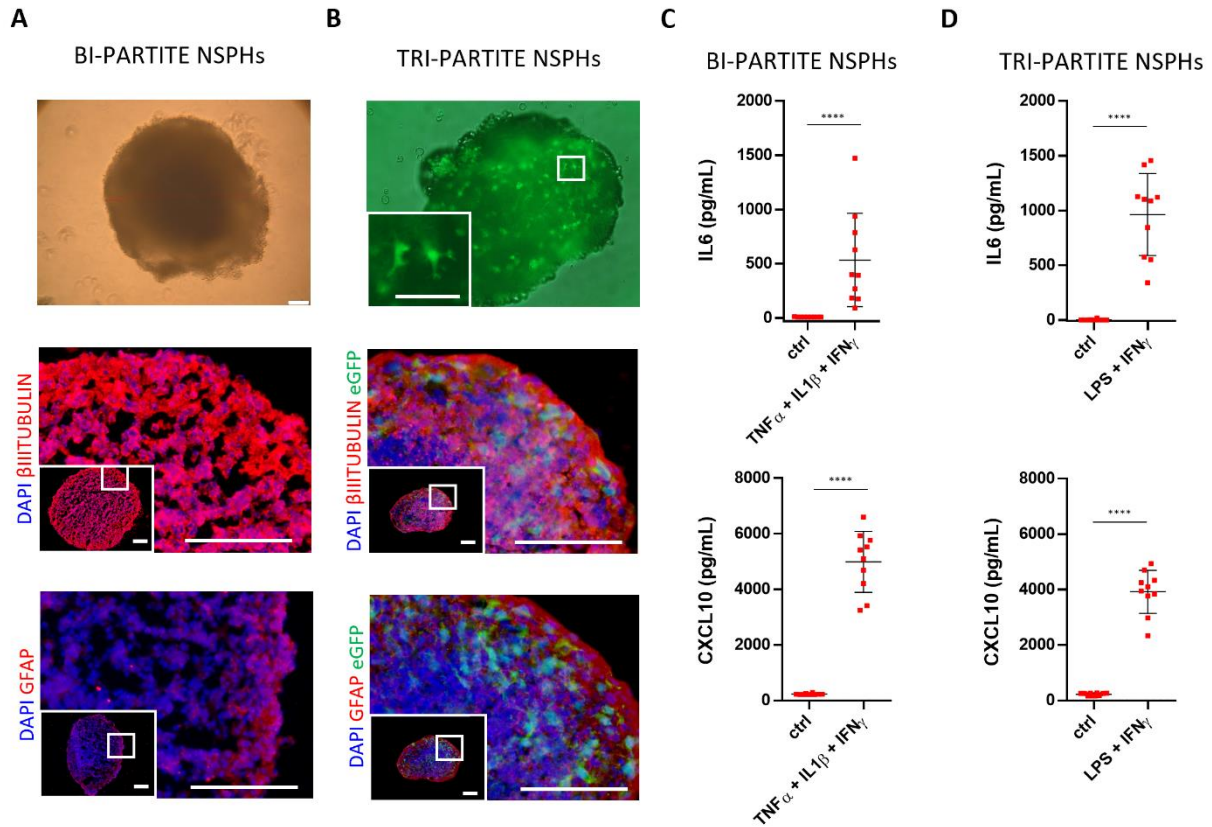


Figure 1: A) Bi-partite and B) tri-partite NSPHs; upper panel: representative live brightfield and eGFP fluorescence image of NSPHs at week 5 of culture. Middle panel: NSPHs immunolabelled for β III-tubulin (in red), nuclei stained with DAPI (in blue) and combined with direct eGFP+/CX3CR1+ fluorescence (in green). Bottom panel: NSPHs immunolabelled for GFAP (in red), nuclei stained with DAPI (in blue) and combined with direct eGFP+/CX3CR1+ fluorescence (in green). C) Dot plots showing IL6 and CXCL10 cytokine secretion (in pg/mL) by bi-partite NSPHs after stimulation with $\text{TNF}\alpha + \text{IL1}\beta + \text{IFN}\gamma$ (n=10 for each). D) Dot plots showing IL6 and CXCL10 cytokine secretion (in pg/mL) by tri-partite NSPHs after stimulation with LPS + $\text{IFN}\gamma$ (n=10 for each). Scale bar microglia magnification, 50 μm ; all other scale bars, 100 μm . **** $p < 0.0001$.

B. Transcriptome-proteome data integration analysis of immune-stimulated bi-partite and tri-partite miPSC-derived neurospheroids

As detailed in Di Stefano et al., 2024, stimulated bi-partite and tri-partite NSPHs, along with control NSPHs, were subjected to transcriptomic and proteomic analyses to identify differentially expressed proteins (DEPs) and differentially expressed genes (DEGs) between unstimulated and stimulated bi-partite or tri-partite NSPHs. The lists of DEGs and DEPs were uploaded into the Ingenuity Pathway Analysis (IPA) tool (Qiagen, Hilden, Germany) to conduct separate gene ontology and functional enrichment analyses. This analysis utilized a matrix containing each quantified protein or gene, its expression ratio, and the associated significance p-value. IPA was employed to predict the activation ($z\text{-scores} \geq 2.0$) or inhibition ($z\text{-scores} \leq -2.0$) of canonical pathways, diseases and bio-functions (downstream pathways), and upstream regulators within the dataset (DEGs or DEPs) based on curated published literature (21). Furthermore, a combined gene ontology and functional enrichment analysis was performed using a comprehensive global matrix containing all quantified proteins and genes to compare stimulated versus unstimulated bi-partite and tri-partite NSPHs. In this context, as previously discussed, we addressed the challenges associated with multi-omics data integration. DEGs and DEPs were consolidated into a single matrix, which required prior standardization based on protein expression abundance ratios and gene expression fold changes. Following this, we applied selection filters to the DEPs and DEGs based on their p-values ($p\text{-value} < 0.05$) to prepare the matrix for subsequent Gene Ontology and functional enrichment analysis using IPA tool. This analysis revealed significant modulation of canonical pathways associated with immune signaling, cell survival, metabolism, and neuronal functioning. Notably, tri-partite NSPHs exhibited activation of the myeloid TREM1 and neuroinflammation signaling pathways, likely due to LPS-stimulated microglia, whereas bi-partite NSPHs uniquely activated the IL1 signaling pathway, reflecting IL1 β -specific stimulation. Both NSPH types showed interferon signaling activation and iNOS signaling, implicating pathways related to apoptosis and pyroptosis. Tri-partite NSPHs also demonstrated necroptosis activation, linked to microglial cytokine secretion, and significant metabolic deregulation. Remarkably, only tri-partite NSPHs showed deactivation of neuronal

function pathways, underscoring the critical role of microglia in inflammation-mediated neurodegeneration and emphasizing their necessity in NSPH models for studying neuroinflammatory processes (Figure 2).

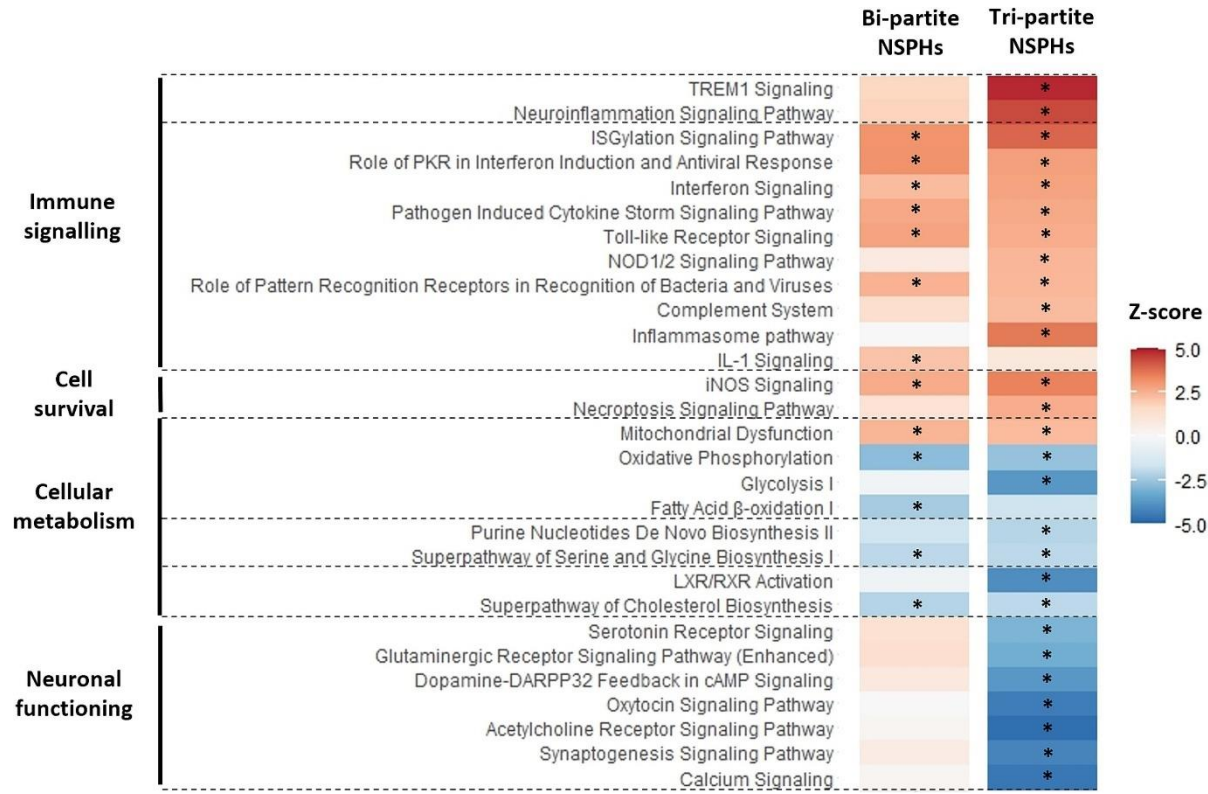


Figure 2: Canonical pathways modulated in stimulated bi-partite and tri-partite NSPHs. Visual representation of the activation z-scores for selected canonical pathways in stimulated bi-partite and tri-partite NSPHs, with color scaling from 5 (dark red) to -5 (dark blue). Asterisks show significantly affected canonical pathways (z-score > 2 or < -2).

Following “canonical pathways” analysis, IPA was utilized to predict diseases and biofunctions induced in stimulated bi-partite and tri-partite NSPHs, based on DEG and DEP datasets. This approach identifies affected biological processes and predicts their directional changes, with results categorized into four main subcategories: immune response, viability, homeostasis, and development. Bubble plots were generated to visualize z-scores, counts, and $-\log_{10}(\text{p-value})$, with bubble size representing the number of genes/proteins involved and color indicating statistical significance (Figure 3). In bi-partite NSPHs, stimulation with $\text{TNF}\alpha$ + $\text{IL1}\beta$ + $\text{IFN}\gamma$ led to significantly increased necrosis, DNA damage, and apoptosis, alongside decreased cell survival. No significant effects were observed in immune response, homeostasis, or development. Conversely, stimulated tri-partite NSPHs exhibited broader downstream effects following LPS + $\text{IFN}\gamma$ stimulation,

including enhanced inflammatory responses (e.g., phagocytosis, chemotaxis, cell adhesion), disrupted cellular homeostasis (e.g., signaling, metabolism, organization), and impaired neuronal development. These effects likely stem from microglial activation by LPS + IFN γ . Overall, tri-partite NSPHs displayed a significantly greater number of affected diseases and biofunctions compared to bi-partite NSPHs, emphasizing the added complexity and biological relevance conferred by microglia integration.

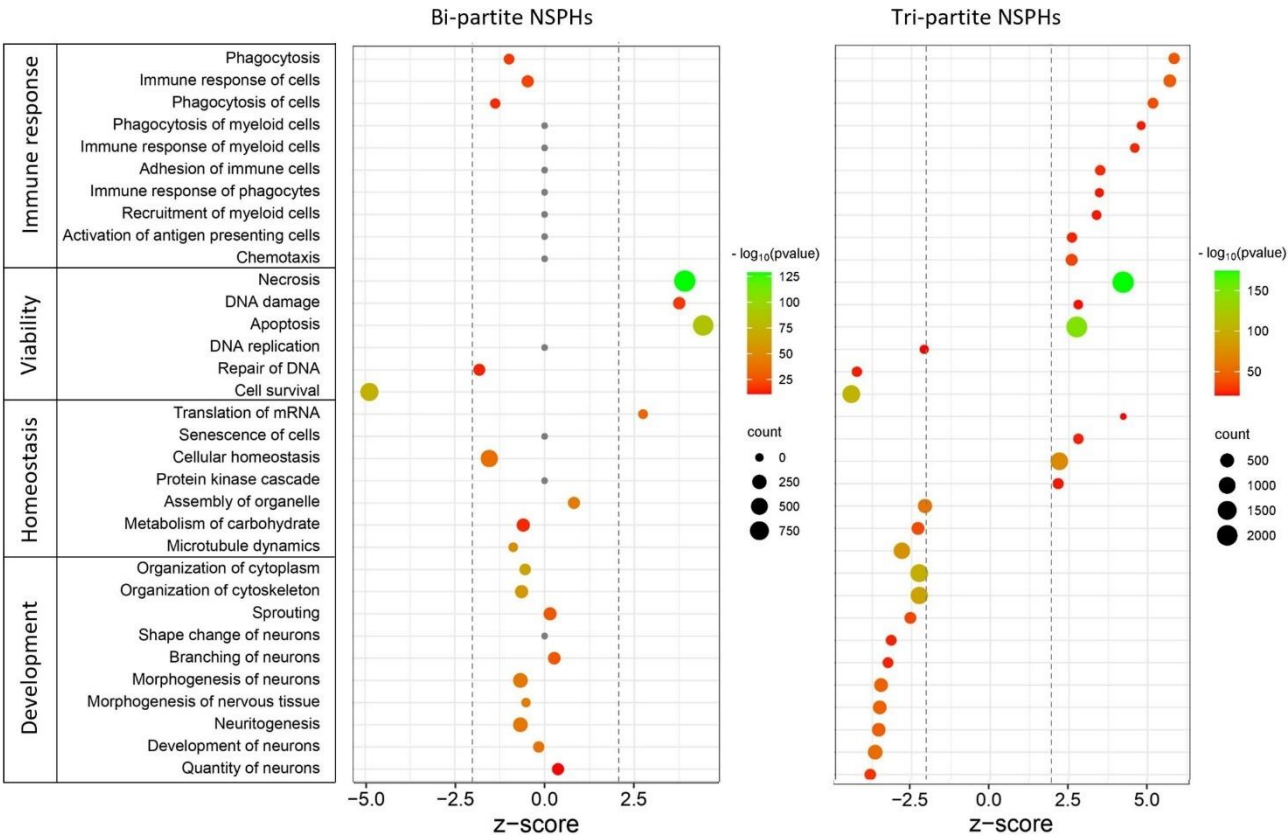


Figure 3: Diseases and biofunctions triggered in stimulated bi-partite and tri-partite NSPHs based on combined transcriptomic and proteomic analysis. Bubble plot representation of the predicted diseases and biofunctions in stimulated bi-partite and tri-partite NSPHs. A z-score > 2 or < -2 indicates un up- or down-regulation of the activation state of each selected biological function. The color of the bubbles corresponds to the $-\log_{10}$ p-value (green = most significant) and the size of bubbles refers to the number of gene and protein counts detected within the given biological function.

C. Modulation of Diseases and biofunctions triggered in stimulated bi-partite and tri-partite NSPHs following treatment with hAMSC secretome

We next focused on studying the effect of the secretome from Human Amniotic Mesenchymal Stromal Cells (hAMSC) on reversing the inflammatory profile induced following immune stimulation of bi-partite and tri-partite miPSC-derived neurospheroids. As these analyses are still ongoing, only the results of the proteomic analysis can be shown at this stage (Figure 4).

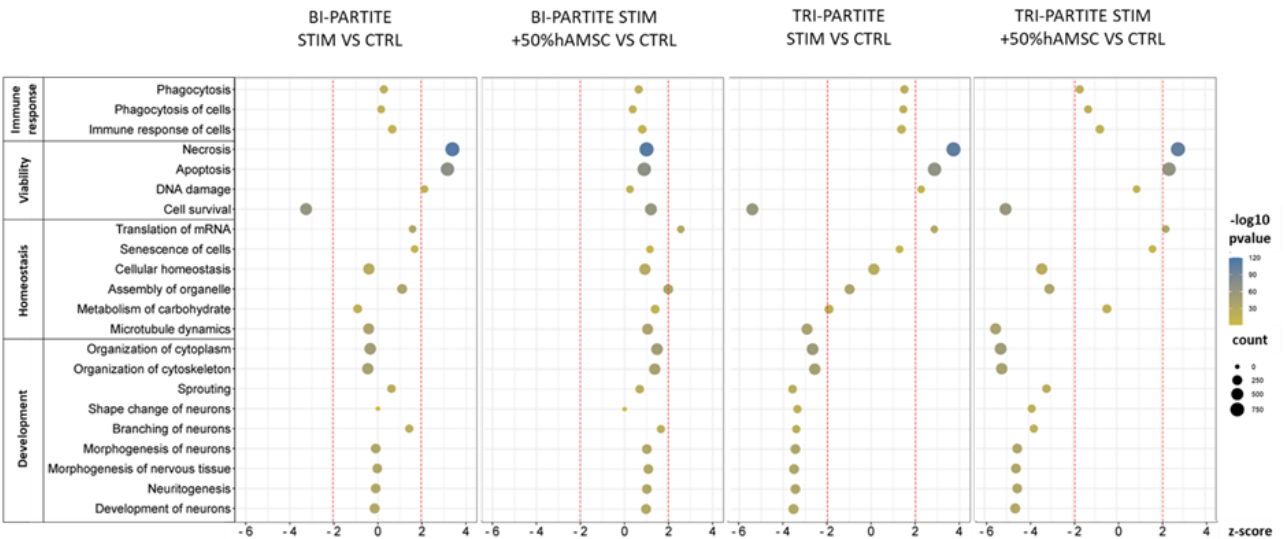


Figure 4: Influence of hAMSC-CM on Diseases and Biofunctions triggered in stimulated bi-partite and tri-partite NSPHs. Bubble plot representation of the predicted diseases and biofunctions providing z-score, counts and $-\log_{10}(\text{p-value})$. Z-score >2 or <-2 indicates an up- or down-regulation of the activation state of each selected biological function. The counts indicate the number of proteins involved in each function, reflected in the size of the bubbles (the bigger the bubbles, the higher the number of proteins involved). The color of the bubbles represents the significance of the p-value (dark blue has the highest significance, yellow the lowest).

Nevertheless, here we present for the first time that unbiased proteomic profiling can detect therapy-associated alterations in disease pathways in both bi-partite and tri-partite NSPHs. We show that hAMSC-CM significantly inhibits cell death-associated signaling pathways in bi-partite NSPHs, with a lesser effect observed in tri-partite NSPHs. This stronger impact on bi-partite NSPHs is likely due to the more pronounced

inflammatory response observed in tri-partite NSPHs, as indicated by the neuronal dysfunction in the latter, which is potentially caused by activated microglia (phenomenon not seen in bi-partite NSPHs). Despite this, hAMSC-CM demonstrates a robust neuroprotective effect on immune-stimulated bi-partite NSPHs, which, hypothetically, may better mirror a more moderate in vivo inflammatory response within the CNS (15). These results are now subject to further validation that will be performed during the PhD research of Julia Di Stefano at LEH-UA.

Discussion and future perspectives

In conclusion, the integration of transcriptomic and proteomic analyses in bi-partite and tri-partite NSPHs highlights the distinct responses and biological complexity of these models under stimulatory conditions. This approach offers valuable insights into the mechanisms driving inflammation-mediated neurodegeneration and highlights the potential of multi-omics integration for advancing our understanding of complex biological systems. Furthermore, we highlighted the distinct immune-related pathways and cellular responses triggered upon immune stimulation in these models, and the neuroprotective effects observed with hAMSC-CM treatment.

Looking ahead, these findings pave the way for further testing immune-modulating compounds as potential therapeutic strategies for neuroinflammatory and neurodegenerative diseases. Future studies could focus on extending this approach to other experimental conditions, incorporating additional immunomodulatory molecules and compounds: exploring the effects of other immune-modulating agents, could provide deeper insights into their therapeutic potential and mechanisms of action. Integrating proteomic and transcriptomic data from multiple experimental conditions will enable a more comprehensive understanding of how these agents influence cellular pathways, immune responses, and disease progression.

However, we do realize that the above-describe work made use of murine iPSC-derived neurospheroids. As such, its translational value for evaluating therapeutic compounds to be used in (i) pre-clinical mouse studies, as well as in (ii) a human context, needs to be further validated.

- For the first, in the next section we will demonstrate that a therapeutic molecule validated by proteomic analysis of treated immune-stimulated bi-partite neurospheroids is also neuroprotective in vivo in a mouse model of neuro-inflammation/degeneration.
- For the seconds, based on the knowledge gained in this first part, and as a new advancement in the "omics" study of organoids, we decided to continue our analysis by generating and studying affected biological processes in human iPSC-derived neurospheroids under control and hypoxic condition.

Magnetic Resonance Imaging and transcriptome-proteome integration analysis demonstrate improved disease-outcome in the cuprizone mouse model following AAV-mediated delivery of a proliferation inducing ligand

In collaboration with Leonardo Ricciardi, PhD student at LEH (UA)

Introduction

Multiple sclerosis (MS) is widely recognized as an autoimmune demyelinating disease of the central nervous system (CNS). Throughout the 20th century, several immune-mediated animal models were developed to study MS, including the experimental autoimmune encephalomyelitis (EAE) model and the Theiler murine encephalomyelitis virus model. In addition to these, toxin-induced demyelinating models, such as the cuprizone (bis-cyclohexanone-oxalyldihydrazone, CPZ) model, the lysophosphatidylcholine injection model, and the ethidium bromide injection model, have been extensively employed to investigate the molecular mechanisms underlying demyelination and remyelination processes (22). Four distinct MS lesion subtypes are described in this disease, linked to autoimmune mechanisms and primary oligodendroglipathy. CPZ treatment also induces axonal swelling due to Na⁺ channel redistribution and calcium homeostasis disturbances. Both MS and CPZ-induced lesions result in motor and cognitive impairments. CPZ-induced demyelination in the corpus callosum correlates with motor deficits, while hippocampal and basal ganglia lesions are associated with memory and motor impairments. Hormonal influences, such as estrogen and testosterone, significantly impact remyelination in the CPZ model. Estrogen protects oligodendrocytes and enhances remyelination via estrogen receptor pathways, while testosterone promotes remyelination through androgen receptor signaling. Interestingly, sex-based differences in remyelination are absent in CPZ-treated mice, possibly due to CPZ's impact on hormonal signaling. The CPZ model addresses limitations of the EAE model, particularly its relevance to non-immune-mediated demyelination. While treatments like anti-TNF α , glucocorticoids, and statins alleviate inflammation in MS, they can impair remyelination, highlighting the

need for alternative therapies. Recent studies, such as those investigating benztropine, demonstrate its potential to stimulate remyelination without affecting the immune response, validating the CPZ model as a valuable tool for testing remyelination-focused therapies. Moreover, the CPZ model's ability to control lesion chronicity through the duration of intoxication offers a significant advantage for studying remyelination, aligning closely with therapeutic goals in MS, where failure to remyelinate is a hallmark feature. This adaptability makes the CPZ model critical for advancing MS treatment strategies (22). As described in Praet et al., 2014, acute/chronic demyelination and remyelination, as well as micro- and [astrogliosis](#), are important signature of CPZ treatment (Figure 1).

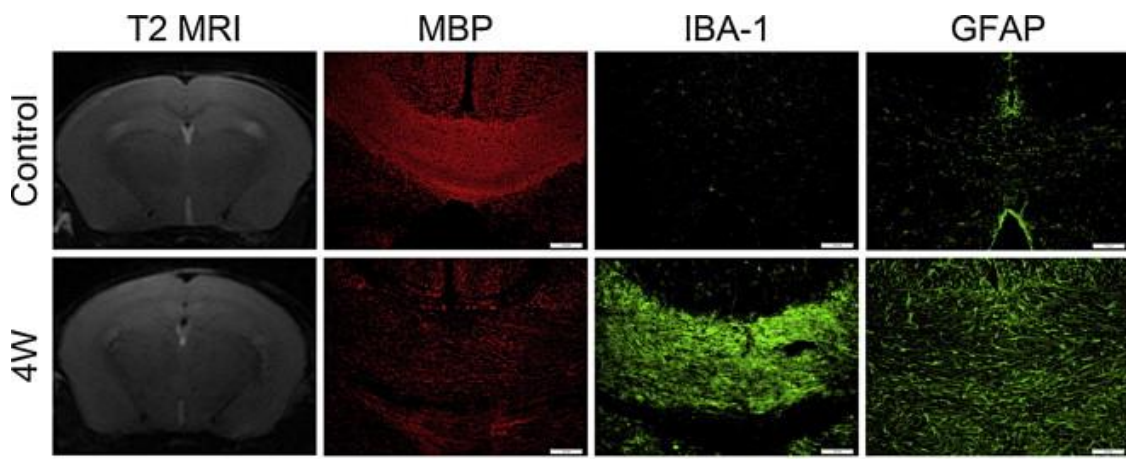


Figure 1: Representative overview showing myelin content (T2 MRI image and MBP staining), microgliosis (IBA-1 staining) and astrogliosis (GFAP staining) at different time points following feeding of 8-week-old female C57BL/6J mice with a 0.2% cuprizone (CPZ) supplemented diet. During acute demyelination (week 4, 4 W), extensive microgliosis and astrogliosis can be observed. (Scale bars = 100 μ m).

A Proliferation Inducing Ligand (APRIL) is a member of the tumor necrosis factor (TNF) superfamily and has recently been shown to modulate pro-inflammatory astrocyte responses, as well as to ameliorate disease outcome in the experimental autoimmune encephalomyelitis mouse model for multiple sclerosis (MS) (23), (24), (25). Therefore, we suggested APRIL to be of interest as a potential immune-modulating molecule in CPZ mouse model. At first, we investigated the neuroprotective role of APRIL in murine bi-partite neurospheroids using proteomic analysis, after which an extensive in vivo study was performed using magnetic resonance

imaging, immunohistochemical and transcriptome-proteome integration analyses, to investigate the immune-modulating and neuro-protective properties of APRIL

Summary of Materials and Methods

The immune modulating and/or neuro-protective properties of AAV-mediated delivery of APRIL were investigated on immune-stimulated murine bi-partite iPSC-derived neurospheroids according to the methods described above, and in the cuprizone mouse model using a combination of magnetic resonance imaging, immunohistochemical analyses, transcriptomics and/or proteomics analyses. For experiments related to the CPZ mouse model, a total of 120 wild-type C57BL/6J mice (RRID: IMSR_JAX:000664, Charles River, Germany) were group-housed under standard conditions, maintained on a 12-hour light/12-hour dark cycle, with ad libitum access to food and water. All experimental procedures adhered to the European guidelines for the care and use of laboratory animals (Directive 2010/63/EU) and received approval from the Committee on Animal Care and Use at the University of Antwerp, Belgium (Approval No: 2021-09). At eight weeks of age, 60 mice were administered standard rodent chow supplemented with 0.2% w/w cuprizone for a duration of four weeks to induce neuroinflammation and demyelination. Of the 120 mice used in this study, 60 underwent MRI, and 60 were used for transcriptomics and proteomics analysis. In both cases, the experimental design and conditions were kept the same. A six-group experiment was performed whereby control, AAV-eGFP injected and AAV-APRIL injected mice were randomly assigned to groups that received either a 4-week regular rodent diet (CTRL, CTRL AAV-eGFP, and CTRL AAV-APRIL; n = 10 each) or groups that received a 4-week CPZ-supplemented diet (CPZ, CPZ AAV-eGFP, and CPZ AAV-APRIL; n=10 each). In this study, for each of the experimental groups described above, we performed unbiased proteomic and transcriptomic analyses to identify DEPs and DEGs, which were then used for subsequent Gene Ontology and functional enrichment analysis by IPA.

Summary of Results

Following our hypothesis that targeted delivery of APRIL to the CNS may be beneficial in terms of neuroprotection, for this study we generated two new rAAV vectors, namely AAV2/7_CMVenhsyn-intron-eGFP and AAV2/7_CMVenhsyn-intron-HA-Fc-MmAPRIL (88-252), further named as AAV-eGFP and AAV-APRIL, respectively. To evaluate the transduction capacity of the generated rAAVs, we applied a previously established in vitro miPSC-derived NSPH model. As such, we first demonstrated that 5-week-old NSPHs can easily be transduced by AAV-eGFP as demonstrated by direct live cell eGFP-fluorescence imaging at day 9 post-transduction (Figure 2A). Likewise, cryosections obtained from AAV-APRIL transduced NSPHs demonstrated successful expression of the APRIL protein following staining for the human Fc-tag linked to the APRIL protein at day 3 post transduction (Figure 2B). In our preceding NSPH studies, we suggested that untargeted proteomic analysis of NSPHs under steady-state, pro-inflammatory and/or treatment conditions is an elegant method to rapidly evaluate the neuroprotective potential of novel CNS targeting interventions within a 3D neural-like environment. Therefore, we here performed a proteomic profiling of (i) control unstimulated NSPHs (CTRL), (ii) NSPHs stimulated with TNFa + IL1b + IFNg (STIM), and (iii) AAV-APRIL transduced NSPHs stimulated with TNFa + IL1b + IFNg (STIM + AAV-APRIL). As already demonstrated in our preceding study, proteomic analysis on the level of 'canonical pathways' (CP) indicated significant upregulation of the mitochondrial dysfunction pathway, as well a significant downregulation of the oxidative phosphorylation pathway in pro-inflammatory stimulated NSPHs (Figure 2C, CPs, STIM vs CTRL). Furthermore, the proteomic profile of pro-inflammatory stimulated NSPHs displays significant upregulation of the 'diseases and biofunctions' (DF) pathways related to DNA damage, apoptosis and necrosis, the latter being associated with significant downregulation of the cell survival pathway and significant upregulation of the neurodegeneration pathway (Figure 2C, DF, STIM vs CTRL). When the same analysis was performed on immune-stimulated AAV-APRIL transduced NSPHs (Figure 2C, STIM + AAV-APRIL vs CTRL), our results demonstrate that although no direct effect was observed on the affected CPs, none of the identified DFs are significantly altered in immune-stimulated AAV-APRIL transduced NSPHs. Based on this last observation, we conclude that AAV-mediated expression of APRIL in an in vitro 3D neural-like environment is able to interfere

with cellular pathways related to cell death following pro-inflammatory stimulation of NSPHs. Therefore, further in vivo studies to evaluate the therapeutic potential of AAV-APRIL administration were justified.

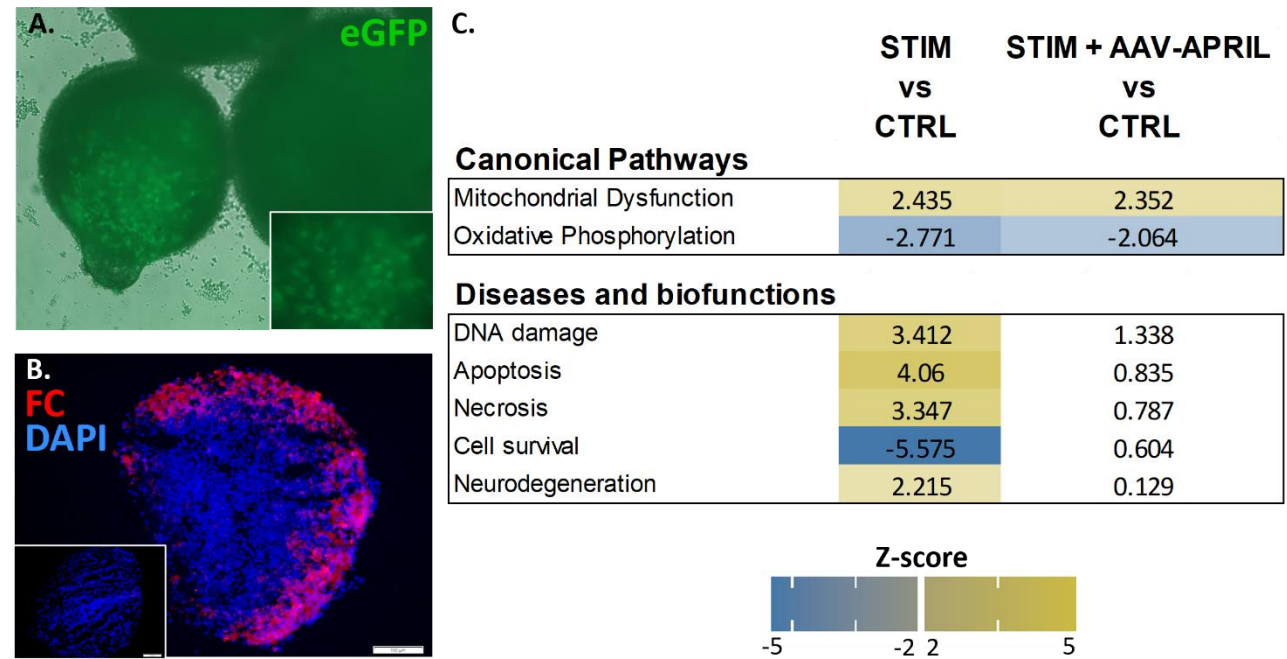


Figure 2: APRIL confers neuroprotection in immune-stimulated miPSC-derived NSPHs. (A) Representative fluorescence live cell image of miPSC-derived NSPHs following AAV-eGFP transduction. AAV-eGFP is green. (B) Representative IHC image of miPSC-derived NSPHs following AAV-APRIL transduction, immunolabelled for APRIL (in red). Nuclei are stained with DAPI (in blue). Scale bar: 100 μ m. IPA analysis outputs: (C) “canonical pathways” and “diseases and biofunctions” affected in immune-stimulated (STIM vs. CTRL), and AAV-APRIL transduced immune-stimulated (STIM + AAV-APRIL vs. CTRL) miPSC-derived NSPHs. Visual representation of the activation z-scores for selected pathways; significantly affected pathways (z-score > 2 or < -2) are represented with the color scaling from 5 (yellow, significantly upregulated) to -5 (blue, significantly downregulated). Non-significant pathways are represented in white.

Next, we investigated whether adeno-associated virus (AAV)-mediated secretion of APRIL by cortical neurons could influence CPZ-induced neuro-inflammation and/or demyelination in mouse splenium of the corpus callosum. For a detailed description of the animal groups included in this study, see the summary of the Materials and Methods section. At first, all experimental groups were analysed using T2-weighted magnetic resonance imaging (MRI). Following MRI data processing, T2 maps were reconstructed for condition CTRL, CPZ diet, CPZ diet with AAV-eGFP injection and CPZ diet with AAV-APRIL injection (Figure 3). Following

four weeks of CPZ diet, AAV-eGFP-injected mice exhibited a significant increase in T2 values (Figures 3A and 3B), indicative of inflammation and demyelination. In contrast, CPZ-treated mice injected with AAV-APRIL did not show a significant increase in T2 values in the splenium during the observation period, suggesting a protective effect of APRIL against CPZ-induced inflammatory and/or demyelinating events. These findings indicate that cortical administration of AAV-APRIL mitigates the effects of CPZ, as reflected by T2 metrics in the splenium, supporting its potential therapeutic benefit in this model.

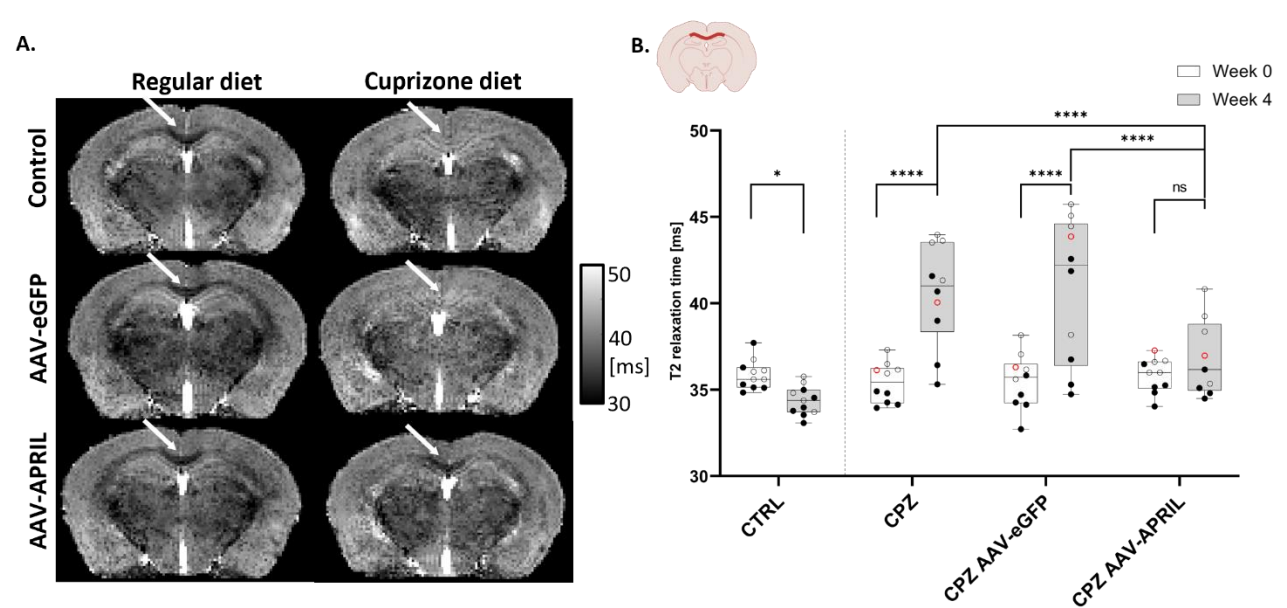


Figure 3: In vivo MRI reveals improved disease outcome in the splenium of CPZ-treated mice following AAV-mediated cortical expression of APRIL. A) Representative images of T2 maps. B) Box plots showing T2-relaxation time. Red circles represent the mice per group used for histological analyses. Bars indicate min to max; the horizontal line represents the median, and the boxes extend from the 25th to the 75th percentile. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

To further document the beneficial effect of APRIL, a transcriptome-proteome integration study (Figure 4) for the splenium revealed the activation of cellular pathways associated with alternative immune cell polarization, cell survival and neurodegeneration. Cortical AAV-APRIL injection alters the neuro-inflammatory environment by modulating cellular pathways involved in alternative macrophage activation and neuro-regeneration in the splenium. As regarding the comparison between the CPZ model and CTRL, inflammatory signaling pathways related to interferon activation, microgliosis, phagocytosis, and endocytosis are

significantly activated. In the comparison between CPZ diet with AAV-eGFP and CTRL, a greater activation of inflammation-related pathways is observed, likely due to the needle injection into the brain, which causes brain damage. This results in a marked increase in the production of reactive oxygen species (ROS) and nitric oxide (NO), which is not seen in the simple CPZ diet model. Finally, in the comparison between CPZ diet with AAV-APRIL and CTRL, significant inflammation persists, but with a shift from a pro-inflammatory to an anti-inflammatory phenotype, as observed in the activation of alternative macrophage polarization. Furthermore, inflammation is modulated, leading to an upregulation of cell survival and repair mechanisms, which reflects the changes in the type of inflammation.

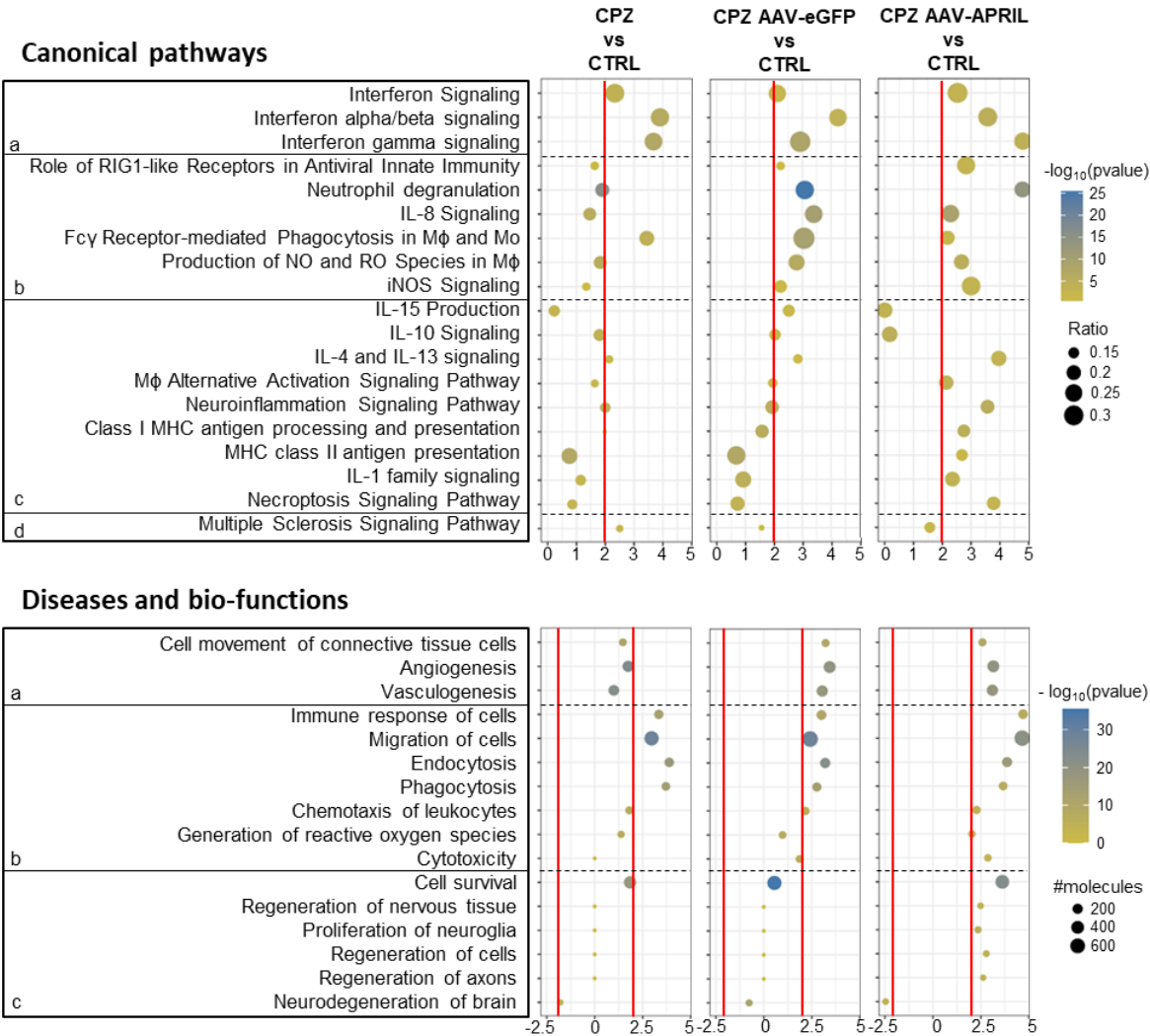


Figure 4: Transcriptomics and proteomics data integration in Gene Ontology and enrichment functional analysis by IPA. This bubble plot illustrates the “canonical pathways”, and “diseases and bio-functions” affected in the splenium of the CPZ mouse model, both under baseline conditions and after AAV-eGFP or AAV-APRIL injection. For “canonical pathways”, the data are divided into: (A) innate immune signaling, (B) neutrophil and macrophage activation, (C) macrophage polarization, and (D) multiple sclerosis signaling pathways. For “diseases and bio-functions”, subdivisions include: (A) wound healing, (B) immune response, and (C) neuroprotection and regeneration. A z-score > 2 and < -2 indicates significant upregulation or downregulation, respectively, of the activation state for each biological function. The color of the bubbles represents the $-\log_{10}(\text{p-value})$ (blue indicating the most significant), while the size of the bubbles corresponds to the ratio of detected gene and protein counts relative to the total number of known genes and proteins associated with the specific pathway.

Discussion and future perspectives

In summary, the results from the CPZ model and its comparison with controls, as well as the administration of AAV-eGFP and AAV-APRIL, highlight significant insights into neuroinflammation and the associated molecular pathways. The CPZ model, representing a toxin-induced demyelination model, activated key inflammatory pathways, including interferon signaling, microgliosis, phagocytosis, and endocytosis, which are pivotal in driving neurodegeneration. The injection of AAV-eGFP exacerbated these responses due to the induced brain injury. In contrast, AAV-APRIL treatment led to a shift from a pro-inflammatory to an anti-inflammatory phenotype, underscoring the therapeutic potential of APRIL in modulating neuroinflammation.

However, performing this extensive transcriptome-proteome integration analysis on mouse brain tissue under disease and treatment conditions, also indicated certain limitations to our analysis approach, as will be discussed further below in the general discussion section of this thesis chapter. Nevertheless, as a future perspective, I am currently working on a more in-depth investigation focusing solely on the CPZ diet model compared to the control group is to better understand the underlying molecular dynamics and to assess the full scope of inflammatory responses and tissue repair mechanisms in this model. This is required as due to the therapeutic intervention with an AAV-based viral vector, the immune environment of the CPZ model itself undergoes dramatic changes, whereby certain aspects of the original disease model are lost during the bioinformatics analysis. This additional study, which I aim to finalize after submitting my PhD thesis, will

help to refine therapeutic strategies and explore new avenues for improving remyelination and neuroprotection in demyelinating diseases, with potential implications for conditions such as MS.

Nevertheless, we would like to stress the importance of this study, whereby we have shown that a molecule identified to be neuro-protective by means of proteomic analysis on treated murine iPSC-derived bi-partite neurospheroids, also displayed neuro-protective features upon delivery in vivo. Hence, we have here shown that miPSC-derived neurospheroid research - and to a further extent – transcriptome-proteome integration analyses – is highly complementary to preclinical animal studies.

Multi-omics analysis reveals stroke-like features in matured hiPSC-derived neurospheroids after oxygen-glucose deprivation and reoxygenation

In collaboration with Siebe Van Calster, PhD student at LEH (UA)

Introduction

Building on the insights gained from the studies described above, we now turn into a new avenue of research by conducting integrated proteomic and transcriptomic analyses on human iPSC-derived brain organoids. This approach represents an important advancement in the "omics" study of organoids, offering a deeper understanding of their molecular responses and potential applications in modeling human brain diseases. Despite the high global burden of ischemic stroke on patients and society, treatment options are limited and currently used models for stroke research often lack physiological relevance. Ischemic stroke resulting from cerebral artery occlusion remains a leading cause of chronic disability worldwide, with limited effective strategies currently available to enhance functional recovery (26). The insufficient oxygen and nutrient supply to the brain due to the impaired blood flow triggers neuronal death, exacerbating the condition. A critical factor underlying ischemic tissue damage is the inflammatory response at the blood-endothelial interface of brain capillaries. This process involves the activation of adhesion molecules, cytokines, chemokines, and white blood cells, all of which play pivotal roles in the pathogenesis of cerebral infarction (27). The pathophysiological alterations following ischemic stroke, including ionic imbalances, neuroinflammation, and dysregulated neuronal functioning, further contribute to neuronal death and tissue injury. In this last study, we therefore investigated whether the optimized transcriptome-proteome integration analysis applied in the above-described studies could be readily applied to human iPSC-derived neurospheroids.

Summary of Materials and Methods

For the generation of hiPSC-derived bi-partite NSPHs, a new bioreactor-based organoid-culture was applied to generate 5-month-old matured hiPSC-derived neurospheroids, containing neurons and astrocytes, to more closely mimic the presence of post-natal neural cell types, as well as their cellular responses under oxygen glucose deprivation (OGD) condition and subsequent re-oxygenation (Figure 1).

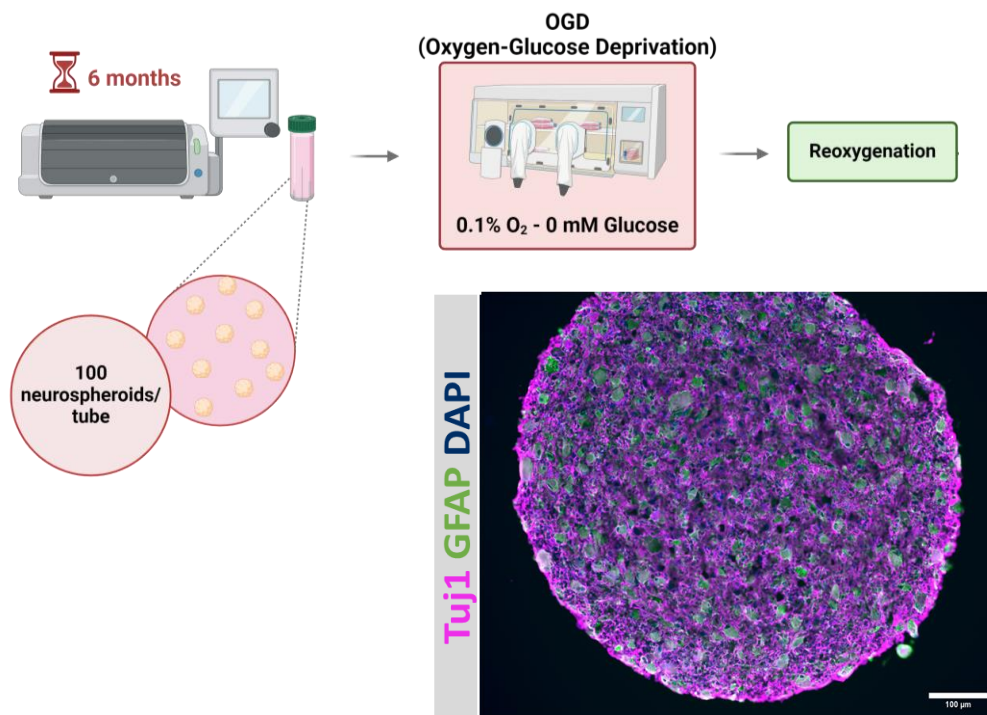


Figure 1: Bioreactor-based culture of 5-month-old matured hiPSC-derived bi-partite neurospheroids containing neurons and astrocytes. To mimic a stroke-like event, neurospheroids were subjected to oxygen-glucose deprivation in a hypoxic chamber, followed by three days of re-oxygenation. Subsequently, a transcriptome-proteome integration study was performed to identify affected cellular pathways.

These human iPSC-derived bi-partite neurospheroids were subjected for 6 hours to oxygen-glucose deprivation in a hypoxic chamber, followed by three days of re-oxygenation, after which both control and OGD-subjected neurospheroids were processed for untargeted transcriptomic and proteomic analysis. Transcriptomics and proteomics analyses, as described in more detail above, identified DEPs and DEGs, which were then used for subsequent Gene Ontology and functional enrichment analysis. Initially, the data from

each omic analysis was considered separately, but integration into a single matrix for a comprehensive analysis was our ultimate goal.

Summary of Results

At first, to demonstrate the that the applied OGD procedure indeed induced cellular stress in 5-month-old hiPSC-derived bi-partite neurospheroids, the supernatant of control and OGD-subjected neurospheroids was analysed for the presence of Neurofilament-I. Results indicate an increased release of Neurofilament-I in the supernatant of OGD-subjected neurospheroids, indicative of neuronal cell death. This highly corresponds to the results of our untargeted transcriptome-proteome integration analysis whereby upregulation of processes related to oxidative stress after 72h of reoxidation, as well as changes in pathways related to cell death, cell cycle progression and ion channel functionality are predicted to be activated and/or altered (Figure 2). Obviously, these results are now subject to further validation that will be performed during the PhD research of Siebe Van Calster at LEH-UA.

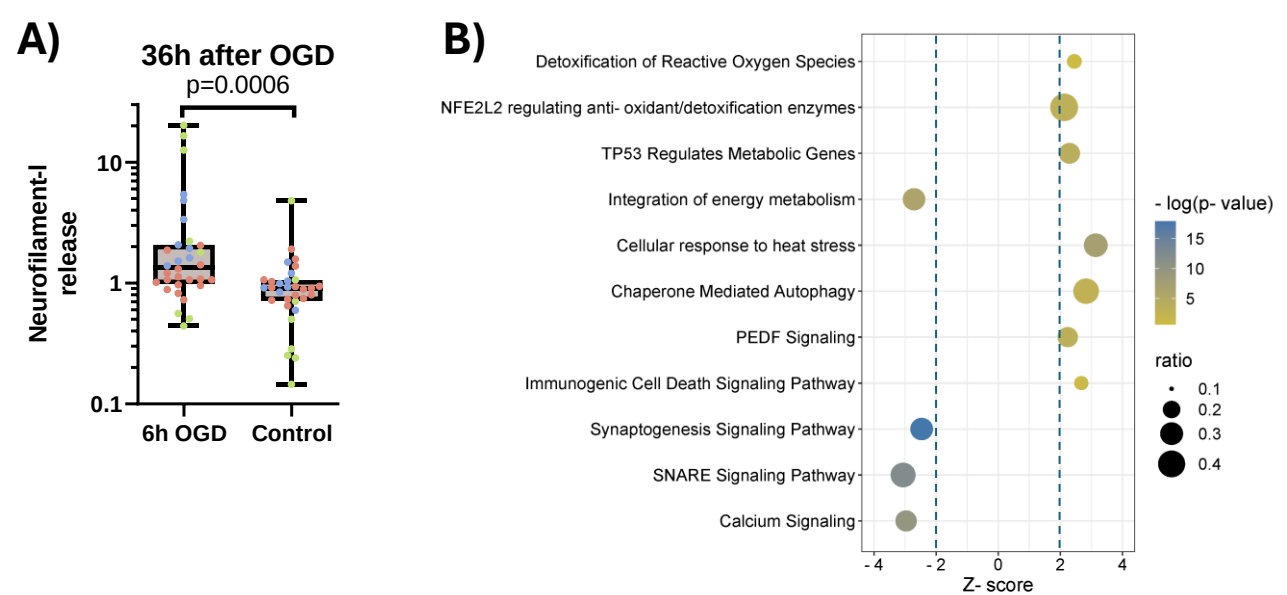


Figure 2: Preliminary results of transcriptomics and proteomics data integration of hiPSC-derived bi-partite neurospheroid under OGD condition. A) Increase in the production of inflammatory neurofilament-I was observed in hiPSC-derived bi-

partite neurospheroid under OGD condition compared to the control. B) Increase in inflammatory pathways and oxidative stress; decrease in synaptogenesis signaling pathway. A z-score > 2 or < -2 indicates an up- or down-regulation of the activation state of each selected biological function. The color of the bubbles corresponds to the $-\log_{10}$ p-value (blue = most significant) and the size of bubbles refers to the number of gene and protein counts detected within the given biological function.

Discussion and future perspectives

Again, we have demonstrated that transcriptome-proteome integration analysis is essential to investigate physiological processes in human iPSC-derived neurospheroids under stroke-like conditions. However, even though the shown analysis on human iPSC-derived bi-partite neurospheroids has provided valuable insights into cellular processes, such as oxidative stress, cell death, and ion channel dysfunction that are critical for understanding stroke-like pathologies, my work performed here will be highly valuable for future research within LEH-UA. Notably, similar to their research on murine iPSC-derived bi- and tri-partite neurospheroids, they now also optimized culture conditions for tri-partite human iPSC-derived neurospheroids, incorporating not only neurons and astrocytes but also hiPSC-derived microglia. This advanced model would enable an even more thorough characterization of metabolic alterations and the involvement of the immune response, particularly in conditions such as oxygen-glucose deprivation (OGD), which mimics ischemic stroke. The incorporation of microglia into the neurospheroid model thus allows for a more complete study of neuroinflammation and its effects on neuronal function, providing deeper insights into stroke pathophysiology and potential therapeutic strategies. For sure, the optimized techniques for transcriptome-proteome integration analysis during my PhD research will be highly instrumental for further research at LEH-UA.

General discussion

During my collaboration with the research group of Prof. Peter Ponsaerts at LEH-UA, before, during and after my research stay at LEH-UA, my knowledge gained at CAST has strongly contributed to the establishment of novel approaches to perform comprehensive analyses of omics data. Hereby, it was aimed to integrate both transcriptomics and proteomics data obtained from the same specimens, to better understand the molecular pathways involved in neuroinflammation in both in vitro cell culture models of murine and human iPSC-derived multi-cellular neurospheroids, as well as experimental models of the central nervous system (CNS) inflammation and demyelination. Through this integrative analysis, we were able to identify key changes in gene expression and protein levels associated with the activation of inflammatory signaling, cell survival mechanisms, and neuronal repair processes, both in vitro and in vivo. The combinational analysis of both transcriptomics and proteomics analysis thus allowed for a more holistic view of the biological systems at play, highlighting the importance of cross-layer data integration to uncover complex molecular interactions that may be overlooked when considering each “omic” data set separately. The integration of transcriptomic and proteomic data proves to be an invaluable tool in dissecting the complexities of neuroinflammatory diseases and in evaluating potential therapeutic interventions.

Being engaged in this field of research, it is noteworthy to mention that this work is only the beginning of a much larger concept of multi-omics analysis of brain organoids and/or brain tissue, including the extension towards the inclusion of metabolomics (including lipidomic analysis). As already briefly mentioned above in the discussion of the APRIL animal study, a limitation of the followed approach of bulk transcriptomics and proteomics on neurospheroids and brain tissue is that some of the cellular processes may not be detected and/or masked by the fact that we are only detecting an overall image of the ongoing processes. This however should not be a – in first instance – a major limitation as our integrative bulk transcriptome-proteome integration analysis approach nevertheless is able to, both in vitro and in vivo, to detect major alterations in cellular processes. Nevertheless, and here specifically focusing on neurospheroid research, the future lies in

further extending a multi-model organoid interrogation toolbox, even to the stage of single cell analyses (Figure 1), according to Di Stefano et al.

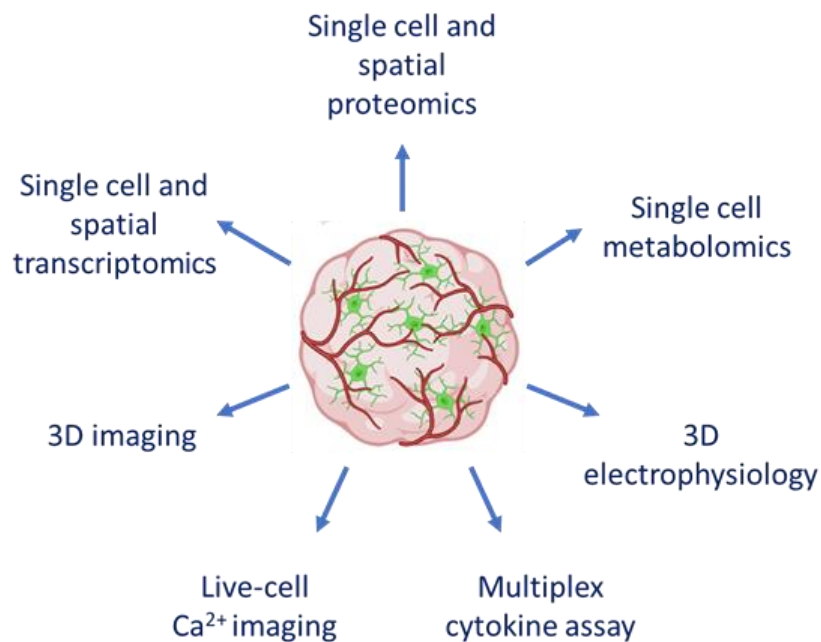


Figure 1: The future brain organoid with microglia and a vasculature and the relative interrogation toolbox.

In fact, the collaborative research between LEH-UA and CAST has been highly successful, with multiple manuscripts in the pipeline, but also has brought us to a whole new era of research whereby multi-omics approaches, either being transcriptomics, proteomics or metabolomics, will need to be extended to the single cell level. The latter is a strong future goal given the increasing complexity of neurospheroids with multiple cell types acting different on certain stimuli and or treatments. In addition, LEH-UA is also continuously developing novel live cell imaging modalities, including the detection of electrophysiologic network activity, to validate predictions from our multi-omics analyses.

Concluding, as the field of neuro-inflammation research will continues to evolve, the application of integrated omics – and subsequent validation strategies - will undoubtedly play a pivotal role in advancing personalized medicine approaches and improving therapeutic outcomes for neuroinflammatory and neurodegenerative diseases.

References

1. Subramanian I, Verma S, Kumar S, Jere A, Anamika K. Multi-omics Data Integration, Interpretation, and Its Application. *Bioinform Biol Insights*. gennaio 2020;14:117793221989905.
2. Yan J, Risacher SL, Shen L, Saykin AJ. Network approaches to systems biology analysis of complex disease: integrative methods for multi-omics data. *Briefings in Bioinformatics* [Internet]. 30 giugno 2017 [citato 6 dicembre 2024]; Disponibile su: <https://academic.oup.com/bib/article-lookup/doi/10.1093/bib/bbx066>
3. Bersanelli M, Mosca E, Remondini D, Giampieri E, Sala C, Castellani G, et al. Methods for the integration of multi-omics data: mathematical aspects. *BMC Bioinformatics*. dicembre 2016;17(S2):S15.
4. Hasin Y, Seldin M, Lusi A. Multi-omics approaches to disease. *Genome Biol*. dicembre 2017;18(1):83.
5. Rappoport N, Shamir R. Multi-omic and multi-view clustering algorithms: review and cancer benchmark. *Nucleic Acids Research*. 16 novembre 2018;46(20):10546–62.
6. Tini G, Marchetti L, Priami C, Scott-Boyer MP. Multi-omics integration—a comparison of unsupervised clustering methodologies. *Briefings in Bioinformatics*. 19 luglio 2019;20(4):1269–79.
7. Chauvel C, Novoloaca A, Veyre P, Reynier F, Becker J. Evaluation of integrative clustering methods for the analysis of multi-omics data. *Briefings in Bioinformatics*. 23 marzo 2020;21(2):541–52.
8. Sun YV, Hu YJ. Integrative Analysis of Multi-omics Data for Discovery and Functional Studies of Complex Human Diseases. In: *Advances in Genetics* [Internet]. Elsevier; 2016 [citato 6 dicembre 2024]. p. 147–90. Disponibile su: <https://linkinghub.elsevier.com/retrieve/pii/S0065266015000516>
9. Gilbert PM, Havenstrite KL, Magnusson KEG, Sacco A, Leonardi NA, Kraft P, et al. Substrate Elasticity Regulates Skeletal Muscle Stem Cell Self-Renewal in Culture. *Science*. 27 agosto 2010;329(5995):1078–81.
10. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FDP. 3D Cell Culture Systems: Advantages and Applications. *Journal Cellular Physiology*. gennaio 2015;230(1):16–26.

11. Imamura Y, Mukohara T, Shimono Y, Funakoshi Y, Chayahara N, Toyoda M, et al. Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer. *Oncology Reports*. aprile 2015;33(4):1837–43.
12. Langhans SA. Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol*. 23 gennaio 2018;9:6.
13. Quarta A, Le Blon D, D’aes T, Pieters Z, Hamzei Taj S, Miró-Mur F, et al. Murine iPSC-derived microglia and macrophage cell culture models recapitulate distinct phenotypical and functional properties of classical and alternative neuro-immune polarisation. *Brain, Behavior, and Immunity*. novembre 2019;82:406–21.
14. Quarta A, Berneman Z, Ponsaerts P. Functional consequences of a close encounter between microglia and brain-infiltrating monocytes during CNS pathology and repair. *Journal of Leukocyte Biology*. 29 giugno 2021;110(1):89–106.
15. Di Stefano J, Garcia-Pupo L, Di Marco F, Motaln H, Govaerts J, Van Breedam E, et al. Transcriptomic and proteomic profiling of bi-partite and tri-partite murine iPSC-derived neurospheroids under steady-state and inflammatory condition. *Brain, Behavior, and Immunity*. ottobre 2024;121:1–12.
16. Pianta S, Bonassi Signoroni P, Muradore I, Rodrigues MF, Rossi D, Silini A, et al. Amniotic Membrane Mesenchymal Cells-Derived Factors Skew T Cell Polarization Toward Treg and Downregulate Th1 and Th17 Cells Subsets. *Stem Cell Rev and Rep*. giugno 2015;11(3):394–407.
17. Giampà C, Alvino A, Magatti M, Silini AR, Cardinale A, Paldino E, et al. Conditioned medium from amniotic cells protects striatal degeneration and ameliorates motor deficits in the R6/2 mouse model of Huntington’s disease. *J Cellular Molecular Medi*. febbraio 2019;23(2):1581–92.
18. Cargnoni A, Ressel L, Rossi D, Poli A, Arienti D, Lombardi G, et al. Conditioned medium from amniotic mesenchymal tissue cells reduces progression of bleomycin-induced lung fibrosis. *Cytotherapy*. febbraio 2012;14(2):153–61.

19. Parolini O, Souza-Moreira L, O'Valle F, Magatti M, Hernandez-Cortes P, Gonzalez-Rey E, et al. Therapeutic Effect of Human Amniotic Membrane-Derived Cells on Experimental Arthritis and Other Inflammatory Disorders. *Arthritis & Rheumatology*. febbraio 2014;66(2):327–39.
20. Onishi R, Ohnishi S, Higashi R, Watari M, Yamahara K, Okubo N, et al. Human Amnion-Derived Mesenchymal Stem Cell Transplantation Ameliorates Dextran Sulfate Sodium-Induced Severe Colitis in Rats. *Cell Transplant*. dicembre 2015;24(12):2601–14.
21. Rossi C, Cicalini I, Cufaro MC, Consalvo A, Upadhyaya P, Sala G, et al. Breast cancer in the era of integrating “Omics” approaches. *Oncogenesis*. 14 aprile 2022;11(1):17.
22. Praet J, Guglielmetti C, Berneman Z, Van Der Linden A, Ponsaerts P. Cellular and molecular neuropathology of the cuprizone mouse model: Clinical relevance for multiple sclerosis. *Neuroscience & Biobehavioral Reviews*. novembre 2014;47:485–505.
23. Ullah MA, Mackay F. The BAFF-APRIL System in Cancer. *Cancers*. 16 marzo 2023;15(6):1791.
24. Baert L, Manfroi B, Casez O, Sturm N, Huard B. The role of APRIL - A proliferation inducing ligand - In autoimmune diseases and expectations from its targeting. *Journal of Autoimmunity*. dicembre 2018;95:179–90.
25. Magliozzi R, Marastoni D, Calabrese M. The BAFF / APRIL system as therapeutic target in multiple sclerosis. *Expert Opinion on Therapeutic Targets*. 1 novembre 2020;24(11):1135–45.
26. Campbell BCV, Khatri P. Stroke. *The Lancet*. luglio 2020;396(10244):129–42.
27. Al-Bahrani A, Taha S, Shaath H, Bakhiet M. TNF- α ; and IL-8 in Acute Stroke and the Modulation of these Cytokines by Antiplatelet Agents. *CNR*. 1 febbraio 2007;4(1):31–7.

