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**LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY,
BIOMATERIALS AND 3D PRINTING
IN ANALYTICAL APPLICATIONS**

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PHD COURSE IN DRUG SCIENCES

XXXV CYCLE

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BIOMATERIALS AND 3D PRINTING
IN ANALYTICAL APPLICATIONS**

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PREFACE: General Purpose of the Research Project

Liquid chromatography-mass spectrometry (LC-MS) is a powerful and versatile analytical technique widely used in life sciences. This thesis work aims to group different analytical applications, where LC-MS is the technique of choice to investigate biomarkers of biomedical interest. In this context targeted and untargeted LC-MS/MS approaches were adopted using different analytical instruments such as HPLC, nano-HPLC, electrospray (ESI), triple quadrupole, orbitrap and trapped ion mobility spectrometry.

The other technology investigated and involved in some of the analytical applications reported in this work is three-dimensional (3D) printing, used for the fabrication of 3D hydrogels based on polysaccharide biomaterials, particularly alginate and hyaluronic acid. In detail, an extrusion 3D printer was used, which, by depositing the biomaterial-based ink, layer upon layer, enabled the construction of porous polymeric scaffolds with customized and tuneable properties. These 3D polymeric networks, sometimes functionalized with drugs, bacterial lysates, nanoparticles, have been used in applications of regenerative medicine, tissue regeneration, treatment of infected wounds and drug delivery.

This thesis paper has therefore been divided into 10 chapters.

The first chapter is a general introduction on the technologies used in the analytical applications reported; therefore, there are two sections: the first deals with LC-MS in proteomics and metabolomics, respectively; the second deals with the use of 3D printing extrusion-based technique for the manufacturing of natural polysaccharide hydrogels for applications in the biomedical field.

From Chapter II to Chapter IX the analytical applications with biomedical interest, developed during the PhD period started in 2019 and ended in 2022 under the supervision of Prof. Lisa Elviri (Food and Drug Department) and Prof. Roberto Toni (Medicine and Surgery Department) from the University of Parma, are described.

Chapter II focuses on the European Horizon 2020 SCRENEED project (#825745) that aim to develop 3D *in vitro* assays for screening the effect of low doses of Endocrine Disruptors (EDs) on thyroid cell function in a sex-specific manner ([The H2020 SCRENEED project \(screened-project.eu\)](https://screened-project.eu)). Our group, coordinated by Prof. Roberto Toni, deals with the investigation of rat cells as a cell model with the ultimate aim of developing 3D organoids to be used in the screening of EDs. EDs were selected and tested at the lowest concentrations, having a possible effect on thyroid function. In this frame, LC-MS targeted analysis was chosen as the analytical technique for the characterization of the developed cellular models and in particular, for the study of specific protein and hormonal biomarkers involved

in thyrocytic differentiation. Finally, untargeted LC-MS analysis was also carried out to achieve a complete overview of the proteomic profile of the developed 3D organoids. This work was reported here as: *SCREENED: Development and characterization of a sex-specific, 3D rat organoid system for the in vitro screening of Endocrine Disruptors on thyroid function*.

Chapter III reports the use of untargeted and targeted LC-MS for extracellular matrix (ECM) proteins investigation. In particular, porcine kidney, liver, pancreas, and skin were decellularized using a quick thawing / quick microwave-assisted decellularization protocol and two different detergents, sodium dodecyl sulfate (SDS) or Triton X-100 (TX100); variations in matrix conservation of stromal proteins were detected by LC-MS coupled to light and scanning electron microscopies, in dependence on each detergent. Since porcine matrices can be used in transplant therapy as natural scaffolds for engineering of human organs, and each detergent allows for saving selective morphoregulatory and mechanochemical ECM patterns, suggestions have been provided to optimize the decellularization of 3D matrices to be used as tools for the recellularization of 3D bioartificial human organs. This work was published in *Tissue Engineering Part C: Methods* with the title of *Decellularization Detergents As Methodological Variables in Mass Spectrometry of Stromal Matrices* in 2022 [1].

Chapters IV and V concern the development of simple and reliable analytical methods based on targeted LC-MS, for the quantification of the protein insulin like growth factor-1 (IGF-1) in bovine milk samples and, for the quantification of cholesterol metabolites 24-, 25- and 27-hydroxycholesterol (OHC) in mouse brain and sera, respectively. The first work was published in 2022 in the journal *Food Chemistry: X* with the title of *Development and single laboratory validation of a targeted liquid chromatography-triple quadrupole mass spectrometry-based method for the determination of insulin like growth factor-1 in different types of milk samples* [2]; the second has been prepared for submission to the journal *Molecular Neurodegeneration* as *PCSK9 ablation attenuates A β pathology, neuroinflammation and cognitive dysfunctions in 5XFAD mice*.

Chapters VI, VII, VIII, IX, share both the use of targeted LC-MS for the analysis of proteins or lipid biomarkers, and the use of 3D printing to fabricate alginate-based hydrogels crosslinked by ionotropic external (chapters VI and VII) or internal (chapter VIII and IX) gelation.

Chapter VI focuses on the development and characterization of 3D printed hydrogels based on alginate, nanocrystalline cellulose and silver nanoparticles to promote antimicrobial and cytotoxic effects. Here, LC-MS technique was used for the targeted analysis of pro-apoptotic initiation and executive caspases, anti-apoptotic and proliferative proteins and hepatocyte growth factor and provided insights into the molecular mechanisms involved in cell death induction. In 2020 this study

was published on *Nanomaterials* as *Three-dimensional (3D) Printed Silver Nanoparticles / Alginate / Nanocrystalline Cellulose Hydrogel: Study of Antimicrobial Efficacy and Cytotoxicity* [3].

Chapter VII reports the work published in 2021 on *BBA - General Subjects*, concerning *The adaptation of lipid profile of human fibroblasts to alginate 2D films and 3D printed scaffolds* [4]. The investigation of the interactions between cells and active materials is pivotal in the emerging 3D printing-biomaterial application fields. In this frame, LC-MS based lipidomics has been used to explore the early impact of alginate hydrogel architecture (2D films or 3D printed scaffolds) and the type of gelling agent (CaCl_2 or FeCl_3) on the lipid profile of human fibroblasts.

In chapter VIII, the development of new self-crosslinking alginate-based inks for 3D printing is described; this method, by exploiting the internal gelation of alginate, avoids the post-printing crosslinking process and allows the loading of water-soluble drugs, such as epirubicin-HCl (EPI). The critical combinations of alginate, calcium carbonate and d-glucono- δ -lactone (GDL) in relation to the scaffold production parameters (extrusion time, temperature, and curing time) were evaluated and discussed. Drug release from 3D printed hydrogels was monitored by targeted LC-MS analysis, technique involve also to study the change in the expression pattern of anti-apoptotic, pro-apoptotic, and autophagy protein biomarkers after exposure of MCF-7 cells to the EPI -loaded hydrogels. These outcomes have been published in 2022 in *Molecules* as *Alginate Self-Crosslinking Ink for 3D Extrusion-Based Cryoprinting and Application for Epirubicin-HCl Delivery on MCF-7 Cells* [5].

Chapter IX reports the development of 3D printed hydrogels based on alginate and hyaluronic acid, obtained with the internal gelation procedure described in chapter IX; these devices were functionalized with two strains of *Lactobacillus delbrueckii* subs. *bulgaricus* derivatives and have been investigated for their potential in wound healing as advanced therapeutic solutions. In this work, untargeted and targeted LC-MS proteomics were used, respectively to characterize bacterial derivatives and, to study their effect on treated human fibroblast cells (in particular, the expression of proteins involved in extracellular matrix modeling, proliferation, wound healing, and apoptosis phenomena were evaluated). LC-MS based lipidomics was chosen as technique to investigate the lipid profile of bacterial lysates. Finally, *L. bulgaricus* derivatives have also been characterized in terms of exopolysaccharides (EPS) and studied *in vitro* on human fibroblast cells (cytotoxicity test, migration test, proliferation test) showing promise for applications in regenerative medicine. This work has been submitted with the title of *Lactobacillus delbrueckii* subs. *bulgaricus* derivatives for 3D printed alginate/ hyaluronic acid self-crosslinking hydrogels: manufacturing and wound healing potential to *International Journal of Biological Macromolecules*.

In chapter X both conclusions and future perspectives of the various analytical applications discussed in this thesis have been summarized.

CHAPTER I: Introduction

1. Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) is a powerful analytical technique with very high sensitivity and specificity. The technique combines the physical separation function of liquid chromatography with the mass analysis capabilities of mass spectrometry. It is a relatively recent analysis technique, which has developed thanks to the evolution of MS (Figure 1). Today, MS is mostly under the purview of analytical chemistry but was born in the field of physics. In 1913, physicist J. J. Thomson built what was later recognized as the first mass spectrometer to measure the masses of charged atoms. The ions were generated using gas discharge tubes, and then passed through parallel electric and magnetic fields. Finally, once deviated into parabolic trajectories, the ions were detected on a photographic plate. From here there followed numerous discoveries including the first electron impact sources, solids in 1918 by A. Dempster and gases in 1929 by W. Bleakney respectively, which allowed the first mass spectrometers to be commercially available starting from the 1940s. Later in 1953, F. Sanger completed the first primary structure of the insulin protein and J. Watson and F. Crick proposed the double helical structure for DNA attracting the interest of the whole scientific community. Finally in 1984, M. Yamashita and J. Fenn developed the electrospray ionization (ESI) technique and used it to ionize biomolecules; hence the barriers between LC, which works with liquid samples at atmospheric pressure and MS which works with gaseous samples at high vacuum, disappeared and LC-MS began to establish itself as the analytical technique of choice for numerous applications. LC-MS is now a hyphenated analytical technique used for various applications from routine analysis to basic research, for quantitative and / or qualitative purpose, in different sectors as the medical pharmaceutical, the agrochemical, food and the environmental [6–9]. In this PhD thesis the use of LC-MS mainly in proteomics, and secondarily in metabolomics has been considered and application examples provided.

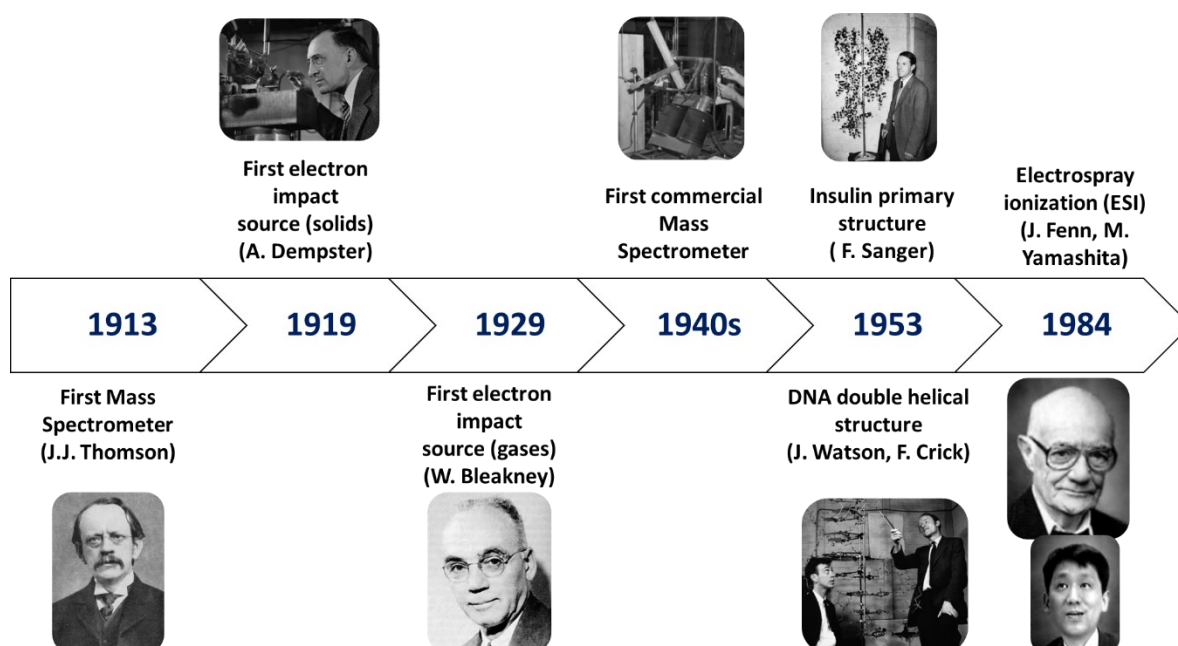


Figure 1: Main discoveries in Mass Spectrometry development and in coupling with Liquid Chromatography for biomolecules analysis.

1.1. Liquid Chromatography-Mass Spectrometry in Proteomics

Proteomics is the large-scale study of proteins, key molecules in cellular processes. Since the dawn of research, the fundamental role of these molecules in life has been highlighted, so much so that in 1838 J. Berzelius defined these molecules "protein", a biological term that derives from the Greek *proteios*, "the first degree". The complex of proteins expressed by a cell or by an organism, thus produced by the genome of the biological system in question, is defined as a proteome; term first used by M. Wilkins in 1994 to define "protein complement of a genome". Proteomics deals with the study of the proteome and therefore with protein identification and characterization in terms of structure, function, activity, quantity and molecular interactions. Proteomics provides us with the most relevant data set to characterize a biological system, in fact proteins are the effectors of biological functions and their levels do not correspond to those of the relative mRNA, but also from the control and regulation of the host [10–13]. The first proteomic study dates back more than 40 years, when two-dimensional gel electrophoresis was used to separate and quantify whole cell lysate proteins from *Escherichia coli*. From here numerous proteomic studies have been carried out with different technologies such as genomic biochemistry, proteomic chips, reverse-phase protein microarrays, activity-based protein profiling, antibody techniques and MS. The latter has only been applied in proteomics in the last decades since 1988, when K. Tanaka and colleagues using Matrix-Assisted Laser Desorption Ionization (MALDI) and a Time-Of-Flight mass spectrometer (TOF) analysed the proteins. Since then, significant improvements have been made in MS-based proteomics which are still evolving, and which have made and make MS-based proteomics a reliable and essential tool for

elucidating biological processes at the protein level. Thousands of proteins can be simultaneously detected and quantified (measurement of the absolute or relative abundance) with MS-based proteomics, with better specificity, sensitivity and robustness than other methods [14]. A general workflow in a LC-MS proteomic experiment, consists of sample preparation, protein digestion into peptides, HPLC separation of peptides, MS analysis and data elaboration. This proteomic approach is generally called bottom-up and differs from the proteomic approach defined as top-down for strategy. In bottom-up proteomics, proteins are enzymatically digested into peptides prior to MS analysis; while in top-down proteomics the whole proteins are analysed in MS, allowing the identification of protein isoforms and possible post-translational modifications within proteins. As shown in Figure 2, there is a further approach, shotgun proteomics, a variant of the bottom-up one, in which a complex mix of proteins is enzymatically digested into peptides that are fractionated by one-dimensional or multidimensional chromatography and then analysed in MS. The main difference with the bottom-up strategy is that bottom-up experiments do not necessarily require LC separation of peptides prior to MS, whereas in shotgun experiments LC separation (mostly multidimensional) is always used. Sometimes a combination of bottom-up and top-down approaches can be involved to optimize protein identification and characterization [13,15,16]. In particular, in this thesis, the bottom-up proteomic approach, used in the analytical applications reported, will be considered.

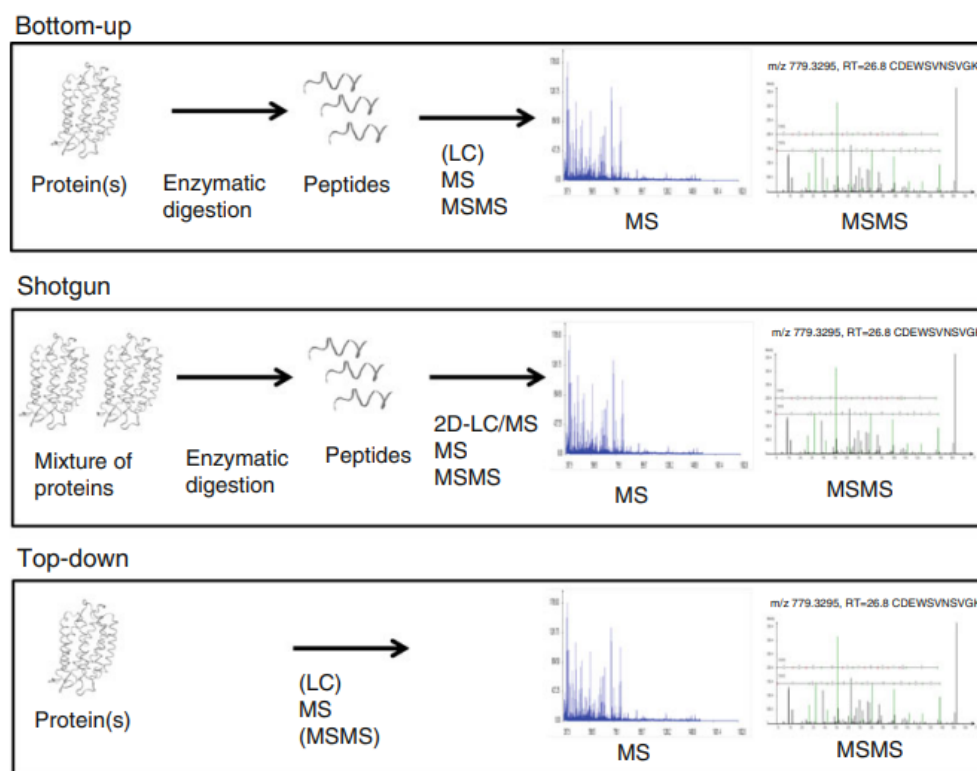


Figure 2: Schematic workflow of bottom-up, shotgun and top-down proteomic strategies. Figure by [16].

1.1.1. Sample preparation and protein digestion

As mentioned above and shown in Figure 3, a proteomic project starts with the sample preparation for MS study. This step is crucial for the success of the analysis as the results will depend on the starting material conditions. Therefore, the choice of the protein extraction method, suitable for the sample under consideration, is fundamental to obtain specific and reliable information, especially in comparative proteomic studies in which small differences between experimental and control samples are sought. Proteins are extracted from samples of various types such as animal tissues, cell cultures, body fluids (e.g. blood, serum, plasma, cerebrospinal fluid, saliva, synovial fluids), plants, bacteria, viruses. Generally, sample homogenization, which consists of cell disruption, is the starting point: the sample should change its physical properties without altering the chemical ones. Depending on sample consistency and the aim of the analysis, homogenization can be mechanical, ultrasonic, with pressure, with lysis and / or osmotic detergents, with freezing-thawing but also with a combination of two or more of these procedures. Once isolated, proteins in their native state are often insoluble, therefore they are solubilized in order to disrupt the interactions involved in protein aggregation such as hydrogen / disulfide bonds, van der Waals forces, ionic and hydrophobic interactions. This solubilization process, of primary importance, mainly involves the use of chaotropic agents (e.g. urea and / or thiourea), but also of detergents (e.g. 3 - [(3-Cholamidopropyl) -dimethyl-ammonium] -1-propane sulfonate (CHAPS), Triton X-100, Sodium Dodecyl Sulphate (SDS)), reducing agents (dithiothreitol / dithioerythritol (DTT / DTE) or tributylphosphine (TBP)) and protease inhibitors in a sample buffer. The combination of these agents with appropriate homogenization techniques can be optimal for protein solubilization. Finally, for efficient sample preparation it is necessary to remove possible contaminants, such as buffers, salts and detergents previously added to solubilize the proteins. This sample clean-up procedure is essential to avoid interference in the subsequent steps of protein separation, digestion, and MS analysis. Among the main interferents generally removed there are salts, detergents, lipids, polysaccharides, abundant proteins (e.g. albumin and immunoglobulins in blood samples), nucleic acids and other substances such as nucleotide metabolites, endogenous ionic molecules. Ultrafiltration and dialysis, which involve the use of membranes, combined with high-speed ultracentrifugation, are the main techniques used for the removal of these contaminants; to these are added precipitation with TCA, acetone, chloroform / methanol, hexane / isopropanol, ammonium sulfate or combinations of the above, but also digestion with protease-free DNase and RNase in order to eliminate DNA and RNA. The choice of procedure or combination of contaminant removal procedures has to be optimized on the basis of the sample and therefore of the analysis in question. Sometimes the sample is subjected to an enrichment process, aimed at increasing the concentration of the proteins of interest. Protein enrichment methods include

prefractionation, precipitation, centrifugation, chromatographic techniques, electrophoresis and solid phase protein enrichment [17–21]. Once the protein extract has been obtained, this can be quantified in order to know the protein content in the sample and therefore to evaluate the amount of reagents and enzyme to be used, to optimize subsequent protein digestion. Due to their simplicity and cheapness, colorimetric assays are commonly utilized to measure the protein concentration of an extract. These are spectrophotometric protein quantitation assays, which thanks to ultraviolet (UV) and visible spectroscopy allow to determine the protein concentration, using a standard or an assigned extinction coefficient. The Bradford assay, the Lowry assay, and the Bicinchoninic Acid (BCA) assay are some examples [22]. A last step before digestion consists in the reduction and alkylation of proteins, that together with solubilization and denaturation is essential to obtain a more effective enzymatic cleavage. In fact, the cysteines in the samples form di-sulfide bonds which help to maintain protein in its native form; therefore, these disulfide bridges are reduced using reagents such as DTT and then blocked thanks to the iodoacetamide (IAA) which alkylates the thiol residues. At this point the protein extract is ready for digestion and then to be cut into peptides. This is the key procedure, as good digestion ensures a high peptide concentration and thus facilitates LC-MS analysis. The most used enzyme for this purpose is trypsin, a serine protease that breaks peptide bonds mainly at the carboxyl side of amino acids lysine and arginine, generating a pool of peptides from about 600 to 2500 Da; optimal range size for MS analysis. Generally, trypsin is used in a ratio of 1:20 or 1:50 (enzyme: protein) and is known to work in samples with urea concentrations not exceeding 2M; therefore, the chaotropic agent, typically used at 8M in the protein solubilization process, is diluted. Depending on the purpose of the analysis, other enzymes may also be used; for example, the endoproteinase Lys-C that cleaves peptide bonds C-terminally at lysine and it is not influenced by urea concentration, the endoproteinase Glu-C that cleaves peptide bonds C-terminally at glutamic and aspartic acid or a combination of enzymes. Finally, the digested sample is dried using for example the nitrogen flow or the lyophilization process and then resuspended in a solvent miscible with that used for the subsequent LC analysis [23,24]. Nowadays for standardized processes, such as routine analyses, there are automated sample preparation and digestion systems that allow to quickly process large numbers of samples while minimizing operator errors, However, in basic research, automated systems are rarely used, and depending on the sample and the purpose of the analysis, it is necessary to fine-tune and optimize the sample preparation and digestion procedure, also combining various approaches in order to obtain true and reproducible results [25].

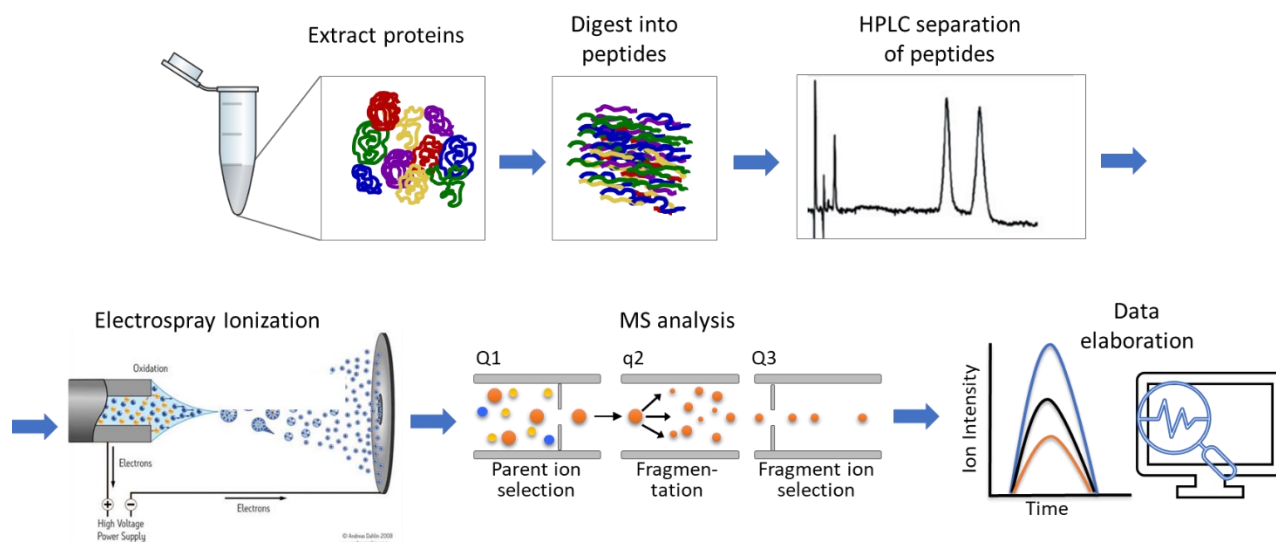


Figure 3: General proteomics workflow: Sample preparation, protein digestion into peptides, HPLC peptide elution, ion source (e.g. electrospray ionization), MS analyzer (e.g. targeted proteomics: triple quadrupole), data elaboration.

1.1.2. LC-MS analysis

Currently, HPLC is the technique of choice for peptides separation in MS based proteomics; in particular reversed-phase (RP) chromatographic separations with C18 column is used. The basis of RP-HPLC separation is the hydrophobic interaction between the analyte and the nonpolar matrix of the C18 stationary phase; while the solvent used for the isocratic or gradient elution is polar (e.g., acidic water mixed with a polar organic modifier such as methanol, isopropanol, or acetonitrile) [16,19,26]. Once eluted, the peptide mixture enters the mass spectrometer. This has three main components: an ion source, a mass analyzer, and a detector. The first step consists in the ionization of the sample; the ions thus produced are separated in the mass analyzer according to their mass-to-charge ratio (m/z) and then detected by the detector. The mass spectrum is the final product, which is a graph of the abundance of ions versus m/z (Figure 4) [27,28].

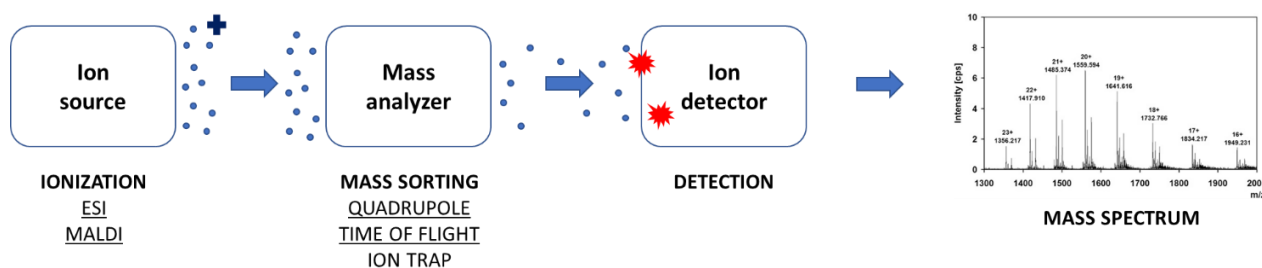


Figure 4: Mass spectrometer components and end product: ion source, mass analyzer, detector and mass spectrum.

The ion source is the interface that connects the two systems, HPLC and MS, which work in two otherwise incompatible dimensions. In fact, there are some differences in the operating environment of HPLC and MS as the solvent flow rate, the incompatibility of common HPLC solvents and nonvolatile additives with mass spectrometry operation and, at the beginning of MS development, the unavailability of ionization methods that could be used for thermally labile and nonvolatile compounds. Over the years a suite of different interfaces has emerged to combine HPLC with MS; currently the most used interfaces in LC-MS systems in proteomics are ESI and MALDI followed by atmospheric pressure chemical ionization (APCI), chemical ionization (CI), and electron ionization (EI)[26,29]. Typically, the ionization of peptides depends on the pH of the solution and on the source electrical potential. The peptides are protonated at low (acid) pH through the amino-containing amino acids as lysine or arginine; the peptides, on the other hand, are deprotonated at a high (basic) pH through the carboxyl-containing amino acid as glutamate or aspartate. The ionization can be in positive or negative mode depending on the positive or negative electrical potential respectively. In positive ionization the peptides are analysed at acid pH and amino acids such as lysine and arginine are protonated; while in negative ionization the pH is basic and amino acids such as aspartate and glutamate are deprotonated. In this paragraph the positive ionization will be considered, as it is mainly applied in the analytical applications reported in this thesis, as well as the most utilized ionization mode in proteomics. Furthermore, the principal enzyme used in reported applications and in proteomics is trypsin, which cuts the peptide bonds between arginine and lysine, obtaining at least doubly charged peptides and producing as predominant fragments the b-ions, derived from the N-terminus of the peptide and the y-ions derived from the C-terminus. ESI ionization, the interface mainly applied in this thesis applications, is an atmospheric pressure ionization (API) technique applicable to a wide range of compounds that are present in liquid matrices. Figure 5 schematically shows an ESI interface. The heart of ESI source is a stainless-steel capillary tube through which a solvent flow continuously at the rate of 2-5 $\mu\text{L}/\text{min}$. By the further introduction of pneumatically assisted ESI, presenting a co-axial capillary flowing an inter gas (i.e. nitrogen), solvent flow-rate can be increased to 200 up to 1000 $\mu\text{L}/\text{min}$. The solvent is a mixture of water and an organic solvent, and generally contains < 1% of acid. A solution of the sample is injected into this solvent stream; a potential difference of 3-5 kV between the tip of the capillary and walls of the surrounding atmospheric pressure region produces an electrostatic field sufficient strong to disperse the emerging solution into a fine mist of charged droplets. A flow of hot-bath gas, usually nitrogen, is added to the interface to assist in the evaporation the solvent from those charged droplets. The ions are transported from the atmospheric pressure region to the high-vacuum region of the mass analyzer via a low-pressure transport region, which consists of two or more successively pumped chambers. Before entry

into a mass analyzer, all sample ions are stripped off the solvent molecules. The calculation of peptide molecular mass of the peptide is performed according to this formula $[M + z]/z$, where z is the charge (e.g. 2 for doubly charged peptides, 3 for triple charged peptides etc.). Using ESI interface, positively and negatively charged ions can be detected and it is possible to switch between the negative and positive modes of operation. ESI-MS is used for qualitative and quantitative studies, for peptides and proteins molecular mass determination, post-translational modifications, and their conformation [30].

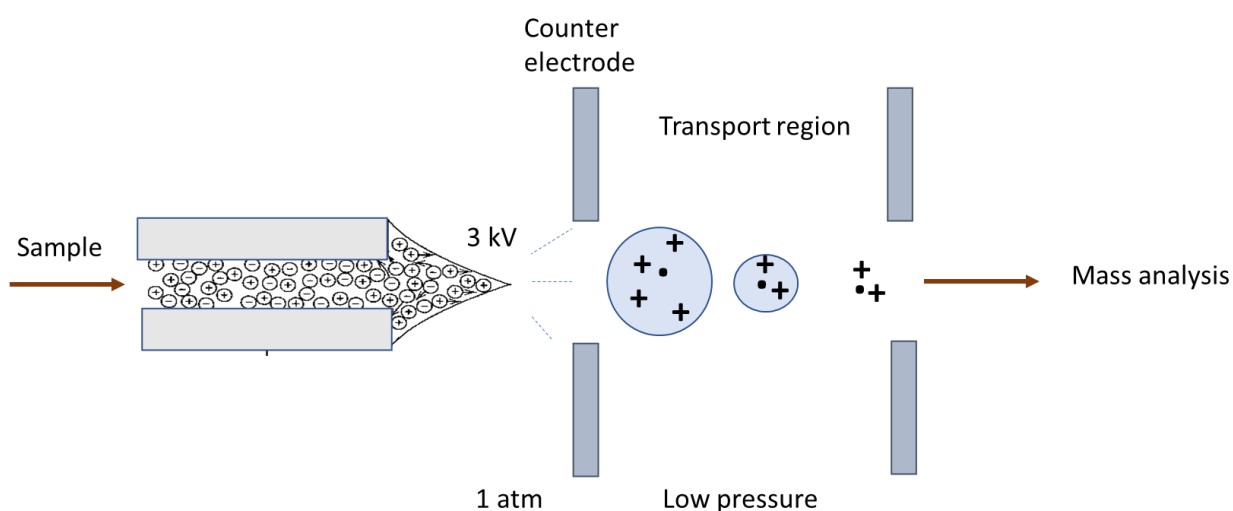


Figure 5: Schematic representation of ESI concepts; positive ionization.

MALDI is the other most applied ionization technique in MS-proteomics, mainly for the determination of the mass of a peptide or a protein and for the identification of a protein using peptide mass fingerprinting. In MALDI, sample, that is in solid phase, is mixed with a matrix solution (e.g. synapic acid, ferulic acid, succinic acid) and the resulting mixture is dried; the solvent evaporates and the analyte co-crystallizes with the matrix. The crystals are bombarded with a laser pulse, the absorption of photons causes a sudden increase in temperature, which results in a vaporization of the crystals and therefore ionization. The ions obtained are single charge; very often the MALDI technique is combined with MS equipped with a time-of-flight analyzer (TOF) [Israr 2020]. More rarely, APCI is also used, for analysis of compounds that do not well ionize in ESI. It is applied for low molecular weight compounds, less than 1500 Da and generates single charge ions. Instead, EI is used for the analysis of all volatile compounds with molecular weight lower than 1000 Da. EI provides good structural information resulting from fragmentation; whereas CI provides good information for the determination of the molecular mass of the molecules; hence EI and CI complement each other [30,31].

The mass analyzers separate ions based on their m/z values; as reported in Figure 3, there are three type of mass analyzer mainly used in proteomics: Quadrupole (Q), Time of flight (TOF) and trapping

type instruments (Orbitrap, linear ion trap_LIT, quadrupole ion trap_QIT, Fourier transform ion cyclotron resonance_FT-ICR). The quadrupole mass analyzer separates ions based on their trajectory in the electric field applied to two charged cylindrical bars. Ions are attracted to a bar by the electric potential between each pair of bars. These instruments are widely used as they are cheap and with good reproducibility, even if the accuracy and resolution are not optimal. The TOF mass analyzer, by applying a certain electric field, accelerates the ions traveling from the source to the detector and measures the time it takes them to pass from the ion source to the detector. TOF mass analyzer is widely used in proteomics due to low cost and high resolution, measurement speed and good mass accuracy. Trapping type mass analyzers initially captured ions in three-dimensional space, the trap; then an electrostatic gate sends pulses to inject the ions into the ion trap. These tools have been widely used in proteomics as they are robust and sensitive; however, their accuracy for precursor and product ions is limited. To overcome this problem there is the FT-ICR which unfortunately is more expensive and less used in proteomics because the peptides do not fragment very well. Mass analyzers can be used alone or in tandem (MS/MS); thus, combining the advantages of multiple analyzers. The Q-Orbitrap, LTQ (linear ion trap quadrupole)-Orbitrap, LTQ-FT-ICR, Q-FTICR, Q-TOF, triple TOF, QQQ, and QQ-LIT are examples of these instruments termed hybrid mass spectrometers which are highly sensitive and have a high resolution. Table 1 summarized the principal mass analysers their features and common applications in proteomics.

Finally, the detectors coupled to MS are generally electron multipliers, microchannel plates, photodiode arrays or image current detectors [13,27,32].

INSTRUMENT	RESOLVING POWER	MASS ACCURACY	SENSITIVITY	APPLICATIONS
LTQ / LIT	2000	+	++	Bottom-up
LTQ-FT-ICR; Q-FT-ICR	600000	++++	++	Bottom-up, top-down, PTM, characterization, quantitation
LTQ-Orbitrap	100000-240000	+++	++	Bottom-up, top-down, PTM, characterization, quantitation
Q-Orbitrap	140000	+++	++	Bottom-up, top-down, PTM, characterization, quantitation
Q-TOF	22500-100000	+++	+++	Bottom-up, top-down, PTM, characterization, quantitation
Triple TOF	40000	+++	+++	Bottom-up, top-down, PTM, characterization, quantitation

QQQ	3000	++	+++	Bottom-up quantitation, targeted quantitation (SRM, MRM, PTM monitoring)
QQ-LIT	2000	++	+++	Bottom-up quantitation, targeted quantitation (SRM, MRM, PTM monitoring)

Table 1: Mass analyzers in proteomics: performances and applications. + indicates performance in a particular aspect, more + means much better performance in that aspect. Abbreviations: PTM, posttranslational modifications; SRM, selected reaction monitoring MRM, multiple reaction monitoring.

1.1.3. Data acquisition and elaboration

Raw data obtained from LC-MS analysis are generally processed with bioinformatics software and output data served for identification or quantification of peptides and proteins. In this frame bioinformatic tools play a fundamental role, progressively increasing with the advent of new high-throughput technologies that generate a large and heterogeneous amount of data. Various algorithms, software have been developed for the analysis of big data obtained from LC-MS proteomics, that allow to process and manage information in a specific and accurate way, helping for example in the identification and quantification of peptides and proteins. Among the most used bioinformatic programs in proteomics, and free of charge, there is the package MaxQuant and Perseus, equipped with its own search engine called Andromeda, Skyline and PeptideAtlas; with these programs it is possible to use the free UniProt protein database. Among the database search engines, there are also Sequest, Mascot, X! Tandem, and Phenyx [28,33–35]. Figure 6 shows the 3 types of acquisition commonly used in MS analysis: data-dependent acquisition (DDA), data-independent acquisition (DIA), and targeted MS approaches (SRM, MRM and Parallel reaction monitoring_PRM). DDA is the traditional method; m/z and selected numbers of fragmentation spectra of peptides are measured and searched in a library containing data on all known peptides, the matched peptides are assigned to proteins. With this approach, the most abundant precursor ions and all the product ions are selected. More recent is the DIA approach, in which all precursor ions, into pre-defined isolation mass windows, are acquired and all precursors in each window are fragmented to generate product ions. The targeted strategies, SRM, MRM and PRM, allow to monitor proteins and peptides of biological relevance in complicated mixtures. In these targeted approaches, peptides are pre-selected to quantify proteins of interest using, in the case of the typical SRM / MRM method, a triple quadrupole (QqQ) mass spectrometer, and in the case of the PRM approach, the third quadrupole is replaced by a high resolution accurate-mass (HRAM) mass analyzer, such as an Orbitrap or TOF analyzer. In details,

the precursor peptides of interest are filtered in the first quadrupole (Q1), fragmented in the second quadrupole (q2) and with the SRM / MRM method, in the third quadrupole (Q3) only selected product ions are monitored for each precursor, generating multiple transition (precursor / fragment ion pair) ion chromatograms, allowing for the detection and quantification of targeted peptides; whereas in PRM mode, all product ions of a pre-determined precursor are recorded by a HRAM analyzer. Both targeted approaches exhibit high sensitivity, selectivity and reproducibility but have limited protein coverage. DDA approach, despite having an accuracy and reproducibility often lower than the targeted methods (due to the selection of the stochastic precursor with a risk of subsampling) has a much wider coverage than the targeted approaches, allowing a global profiling on a protein scale. In fact, in DDA the most abundant precursor ions are isolated in Q1, fragmented in q2, and all the product ions are monitored by an HRAM mass analyzer. An approach that combines the strengths of the above, is DIA, which like the targeted methods has a high sensitivity and reproducibility, combined with a wide protein coverage such as the DDA. In DIA, precursor ions are separated into consecutive small m/z window in Q1, fragmented in q2, and all product ions into a defined window are detected using an HRAM analyzer. The Sequential Windowed acquisition of All THEoretical fragment ion Mass Spectra (SWATH-MS) is a DIA method commonly used with Q-TOF instrumentation. Recent advances in DIA instrumentation include the Trapped Ion Mobility Spectrometer (TIMS) which traps ions in different positions in an ion tunnel by counteracting the source gas flow with the strength of the electric field. By lowering the electric potential, the trapped ions are released over time according to their mobility. Thanks to the introduction of TIMS in a Q-TOF system, it is possible to combine the separation of ion mobility with chromatographic and mass separation. This strategy is called Parallel Accumulation-Serial Fragmentation (PASEF), and with this the release of precursor ions is synchronize with the selection of the quadrupole for fragmentation. The introduction of PASEF in the DIA approach allows an increase in the selectivity for precursor identification, a high reproducibility and accuracy also for the analysis of complex biological samples, however reducing the complexity of the analysis, thanks to the introduction of separation with ion mobility in DIA.

In the past years DIA approach was mainly used for discovery study, however thanks to the recent improvements described above, this approach is starting to be used also in quantitative relative / absolute and global / targeted studies with performances comparable to the targeted approaches (SRM / MRM and PRM) that have represented and still represent a gold standard for MS-based protein quantification [14,36–39].

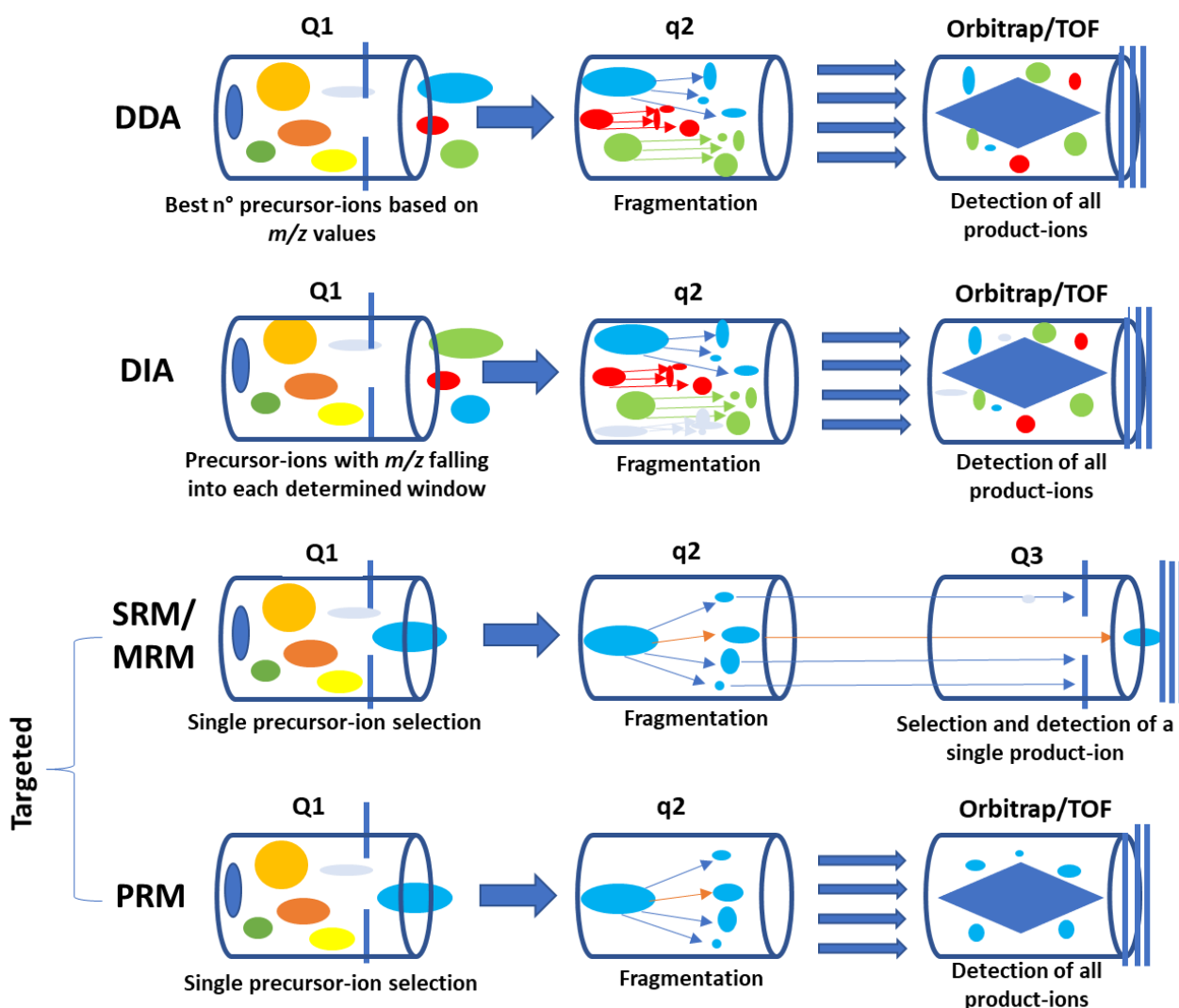


Figure 6: MS acquisition approaches: DDA, DIA and targeted (SRM, MRM and PRM).

1.1.4. Quantitative proteomics

Mass spectrometry, thanks to the great advances in recent years, has become the tool of choice for qualitative proteomics, and therefore for the identification of proteins but also for their quantification. Among the advances we mention the improvement of the sensitivity, the accuracy of the resolution of the mass spectrometers accompanied by that in the efficiency of the HPLC and in the development of increasingly advanced bioinformatics software that have allowed the use of the LC-MS / MS (SRM / MRM, PRM or DIA) for the quantification of thousands of proteins in complex biological matrices. Depending on the analysis aim and on the sample in question, a global protein quantification can be performed by studying the protein changes between different conditions (i.e., healthy and diseased) allows to uncover pathological mechanisms on the phenotypic level, or a targeted quantification for example of a specific biomarker, a drug targeted or a PTM during the cell cycle. Accurate and reliable MS quantification of protein biomarkers and their PTMs plays an increasingly important role in

disease diagnostics, monitoring and treatment. Traditional quantitative analysis uses tools capable of MS / MS fragmentation (i.e. triple quadrupole, ion trap MS) because many compounds have similar masses and it may be difficult to detect them in complex samples. Thanks to the combination of MS and MS / MS it is possible to use a set of precursor ions from MS and fragment ions from MS / MS, improving the selectivity of peptide / protein quantity monitoring. Figure 7 schematically summarizes MS quantitative proteomic strategies. According to the purpose of the analysis, there are two strategies, the untargeted (global) and the targeted (single- or several-component); in the first case hundreds or thousands of proteins are quantified for protein profile comparison studies, in the second case only a few components are quantified, generally isolated from a sample and quantified. Depending on the methodology, label free or label-based techniques can be used for protein quantitation. In label-free quantification, a simple and low-cost method, the sample maintains its native isotope composition; the approaches used include spectral counting and MS ion intensity measurement (chromatographic peak area). Label-based quantification, more expensive but more accurate method, includes stable isotope labels incorporated within the peptides / proteins, introducing mass difference within different experimental conditions; stable isotopes can be incorporated into proteins or peptides metabolically (e.g. labeling by amino acids in cell culture _SILAC), enzymatically (^{18}O labeling), or chemically (e.g. isobaric tags for relative and absolute quantification_iTRAQ, tandem mass tags_TMTs). Finally, based on the information provided by quantification methods, quantification can be relative or absolute. The relative quantification is based on a comparison between single proteins or entire proteomes between the samples, the information obtained therefore concerns protein quantitative ratio or relative change. Absolute quantification, on the other hand, provides information on the absolute quantity or concentration of a given protein in a sample and needs of an appropriate “internal” standard [13,40,41].

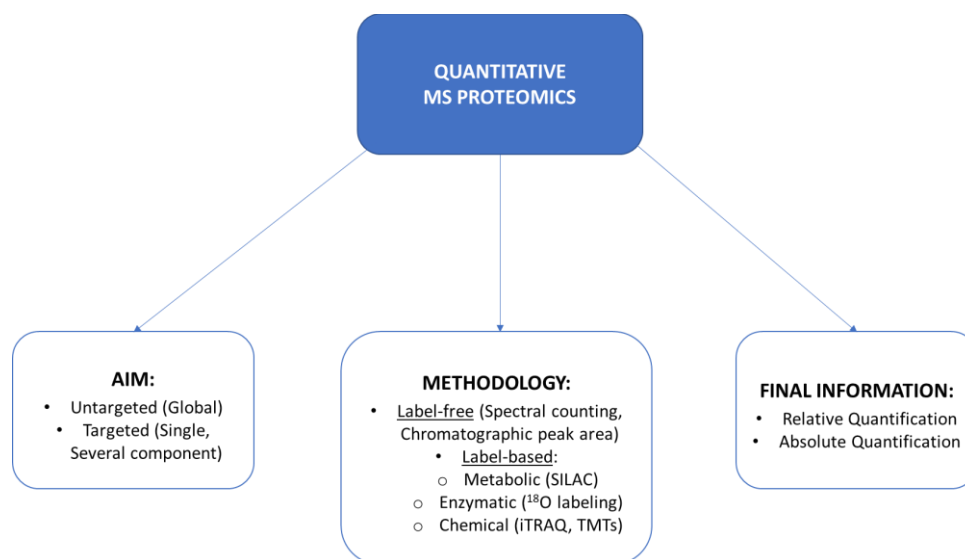


Figure 7: Scheme of MS-based protein quantification approaches.

Rapid advances in label free quantification (LFQ) have enabled fast, efficient, and low-cost protein analyzes. As mentioned above and indicated in Figure 7, the two most used approaches, especially in relative quantification, are spectral counting and MS ion intensity measurement and the results obtained are often expressed as "fold changes." Spectral counting consists of counting the number of MS / MS spectra that identify a particular peptide or protein. The rationale behind this method is that an increase in protein abundance is related to an increase in the number of its proteolytic peptides, and vice versa. In DDA mode, a survey MS scan is acquired followed by the selection of 10 most abundant ions in the initial MS scan, for subsequent MS / MS analysis. The spectral counts of the peptides of a given protein are summed up for the relative quantification of the protein; the limiting factor is that the linearity and number of quantified proteins will depend on the details of the DDA setting, furthermore the spectral counts for proteins present in low quantity provide less robust information due to statistical limitations and the need to limit the false discovery rate so as not to "count" lower quality spectra. MS ion intensity measurement (chromatographic peak area) is based on experimental evidence that in LC-MS, an ion with a particular m/z is detected and recorded with a specific intensity and retention time. In LC-ESI-MS the signal intensity from ESI correlates with ion concentration. So, the quantitation corresponds to the area under the curve or peak height calculation for each peptide that elutes from the LC column at a specific retention time. Specifically, peptides are identified by matching the accurate mass characteristics and normalized elution time with those stored in a previously established peptide reference database. With this method, subsampling problems are alleviated and measurements with high precision and wide dynamic range are obtained, thanks to the use of high-resolution mass spectrometers. There are further similar approaches based on direct LC-MS measurements, peak identification and feature alignment. In LFQ it is important to normalize the data to correct, as much as possible, the systematic variations. In fact, even the slightest variation in sample preparation, in the reproducibility of LC-MS, in the ionization efficiency influenced by ion suppression (the intensity of the peptides depends on the quantity of ionized peptide and therefore on the ionization efficiency and sometimes also on the properties of coeluting peptides), and in possible other sources of instrument error, can lead to an increased measurement error. There are some bioinformatics software, mentioned above (e.g. MaxQuant-Persues, Skyline) capable of performing alignments of LC-MS characteristics, peak matching, normalization, and statistical analysis of LFQ data. Although LFQ is widely used, it is necessary to consider that due to the limited reproducibility (sample preparation, LC-MS platform etc.), its accuracy and reliability are not comparable to those of label-based quantification [42,43].

Label-based quantification, on the other hand, is based on the concept of stable isotope dilution. In fact, a peptide labeled with a stable isotope maintains the chemical properties unaltered, behaving

like its equivalent native during elution, and ESI but thanks to the different mass it can be separated in MS from the native one, allowing an accurate quantification of the peptides (but not necessarily of proteins). Differences in relative abundance measured by LC-MS analysis between the labeled peptide correspond to differences in abundance within the sample. The stable isotopes commonly used and incorporated into proteins or peptides are ^{13}C , ^{15}N and ^{18}O ; less used is ^2H . With this approach the samples are combined after labeling and analyzed, thus avoiding the uncertainty induced by variations in instrument performance between measurements, improving accuracy and precision compared to LFQ. Stable isotope can be incorporated metabolically as in SILAC mode, where amino acids such as arginine or lysine are labeled with "heavy" isotopes (e.g. ^{13}C and / or ^{15}N) and added to the growth medium; a limitation of this approach is that it can only be used for cell cultures and not for *in vivo* studies. Another method is enzymatic isotope addition, as in the case of ^{18}O which is added to the C terminus of all proteolytic peptide during or after protein digestion catalyzed by trypsin, Glu-C, or some other proteases; this approach can be used for all types of samples, however incomplete labeling and ^{18}O back-exchange to ^{16}O , limited the application of ^{18}O labeling for global quantitative proteomics. Finally, there is the labeling strategy that exploits chemical reactions; some examples are the iTRAQ and TMTs approaches where labels react specifically with primary amine groups of tryptic peptides, as N-termini and the side chains of lysine residues. For these types of labels, it is necessary to use high resolution mass spectrometers as low m/z fragment ions are obtained, furthermore, since the quantification takes place at the MS / MS level, higher signal / noise is obtained than those obtained with an MS quantification [41,43].

According to final information provided by LC-MS proteomic analysis, the quantification can be relative when provides calculation of abundance ratios between peptides and proteins by comparing their signals deriving from different samples. It can be based on both label-free and label-based quantification using all the types listed above. If, on the other hand, the final information reported by the LC-MS proteomic analysis concerns the absolute quantity of a protein in a mixture it is absolute quantification. These results are very informative and take advantage of both label-free (however less accurate) and label-based methods such as iTRAQ and TMT [41].

It is interesting to mention the use of the SRM strategy for the relative and absolute quantification of proteins, a technique also used in the analytical applications reported in the following chapters of this paper. As mentioned in paragraph 1.1.3, SRM is usually performed with a triple quadrupole tandem mass spectrometer (selection quadrupole for precursor ions, fragmentation quadrupole, selection quadrupole for product ions). In an SRM assay, there is a list of m/z of precursors and products sometimes associated with specific retention times; so, the instrument is set up for exclusive monitoring of these ion transitions and a specific ion is detected and quantified when it and its

fragments have the expected m/z values. With this simple mode it is possible to carry out numerous targeted studies, and to obtain a relative label-free protein quantification. However, the SRM approach is also used for label-based absolute quantifications using known concentrations of labeled synthetic peptides as internal standards. In this way an excellent reproducibility is obtained, and the approach is less subject to the complexity of the sample as the noise signals are filtered both at the precursor and product level; also, the sensitivity is higher with a wide dynamic range that extends 4–5 orders of magnitude. This technique is then used for the detection and quantification of protein biomarkers of medical, food, environmental interest, as well as particular peptide modifications such as phosphorylation and ubiquitination; however, the number of proteins that can be studied in a single assay is restricted. In detail, initially a target peptide is synthesized in an isotope-labeled form and added to the protein digest at a known concentration, subsequently the concentration of the native peptide is calculated by comparing the ion intensities between the labeled and unlabeled forms. The use of the internal standard can correct errors due to ion suppression and matrix effects; for example, when analyzing more samples, it is advisable to use the internal standard to have a fair comparison between the samples. A limitation of this approach is that the internal standard, a labeled peptide, is added after digestion, and therefore cannot obviate any losses or enrichments that occurred in the previous stages of sample preparation; therefore, the absolute quantification thus obtained may not reflect the true expression levels of the protein in the sample. The problem can be solved for example by using, instead of the labeled peptide, a protein labeled with isotopes as an internal standard which will then undergo the entire sample preparation procedure. However, the identification and synthesis of adequate internal standards is neither trivial nor economical, factors that limit the absolute quantification approach with labeled standards to a restricted set of proteins. An alternative to the use of the internal standard can be the choice of a reference protein with chemical characteristics as similar as possible to the protein of interest, to be added to the sample before digestion and to be used as a reference in the quantification which, however, will be less accurate than that obtained with the labeled internal standard.

In conclusion, quantitative proteomics based on LC-MS includes various strategies, from global to targeted measurements, exploiting or not the isotope labelling, providing information of relative or absolute abundances. There isn't a single standard, universally recognized procedure that can solve various analytic problems; scientists must choose and optimize the most appropriate approach to the analytical problem in question. Although LC-MS-based quantitative proteomics is continuously improving, thanks also to the commercialization of ultra-performance LC (UPLC) and high-mass accuracy / resolution mass spectrometers, its potential has not yet been fully realized. There are still some factors to improve from sample preparation to data analysis where the missing value problem

has not yet been completely overcome. With the most recent instrumentation, better in resolution and accuracy, the target peptides are differentiated from the coeluted molecules with similar mass and, low abundant proteins quantified thanks to improved instrument sensitivity; faster scanning rates also help quantify proteins based on multiple peptides. Finally, to facilitate the application of quantitative proteomics also in routine analyzes, and to reduce the number of qualified operators, the introduction of automated sample preparation-digestion procedures as well as more intelligent and intuitive software are necessary [43–45].

1.1.5. Conclusions and future perspectives

Over the years, LC-MS has established itself as the analytical technique of choice for proteomics studies. MS was initially used in proteomics at a qualitative level and subsequently also at a quantitative one. LC-MS technology has become available to a wider community, although the costs of analysis are still quite high, and require specially trained personnel, as automated approaches are still in their infancy. Today LC-MS based proteomics is involved for the identification of proteins and peptides, for their structural characterization, for the study of their functions, interactions, quantities as well as for studying their localization and PTMs; the targeted approaches are exploited both in research and in routine analysis for the discovery and monitoring of protein biomarkers of medical, food and environmental interest, while the DIA approach, thanks to continuous instrumental and bioinformatics advances, is establishing itself as the MS strategy for the “future”.

Although, thanks to the huge advances in MS-proteomics, it has been possible to identify for example all (or almost) the proteins expressed in a specific bacterial cell at a particular moment of growth, the human proteome has not yet been fully identified. While the human genome contains only 30,000 genes, the proteome is much larger in size; in fact, these genes encode about 100,000 unique protein sequences, a number that increases considerably if truncated proteins, PTMs, splice isoforms and mutated proteins are considered. Realizing the fundamental importance of knowledge of the human proteome, in 2010 the Human Proteome Project (HPP) was promoted by the Human Proteome Organization (HUPO); an international framework was created for global collaboration, data sharing, quality assurance and enhancing accurate annotation of the genome encoded proteome. In 2020, on the 10th anniversary of HPP, the completion of 90.4% human proteome HPP’s list was reported. This important milestone opens up the prospects for future progress to improve the well-being of the world community in post SARS-CoV2 pandemic period. A precision and personalized medicine based on multidisciplinary approaches, on omics sciences (genomics, proteomics, metabolomics) is desirable, in order to develop, for example, new diagnostic tests and personalized therapies [46].

1.2. Liquid Chromatography-Mass Spectrometry in Metabolomics

Metabolomics is the systematic study of all metabolites in a biological system at a defined time. Metabolites are the end products of the gene expression of a biological organism, therefore the metabolomic profile reflects the cell physiology, and is a dynamic and sensitive representation of the cellular phenotype at the molecular level. Metabolic profiling has been used for centuries in the clinical detection of human diseases using blood and urine samples. The Urine Wheel by Ullrich Pinder (published in his book *Epiphanie Medicorum*) dates back to 1506 and was used to diagnose diseases based on patient's urine smell, color and taste. From here numerous improvements have followed over the centuries until the introduction of advanced chromatographic separation techniques which led to the conception of "modern" metabolomics with L. Pauling, who in the 1970s together with his collaborators, developed methods based on Gas Chromatography-Mass Spectrometry (GC-MS) to monitor metabolites present in urine. Only in 1995 the LC-MS was used for metabolomic studies by B. Cravatt and coworkers, to analyze the cerebrospinal fluid from sleep deprived animals, leading to oleamide discovery, a molecule which was later associated with the ability to sleep induction. Hence various advances have followed, and today metabolomics uses LC-MS, GC-MS, Capillary-Electrophoresis-Mass Spectrometry (CE-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy. Today, metabolomics is applied to study a wide variety of compounds such as amino acids, hormones, carbohydrates and lipids, from biological matrices, fluids, tissues and a variety of cellular fractions. Unlike DNA, RNA and protein, metabolites exhibit a greater range of chemical diversity and complexity and require the combination of different analytical approaches for complete coverage. An example is lipids, which despite being considered representative of a potentially homogeneous group of molecules, show a wide structural diversity due to different combinations of acyl groups, head groups and further modifications. Metabolites, as proteins, are: indicative of what has been encoded by the genome and modified by environmental factors and (unlike DNA and RNA) they cannot be amplified, further aggravating their analysis [47–51].

Currently, LC-MS is the most widely used technique for the development of metabolomic analysis, mainly for its capacity to separate a wide range of metabolites before detection as well as to provide information with high sensitivity and selectivity in a single measurement. LC-MS based metabolomics analysis's workflow is similar to that of a LC-MS based proteomics: it starts from sample preparation which consists in the extraction of the metabolites of interest, followed by HPLC metabolite separation, MS analysis and data processing. In order to achieve accurate and reproducible results, the first step of metabolite extraction needs to be performed in a very meticulous way. There are various methods for metabolite extraction which can be optimized according to the matrix, the analytes of interest and therefore the purpose of the study. Typically, in metabolomics, sample

preparation is performed maximizing metabolite coverage and minimizing metabolite loss/degradation and matrix effect. For example, one of the simplest and cheapest methods to "clean" the sample from proteins, is proteins precipitation in organic solvents; solid-phase extraction (SPE) is often used to reduce matrix effects from proteins, but also from salts and lipids and to concentrate metabolites. For the extraction of lipids and their derivatives, the classic technique used is liquid-liquid extraction (LLE) in which combinations of solvents such as water: methanol: chloroform (Bligh-Dyer and Folch methods), or butanol: methanol (BUME) or even hexane: isopropanol are used [52,53].

Metabolites, once extracted, are chromatographically separated by passing through a column together with the mobile phase. RP-HPLC is one of the chromatographic techniques employed, there are also hydrophilic interaction chromatography (HILIC) and supercritical fluid chromatography (SFC) used to analyze hydrophilic and lipophilic molecules, respectively. As in LC-MS proteomics, to optimize analytes separation and chromatographic peak resolution, as well as to reduce the ion suppression, it is possible to intervene on the mobile phase composition, on the gradient, and on the column temperature. Finally, even in metabolomics, the use of UPLC columns guarantees better sensitivity and resolution. ESI is the main ionization technique applied in LC-MS metabolomics, followed by the APCI. This mild ionization technique (ESI) provides a good balance between ionization and vaporization for a wide range of polar compounds and allows for multiple detection of biomolecules in a single assay [54].

Untargeted and targeted approaches are the two complementary strategies typically used for MS-based metabolomics studies. Untargeted approach aims to provide the widest coverage of the metabolome but also to discover new biomarkers; the methods used can be the DDA or the DIA and the mass spectrometers involved are the high-resolution ones, such as Q-TOF, LTQ-Orbitrap and FT-ICR. A combination of the mass fragmentation models, and accurate mass determination (<1-10ppm) are used for metabolites identification. The alternative to this approach is the targeted one, which mainly exploits the SRM / MRM strategy with the aim, for example, of quantifying a predetermined set of specific metabolites or verifying and validating biomarkers. In the target approach, each metabolite of interest is studied by monitoring one or more specific m/z transitions, using low resolution mass spectrometers such as QqQ, QqQ-ion trap (QTrap), or quadrupole linear ion trap (QLIT). Qualitative and quantitative metabolomics are carried out with both untargeted and targeted approaches, although for quantitative studies (relative or absolute) the targeted approach is preferred, in which analytes are often derivatized [52]. The derivatization strategy is applied to improve the sensitivity and separation efficiency in targeted metabolomics studies, in the case of low-abundance or poorly ionized metabolites. Derivatization techniques increase the hydrophobicity of polar

metabolites to avoid coelution with polar molecules, reduce the formation of dissociated species, to unify the forms of the metabolites and improve the LC resolution. The analytes increased hydrophobicity also serves to increase the droplets surface affinity, and therefore to enhance the ESI surface activity of the analyte and the ESI response. Depending on the functional groups present in the metabolites of interest, there are 7 types of chemical reactions that can be used for derivatization such as sulfonylation, acylation, alkylation etc. Thanks to these strategies, derivatives are obtained, better than the corresponding native metabolites, because they allow to improve LC separation, MS ionization, structural elucidation, to reduce exogenous interference and to facilitate the determination of the isomer [53].

As in proteomics, also in metabolomics the use of bioinformatics software is fundamental above all in management and processing of a vast and variety amounts of data. Among the free databases commonly utilized, there are for example HMDB, Massbank and KEGG (also used in proteomics). Finally, machine learning has recently been introduced in LC-MS metabolomics studies to facilitate data processing and automation, and to increase the accuracy of final information (even obtained by multi-omics approaches) such as disease classification or clinical outcome prediction [52,53,55].

1.2.1. LC-MS in Lipidomics

Lipidomics is a sub-category of metabolomics that deals with cellular lipid species. Lipids play a fundamental role in many physiological processes by acting as constituents of biomembranes, signal molecules and sources of energy. Lipids are generally classified on the basis of fatty acyl composition and stereochemical properties in phospholipids, glycerolipids, sphingolipids, sterols and fatty acids. Abnormal lipid metabolism is associated with various diseases such as atherosclerosis, diabetes, infectious diseases, cancer and obesity. Therefore, the development of suitable analytical methods for lipid profiling in biological samples is fundamental in biomedical research [56,57]. The term “lipidomics” was introduced in 2003 by X. Hann and R. Gross and was followed by the development of more accurate and efficient lipidomics studies attracting the interest of numerous researchers. Today in lipidomics, untargeted (with HRMS platforms) and targeted (with QqQ) approaches are used; a combination of these two approaches can be also involved to maximize the coverage of lipid species detected and quantified in complex biological matrices. Thus, lipidomics deals with lipids identification, metabolism, composition, structure and quantification in biological samples also with the aim of identifying and monitoring biomarkers and therefore studying the role of these metabolites in various diseases. In order to improve this last point, it is important to adopt a multi-omics approach and link lipidomics to genomics and proteomics studies (Figure 8). LC-ESI-MS is one of the most commonly used tools in lipidomics, and is also the technique adopted for applications reported in the

next chapters including targeted analyzes (SRM approach) of lipids and their derivatives such as oxysterols [55,57,58].

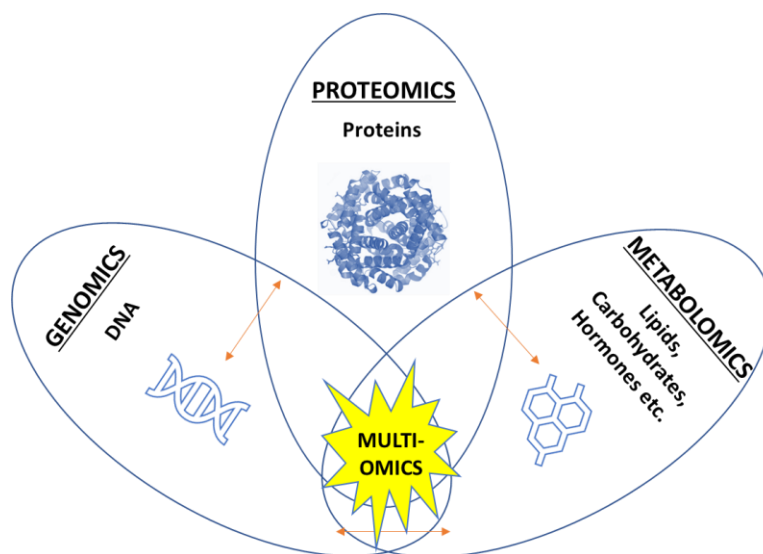


Figure 8: Multi-omics approaches (genomics, proteomics, metabolomics-lipidomics) to better understand the cellular life cycle.

1.2.2. Conclusions and future perspectives

Metabolomics, thanks to continuous advances, has led to interesting and beneficial improvements in biomedical research. This was possible mainly thanks to the application of LC-MS which allowed for example the identification of new biomarkers such as lipids, hormones, carbohydrates, useful for the diagnosis, monitoring and therapy of diseases, also improving the patient's quality of life. However, metabolomics studies a wide variety of molecules with various chemical structures, and including a wide range of concentrations; moreover, there are many molecules still unknown. There is still no single analytical platform capable of characterizing the entire metabolome or lipidome, and although there are established metabolomic analysis procedures, these have not yet been standardized. In the near future we could arrive at the development of standard procedures and analytical platforms that allow the analysis of the complete metabolome, also integrating data from other instrumentations (for example NMR). Another future trend in metabolomics is the miniaturization of the LC-ESI analysis system with the introduction of nano-columns and nano-ESI, already established in proteomics. This strategy will allow to analyze even samples with low amount of starting material. Finally, multi-omics approaches, as already mentioned, will be the flagship, which will allow an increasingly in-depth study of metabolomics, essential to guide new diagnostic and therapy strategies, and also to affirm metabolomics as a technique with analytical validity and clinical utility.

2. Biomaterials and 3D printing

3D printing is a manufacturing technology with great potential in production of biomaterials with tunable properties, used as scaffolds in biomedical applications. With the emergence of this technology, the traditionally used approaches in scaffolds fabrication, such as porogen leaching and gas forming, have been replaced and the associated limitations overcome (e.g. low or no control over pore size and interconnectivity, poor mechanical strength, residual solvents in scaffolds, use of cytotoxic solvents, long production times and large energy consumption). Biomaterials, used as inks in 3D printing, thanks to the flexibility and automation in design, can be involved to manufacture scaffolds with complex and customizable architectures. Among the additive manufacturing processes, extrusion-based approach is probably the most widespread method in 3D scaffold printing; the ink based on one or more biomaterials is deposited, layer by layer, to create scaffolds with a 3D structure. The advent of this technology has transformed the field of regenerative medicine, tissue engineering and drug delivery by providing a tool that enables unprecedented control, precision, flexibility and speed over traditional manufacturing methods. The biomaterials chosen as inks for 3D printing need to have some crucial properties including biocompatibility, biodegradability, good printability (adequate viscosity), mechanical stability, non-toxicity, high availability, and high shape fidelity, after the printing steps. Polymer hydrogels, highly hydrated 3D polymer networks (up to 95% water content but retaining the properties of solids), thanks to their chemical, physical, and biological properties, are ideal biomaterials for creating 3D porous scaffolds. Hydrogel scaffolds mimic the structure of the extracellular matrix (ECM) and facilitate its remodelling; they modulate migration, adhesion, and cell fate by interacting with cells (e.g., as a function of contact time and the size of the 3D construct) [59–64]. Consequently, these active biomaterials have been used in numerous applications; in particular, in the works reported in this thesis, hydrogel scaffolds composed of polysaccharides obtained from natural sources (e.g., alginate, hyaluronic acid) are used in applications of regenerative medicine, tissue regeneration, treatment of infected wounds and drug delivery.

2.1. Biomaterials

Biomaterials are defined as materials able of interfacing with biological systems in order to evaluate, treat, increase or replace tissues, organs or functions of the organism [65,66]. Widely studied in the last 60 years, biomaterials are used to fabricate biomedical implants, and devices for the controlled delivery of drugs or vaccines, with the aim of reducing side effects due to toxicity, as well as exploring new administration routes (e.g. transdermal, pulmonary, nasal, ocular) compared to the more classic oral or injectable formulations. Based on their chemical nature, biomaterials can be: polymeric (e.g. polylactic acid, cellulose, alginate), metallic (e.g. titanium, chromium-cobalt alloys), ceramic (e.g. hydroxyhepatite, alumina, zirconia) and composites [67]. Of particular interest are the polymeric biomaterials which thanks to the various advantages (e.g. ease tunable physical, chemical and mechanical properties) are widely involved in medical field, so that there are international accepted and validated procedures that regulate their production and usage [68,69]. Among the most desirable characteristics for biomedical applications of these biomaterials there are biocompatibility, so the ability not to produce a toxic, harmful, or immunological response in the patient, and biodegradability, that is the degradation of the material in a certain period of time, without the development, in the organism, of toxic products. This latter property is highly sought after in the case of *in vivo* implants, especially in tissue regeneration field; it is not always essential in drug delivery. In the specific case of biomaterials used in devices for vaccines or drugs controlled release, other important properties are: specificity towards the therapeutic target, often achieved through the biomaterial functionalization or thanks to the use of stimuli-responsive biomaterials (variations of pH or activation following oxidation or reduction), ideally allowing to decrease the undesirable effects due to off-targets and also to decrease the amount of active necessary for the treatment; the active component protection achieve by variable factors within the body (e.g. pH, hydration, enzymes or temperature); ability to incorporate a predictable and adequate amount of active; possibility of controlled and predictable release over time, to avoid "peaks and troughs" [67,70]. Natural origin polymers are widely used as biomaterials in both regenerative medicine and drug delivery. In fact, thanks to their similarity with human extracellular matrix (ECM), they are easily recognized and accepted by the organism, ensuring a certain degree of biocompatibility and bioactivity. These polymers are also abundant in nature, easily isolated from renewable sources (e.g., plants, animals, microorganisms), suitable for technologically improving chemical modifications, and do not generate toxic degradation products. Among the most applied biopolymers there are cellulose, collagen, chitin and chitosan, alginate, hyaluronic acid, pullulan, xanthan gum and various glycosaminoglycans (GAGs). These biopolymers show wide differences in terms of molecular weight, presence of fillers and monomer composition, features that determine different levels of hydrophobicity, solubility in

water, bioactivity, biocompatibility and biodegradability. Limits that mainly affect this class of materials concern the variations in composition depending on the origin, possible microbiological contamination, uncontrolled absorption of water, poor rheological properties, and degradation patterns, that are not always predictable. To better control these aspects, it is possible to produce semi-synthetic derivatives, for example by adding monomers as branches of the natural chains, or to exploit various cross-linking techniques in order to limit polymer chains movement. Starting from these biopolymers, it is possible to produce porous scaffolds, fiber matrices, hydrogels, and micro- or nanoparticles that can be involved in a wide range of biomedical applications [71]. In particular, the novel strategies that will be presented and discussed rely on the development of devices based on 3D hydrogel scaffolds obtained with polysaccharides-based materials. 3D hydrogel scaffolds consist on crosslinked hydrophilic polymers, water insoluble but able to interact with aqueous solutions by swelling and depending on their composition they can absorb vary amount of drainage. These devices mimic the ECM composition (providing a physical and biological support to the cells involved in biological process) and can be enriched for example with drugs, nanoparticles, growth factors, bacterial lysates; 3D hydrogel scaffolds are conceived as possible advanced dressings to be combined with traditional ones hoping in better and effective results. Polysaccharides used to develop these devices are presented below.

2.1.1. Alginate

Alginate is a natural polysaccharide found in the cell walls of brown algae and in some bacteria, and from which it is obtained in the form of sodium, magnesium, or calcium salts. Biodegradable, non-toxic and non-immunogenic, it is one of the most used biopolymers for biomedical applications: its peculiar characteristic is the ability to form gels in the presence of multivalent cations in an aqueous environment. Thus, it is also used as a gelling ingredient in food industry. Gelation and cross-linking of sodium alginate occur thanks to sodium-multivalent cations exchange; the resulting hydrogel (3D network of cross-linked hydrophilic polymers in which the liquid component is water) can be used in scaffolds production for tissue regeneration (i.e., bone regeneration, cartilage repair, wound healing) and drug delivery. Figure 2 shows that alginate is an anionic and hydrophilic block copolymer consisting of two different monomers: β -D-mannuronic acid (M) and α -L-guluronic acid (G). The blocks can be of two types: fragments of a single monomer (poly-G, poly-M), or fragments with alternating monomers (MG). G blocks are key in gel formation, while M and MG blocks provide flexibility. On the other hand, an excess of M blocks can cause immunogenicity phenomena [72].

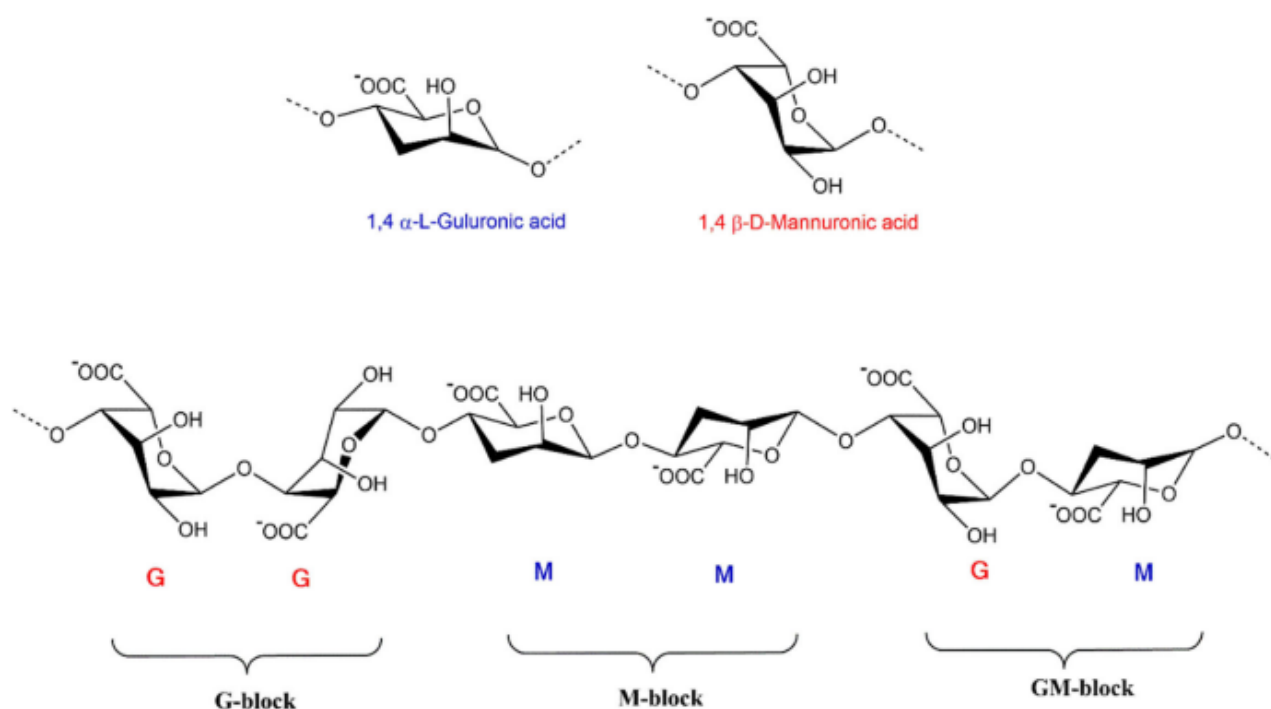


Figure 9: Alginate: above) alginate monomers; below) three different types of alginate constituent blocks: poly-G (G-block), poly-M (M-block) and GM-block (block with alternating monomers). Figure by [72].

Glucuronic acid residues present in the G blocks of two different adjacent polymer chains can be cross-linked by means of divalent cations (such as Ca^{2+} or Ba^{2+}) thanks to the interactions with sugars carboxylic groups, leading to the formation of a gel network, called “egg box ” (Figure 10) [73].

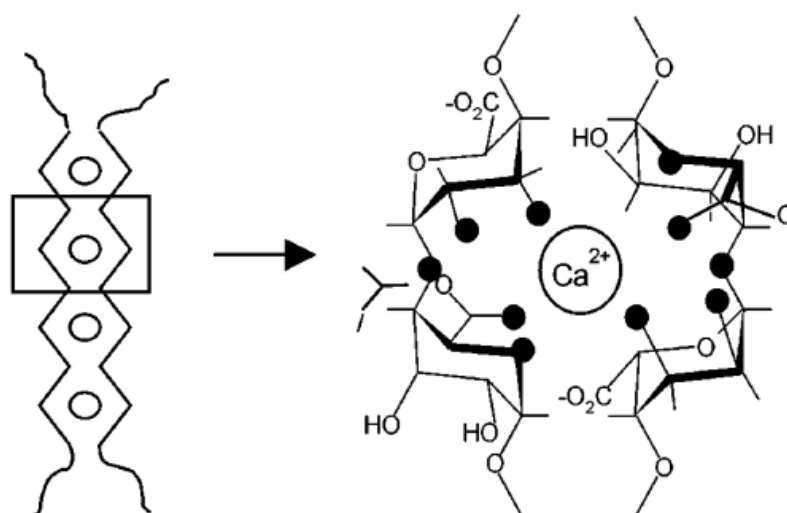


Figure 10: On the left, the diagram of two polymer chains (lines) cross-linked by multivalent cations (circles), right: detail of the interactions between chains and the Ca^{2+} ion. Figure by [73].

Cross-linking method with divalent cations has been investigated in the analytical applications reported in this thesis, however this is only one of the ways in which alginate-based hydrogels can be obtained. Among the other methods of cross-linking are covalent cross-linking, which allows the polymer chains to be cross-linked by forming covalent bonds between them thanks to the use of appropriate cross-linking; incorporation in Poly-alginate (N-isopropylacrylamide) (PNIPAAm), which allows the production of a temperature-sensitive hydrogel; cell-cross-linking, which exploits specific ligand-receptor interactions, following the incorporation of ligands into the polymer structure; radical polymerization, possible following an appropriate functionalization (for example with methacrylate) of the natural polymer; the “Click” reactions, mediated by metals or also in a biocompatible version (reactions with Schiff bases) [74]. Another factor that can influence hydrogel final characteristics is alginate molecular weight, which varies between 32,000 and 400,000 g / mol, and the higher it is, the more viscous the gel obtained will be. For example, in the regenerative field, the properties of alginate are exploited for the development of devices capable of keeping wounds moist and decreasing the risk of bacterial infection [75]. Evidences of some alginate-based products are Algisite MTM [76] and AlgicellTM [77]; the first is a calcium alginate-based soft gel medicament, used in moist wound management for its absorbent properties; the second is a dressing apply to treat ulcers and surgical wounds, it is based on sodium alginate and silver (1.4%) used as an antimicrobial agent.

2.1.2. Hyaluronic acid

Hyaluronic acid (HA) is a linear polysaccharide, a non-sulfated GAG composed of repeated disaccharide units consisting of β -1,4-D-glucuronic acid and β -1,3-N-acetyl-D-glucosamine (Figure 11). It is a polymer widely represented in a large variety of tissues, including dermis and nerve tissue, where it is one of the main components of the extracellular matrix (it is produced by membrane cells). It contributes to the hydrodynamic regulation of tissues, to differentiation, cell migration, is involved in processes such as inflammation, angiogenesis, and tissue regeneration. HA binds to the receptor cluster of differentiation 44 (CD44), involve in tissue formation and in maintaining and anchoring the proteoglycan polymer to the cytoplasmic membrane; thus, inductions of these receptors could be useful in promoting wound healing. HA, thanks to the properties of biocompatibility, biodegradability, non-immunogenicity, ease of production and modification as well as its hydrophilic and non-adhesive character, is widely used in the production of scaffolds for biomedical applications. HA has a water content comparable to that of human tissues and this favors the exchange of oxygen, nutrients and metabolic waste; on the contrary the HA presents weak cell adhesive properties [78–80]. Therefore, this polymer although it can be used alone, is more often combined to other polymers (for example, in this thesis with alginate) in order to produce hydrogels exploiting possible chemical

modification, crosslinking or photo-crosslinking on its carboxylic and hydroxyl groups [80]. Examples of HA-based products available on the market are Hyalogran® (Anika Therapeutics, Inc.) [81], Regenecare® (MPM Medical, Inc.) [82]; the former is a wound dressing based on an HA ester and sodium alginate, used for managing a variety of exuding wounds as ulcers and diabetic wounds; the second is a hydrogel based on various polymers, among which the main one is the HA which has the function of acting as a moisturizing agent in wounds.

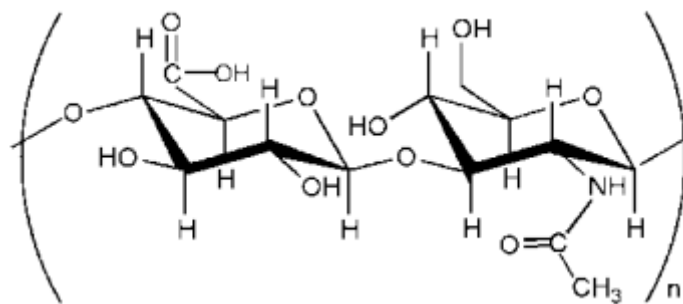


Figure 11: Disaccharide unit constituting hyaluronic acid, composed of glucuronic acid (left) and N-acetylglucosamine (right). Figure by [78].

2.2. 3D printed Hydrogels

Biomaterial-based hydrogels can be produced by 3D printing. Manufacturing procedure plays a crucial role on properties and effectiveness of a device whatever its application. Furthermore, in order to design and subsequently market a device, it is also necessary to take into account the production costs, the possible risks for an operator and consumer, the ease of manufacture and use. 3D printing is a production procedure that responds well to these requirements and for this reason, in recent years, it has been very investigated. Regarding the production of devices in the form of hydrogel for biomedical applications, 3D printing is becoming the technique of choice compared to the more traditional ones (e.g. solvent casting, electrospinning); in fact, as said, this technology stands out for its personal customization, rapid prototyping, flexibility in design and high precision in fabrication of intricate architecture [59,83,84]. 3D printing was introduced in 1986 by Charles (Chuck) W. Hull, who introduced stereolithography [85]; from here it has been developed and applied in various industrial and academic sectors. 3D printing, also known as additive manufacturing, is an additive manufacturing technique, in which the material is deposited layer by layer until a 3D network is formed. In biomedical field, 3D printed hydrogels are exploited as they can mimic the acellular physical structure of a tissue/organ, for example promoting cell proliferation and the execution of functional activities up to the healing of the target tissue; or they can function as drug-delivery devices with personalized drug dosing and so personalized topical treatment devices [86,87]. Generally,

digital files deriving from a computer-aided design (CAD) software provide the desired structures which will then be reproduced in a 3D network by drawing multiple layers of material. Then, a standard STL file (triangle / tessellation file) is created from the digital data to convert the object of interest into thin-sliced horizontal cross-sections for subsequent printing processes based on a layer-by-layer deposition of material [60]. According to ASTM F2792 standard, there are 7 categories of 3D printing: binder jetting, directed energy deposition, material extrusion, material jetting, powder bed fusion, sheet lamination, vat photopolymerization [88]; among these the material extrusion, as mentioned, is the most used technique as it offers an excellent compromise between resolution and cost.

2.2.1. Material Extrusion 3D printing

In material extrusion 3D printing, continuous filaments are produced through an extrusion force that acts by making the ink escape (often from a syringe) through a nozzle. Depending on the needs, compressed air (pneumatically operated extrusion), a piston or a screw (mechanical extrusion) can be used to obtain the force necessary to extrude the material. The material is deposited in its liquid viscous phase and its deposition in X, Y, and Z axes is controlled by actuators with the function of regulating nozzle orientation in three dimensions, thus each layer is built on top of the previous. There are some parameters to consider in material extrusion such as ink physical phase and during printing, the printability of the material, viscosity, stability over time. With this technique, various types of both natural and synthetic polymeric materials can be printed in order to obtain gel-like devices. Finally, this technique has recently also been used to directly print tissue and organ components, through the use of bio-inks (a combination of biomaterials and cells) [60,86,89]. Figure 12 shows the 3D extrusion printer used in the analytical applications reported in this thesis. Polysaccharide biomaterials, particularly alginate in formulation also with other components such as hyaluronic acid, nanoparticles, drugs, and other actives, were used as ink in order to obtain 3D scaffolds in the form of hydrogels. As previously reported [90], with this in-house built 3D printing machine, 3D models of the hydrogels were drawn using CAD software, and then constructed by superimposing two figures that have parallel wires and rotating them by 90°. In detail, a custom low-temperature manufacturing system was developed by combining Peltier cells and liquid/air exchangers with a commercial Direct Ink Writing (DIW) 3D printer. The ink formulation is loaded into a syringe and extruded through a needle by applying controlled pressure on the syringe through a mechanically assisted piston (mechanical extrusion). The 3D printing technique used is called freeze-deposition 3D printing: the liquid-gel ink, thanks to a robotic arm moving along the three axes (x, y, z), is deposited on a stainless-steel Peltier cell at -14°C; this allows a very rapid freezing of the ink and the maintenance during

printing of both macro and micro hydrogel structure. Various layers can be printed in order to form an orthogonal grid with fixed distances between the filaments and creating a 3D mesh-like "gauze" of set dimensions. 3D hydrogels obtained in this way have been shown to exhibit defined chemical-physical properties and controlled porosity; moreover, they have also been studied *in vitro* cell cultures showing an improvement, thanks to production technique, in terms of biocompatibility and proliferation than homologous hydrogels obtained by traditional solvent casting [90].

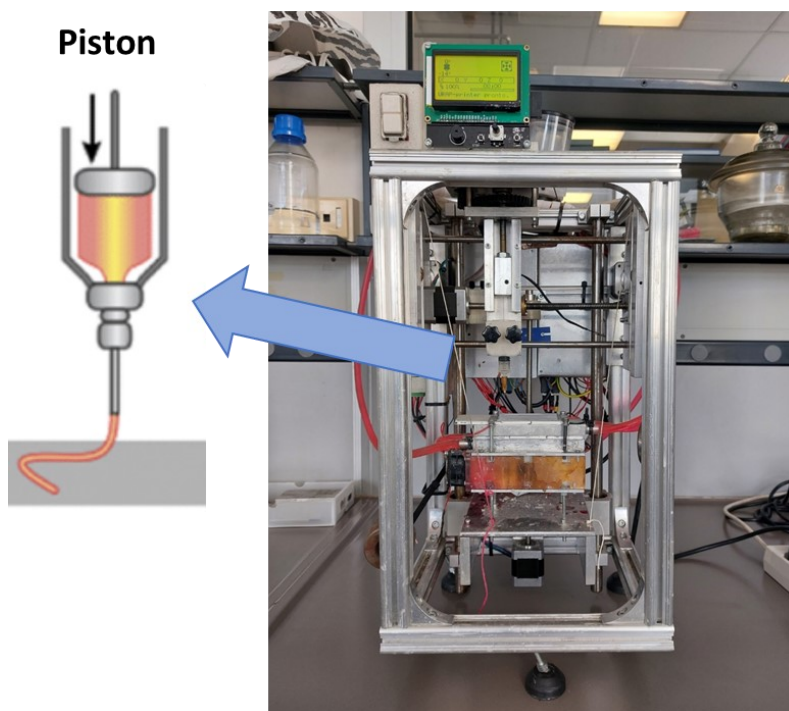


Figure 12: In-house built 3D printing machine used in this thesis; a piston is used to obtain the force necessary to extrude the material.

2.3. Conclusions and future perspectives

As described above, extrusion 3D printing is an excellent technology for fabrication of hydrogels involved in biomedical field. Natural polysaccharides can be used as liquid or semi-liquid inks in order to obtain biocompatible three-dimensional networks with controlled structure and porosity. In this frame, to maximize the effectiveness of the printing process, it is essential to check some parameters such as the type of polymer, the hydrophilic / hydrophobic characteristics, the solution viscosity, the printability, the mechanical properties, the connection processes, the swelling behavior, the presence or absence of cells in the ink.

Among the advantages of 3D printing technology there are the versatility and the possibility of obtaining customized 3D structures for applications in regenerative medicine, tissue engineering or drug delivery. However, there are still some limitations associated with 3D printing polysaccharide-

based hydrogels such as limited ink shelf life, ink preparation times, poor mechanical properties, and difficulty in storage. To overcome these limitations, a continuous optimization and innovation of the biomaterials used is underway; for example, to improve the mechanical properties, polysaccharides can be combined with different substances (for example micro or nanofibers) at different concentrations and / or through a multiple crosslinking approach. Finally, the evolution of 4D printing in the development of polymeric hydrogels opens the doors for a near future of smart hydrogels that change their properties over time, when exposed to specific external stimuli after fabrication [91].

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CHAPTER II: SCREENED: Development and characterization of a sex-specific, 3D rat organoid system for the *in vitro* screening of Endocrine Disruptors on thyroid function

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Personal Contribution

G.R.: liquid-chromatography mass spectrometry, analytical studies and article writing.

Abstract

The Horizon 2020 European SCREENED Project #825745 is focused on developing 3D *in vitro* assays based on 3D organoids engineered with mammalian thyroid cells, for screening the effect of low doses Endocrine Disruptors (EDs). Here, to reach this aim, two models of adult rat cells have been obtained and characterized both morphologically, by microscopy, and molecularly, by liquid-chromatography-mass spectrometry (LC-MS). The first rat cell model is based on certified adult rat thyrocyte cell line FRTL-5 (CLS n.500407), of which the morphology, protein profile and thyroid hormone were studied. These very cells proved to be a suitable and handable surrogate of differentiated, thyroid epithelial cells with a thyroid-specific proteomic profile fully comparable to that of adult male rat primary thyrocytes; therefore, their ability to be magnetized and guided on a thyroid lobe-like 3D collagen scaffold, might be investigated; this proof of concept will be useful for the development of 3D matrices involved as *in vitro* 3D assays for EDs screening. The second rat cell model concerns the use of adult male rat thyroid stem cells / progenitors (TSC/P), suitably cloned as demonstrated by LC-MS targeted proteomic analysis and subsequently differentiated into thyrocytes (TSC/P-T). These cells were used for the development of a new *in vitro* 3D assay based on suitably characterized thyroid follicular organoids, submitted for registration to the European Joint Research Center. The organoids thus obtained were challenged with 3 scalar concentrations of 7 EDs and their proteomic profile were investigated by qualitative LC-MS based proteomics to achieve the first spectrum of molecular effects of each of the tested EDs on male rat thyroid follicular cells.

Keywords: SCREENED, Endocrine Disruptors, 3D *in vitro* assay, rat thyroid cell model; FRTL-5; organoids; liquid chromatography-mass spectrometry

1. Introduction

Endocrine Disruptors (EDs) are environmental toxicants, both synthetic and natural, capable of altering the production, circulating levels, peripheral effect and / or metabolism of hormones secreted by the endocrine axes of all vertebrates, including humans. In the medium and long term, they are the basis of chronic diseases with a high social impact (i.e., obesity, diabetes, infertility, cancer, dystyroidism, alterations in neurobehavioral development including attention deficit syndrome and autism) [1]. In 2015, a median cost for their pathological effects on the European population was estimated at 157 billion euros / year, equal to 1.23% of the GDP (Gross Domestic Product) of the European Union, inducing states members to adopt specific policies for the prevention and treatment of damage from EDs. In this context, the Horizon 2020 program has financed 8 five-year European projects (EURION Consortium) among which the SCREENED project (No. 825745), within which this research is located. SCREENED aims to develop *in vitro* assays for the analysis of EDs effects on thyroid function in a sex-specific manner [2]. The main sources of EDs on the hypothalamus-pituitary-thyroid axis are of food, industrial and environmental origin; for example chemicals that are suspected to be EDs can be found in plastics and cosmetics (e.g. phthalates), in coatings of food cans (e.g. Bisphenol A), in electrical, plumbing, refrigerants and household appliances (e.g. polychlorinated biphenyls), in flame retardants and pesticides (e.g. atrazine and DDT) and also in drinking water and ambient air, especially around industrial areas and natural heat sources such as volcanoes and geysers (e.g. polycyclic aromatic hydrocarbons, heavy metals) [3]. Although between 1998 and 2018 the OECD (Organization for Economic Co-operation and Development) introduced some *in vitro* and *in vivo* tests for EDs screening on thyroid function, these are currently very limited. In particular, *in vitro* assays are based on 2D cultures of thyroid cell lines or primary thyroid cells, which variably suffer from inadequate expression and functionality of basic biomarkers of native thyrocyte activity, such as thyroglobulin (TG), thyroid peroxidase (TPO), or sodium iodide symporter (NIS). In addition, thyroid explant cultures are affected by inherent animal-to-animal variability and assays based on either stem cell-derived thyrocytes or thyroid stem cells/progenitors are lacking. Likewise, animal testing may not offer a complete picture of the changes that are expected to occur in the human thyroid gland. Data on EDs Mode of Action (MoA) at low doses and in combination, and on the sequence of their adverse effects from the molecular to the complete organism (AOP) level, are also lacking. Finally, no available test considers these variables in relation to sex, which effect is preponderant in the context of thyroid function disorders [4,5]. To overcome these limitations, eight European partners (including our group from University of Parma) collaborate in SCREENED project with the aim to develop three different 3D *in vitro* assays for EDs screening, which should also shed light on the effects involving thyroid stem / progenitor cells. These *in vitro*

tests consist of: 1) a 3D organoid based on stem cell-derived thyrocytes, 2) a 3D organoid based on a decellularized thyroid lobe stromal matrix repopulated with stem cell-derived thyrocytes, and 3) a bioprinted organoid based on stem cell derived thyrocytes capable of mimicking the spatial and geometric characteristics of a native thyroid gland. These sex-specific 3D organoids are the basis for the development of a modular microbioreactor to evaluate the effects of different classes of EDs, at low doses and in combination on thyroid function [1]. The possibility of generating the aforementioned 3D *in vitro* models is based on the organomorphogenetic approach, according to which the function of a native endocrine organ can be reliably replicated by reconstructing its 3D replica with stem / progenitor cells induced to differentiation on a biocompatible 3D scaffold, mimicking the 3D geometry of the organ's natural matrix (organomorphism). Therefore, topological inputs, cell surface signals and mechanochemical transductions may take place to guide seeded stem cells / progenitors up to their self-organization, first in a 3D functional organoid and finally, in a replica of an entire thyroid gland. This is due to the strong relationship between structure and function during thyroid development [6,7]. Another aspect of innovation of SCREENED project, lies in the use of qualitative and quantitative liquid chromatography-mass spectrometry (LC-MS) for the analysis of these organoids' proteome, in the presence of EDs, supplemented by transcriptomic analysis and MoA *in silico* simulation, in order to identify the lowest doses of EDs that alone or in combination can modify one or more molecular markers of thyroid function and therefore trace new AOPs. The development of a humanized 3D thyroid organoid prototype, represents, to date, the potentially most sensitive, specific, and accurate system for evaluating *in vitro* combined low-dose effects of EDs on thyroid function, with a patent perspective, regulatory repercussions on tests approved by the OECD and significant commercial potential [1].

In detail, our unit has been focused on 3D organoids engineered with mammalian thyroid cells, in particular rat cells (*Rattus norvegicus*), for screening the effect of low doses EDs. Two cell models have been developed: the first uses the FRTL-5 certified adult rat thyroid cell line (CLS # 500407) and the second uses adult male rat thyroid stem cells / progenitors (TSC/P) differentiated to thyrocytes (TSC/P-T).

FRTL-5 cell line was initially investigated as it is easier to find and use than TSC/P; we investigated FRTL-5, a surrogate of adult male rat TSC/P to test the capacity of a differentiated rat thyroid cell line to grow and colonize geometrically-simple lobar scaffolds of bovine collagen, with the intention to test their eventual capacity to be magnetized and *ad hoc* positioned on the scaffold under an external magnetic field. Thus, our research group performed the molecular characterization and ensuing comparison between primary male rat thyrocytes (PT) and FRTL-5 cells, the latter as a suitable and handable surrogate of differentiated, thyroid epithelial cells. This, with the intent to set

a panel of molecular markers useful to follow the compatibility of mature thyroid epithelial cells, like those normally forming functional follicles, to magnetized particles and their magnetic guidance onto a thyroid lobe-like 3D collagen scaffold, which the unit of the CNR of Faenza (Italy, Dr. Silvia Panseri and Dr. Monica Sandri) is in charge of.

Regarding the second cell model developed, previously isolated and characterized TSC/P cells [8-10], were cloned to provide a suitable source of viable and functional TSC/P able to take the phenotype of mature thyrocytes under adequate differentiation inputs; the clones thus obtained (TSC/P-c) were characterized and the molecular profiles compared with those of the PT. This with the final aim to obtain new 3D thyroid follicular organoids based on an original “sandwich-like” Matrigel transwell system and high density TSC/P-c induced to thyrocyte differentiation; once developed, this bioconstruct was used to set an innovative *in vitro* assay for screening the effect of low doses EDs on thyroid cell function. In particular the 3D thyroid lobe-like organoids were challenged for 48hs with three scaling doses of 7 different EDs (aromatic hydrocarbons, phthalates, polychlorobiphenyls); untreated organoids were used as controls. In collaboration with Atturos Ltd. (Dublin, Ireland, tutors Prof. Stephen Pennington and Dr. James Waddington), proteomic profiles of these samples were obtained, and will be confronted by statistical analysis with untreated controls.

Liquid chromatography-mass spectrometry (LC-MS) was the method of choice for the molecular characterizations of these two cell models. This accurate and sensitive analytical technique was used for the identification and quantification (absolute or relative) of proteins or hormones in thyroid cell samples. Traditional techniques used for the analysis of thyroid biomarkers consisted, for example, of western blot, immunohistochemistry, radioimmunoassay [11-15]; techniques that often have problems of specificity, accuracy, the need for large quantities of sample or high laboriousness.

LC-MS based proteomics for thyroid cells applications has been relatively little explored. For example, LC-MS / MS with triple quadrupole instrumentation has been successfully used to measure TG concentration in patient serum samples [11,16-18]; as regards the qualitative approach, only a few studies have analyzed the thyroid proteome in the entire thyroid tissue [19-24] and thyroid cell cultures [12,25]. Instead, various LC – MS / MS methods have been validated for the quantification of thyroid hormones and their metabolites, mainly for plasma samples, but also in some tissues [14,26-33], also using nano-LC-MS / MS system to facilitate TH measurements in small samples amounts [13].

In this work, using a liquid chromatography-triple quadrupole-tandem mass spectrometry targeted approach (LC- MS / MS), three methods were developed: the first for the protein characterization of FRTL-5, TSC/P-c and comparisons with PT; the second for the absolute quantification of intracellular thyroglobulin in FRTL-5 samples; the third for the absolute quantification of intracellular thyroid

hormones free triiodothyronine (fT3) and free-thyroxine (fT4) in FRTL-5 samples. Finally, an untargeted LC-MS based qualitative approach has been involved for the investigation of the EDs effects on the 3D thyroid organoids entire proteomic profile.

2. Materials and Methods

2.1. Reagents

Sodium dodecyl sulphate (SDS) was from Lisapharma S.p.A. (Erba, Como, Italy). Triton X-100 (TX100), sodium hydroxide (NaOH), ethylene diammino tetraacetic acid (EDTA), trypsin (p.n. T1426), iodoacetamide (IAA), dithiotreitol (DTT), D-(+)-Sucrose, HEPES, bovine serum albumin (BSA), ammonium bicarbonate (ABC), Thyroglobulin-Bovine Thyroid Gland (TGb) (p.n. 609310), 3,3',5-Triiodo-L-Thyronine (fT3) (p.n. T2877), 3,3',5-Triiodo-L-Thyronine $^{13}\text{C}_6$ Solution ($^{13}\text{C}_6\text{-T}_3$) (p.n. T-077), L-Thyroxine (fT4) (p.n. T2376), L-Thyroxine $^{-13}\text{C}_6$ Solution ($^{13}\text{C}_6\text{-T}_4$) (p.n. T-076), insulin, transferrin, somatostatin, glycyl-L-histidyl-L-lysine acetate, hydrocortisone, thyroid stimulating hormone (TSH), Non-Essential Amino Acid solution, Benzo[a]pyrene (BAP), Benzo[k]fluoranthene (BKF), Benz[a]anthracene (BAA), Di-isodecyl phthalate (DIDP), Triphenyl phosphate (TPP), Tris(1,2-dichloro-2-propyl)phosphate (TDCPP) and Polychlorinated biphenyls 153 (PCB 153), Propylthiouracil (PTU), Triethylammonium bicarbonate (TEAB), HeLa Cell Line human and Dispase® II, Eukitt® were all purchased from Sigma-Aldrich® (Darmstadt, Germany). Glutamine, Gentamicin, Penicillin/Streptomycin, Fetal Serum Bovine (FBS), Fetal Horse Serum (FHS) and Dulbecco's Modified Eagle's Medium low glucose (DMEM-LG) were from AUROGENE (Roma, Italy). Dulbecco's phosphate buffered saline (PBS) and Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} were from Euroclone (Milan, Italy). Dimethyl sulfoxide (DMSO) was from PanReac AppliChem (Monza, Italy). Collagenase type IV Gibco™ was from Thermo Fisher Scientific. Corning Matrigel Matrix was from Corning (United States). Protein assay dye reagent concentrate was from Bio-Rad. LC-grade water, glacial acetic acid, 2-propanol, methanol, acetonitrile, t-butyl methyl ether (TBME), analytical reagent grade formic acid (FA), trifluoroacetic acid (TFA), urea, were purchased from Carlo Erba (Milan, Italy).

2.2. Cell cultures

2.2.1. FRTL-5 cells

FRTL-5, a continuous diploid cell line (CLS # 500407), derived from the thyroid gland of the 5 to 6-week-old Fisher rat, was employed in this study and cultured as previously reported [34]. The cells were grown in well-established Thyrogenic Medium (TM): Coon's Modified Ham's F-12 solution medium added with insulin (10 $\mu\text{g/mL}$), transferrin (5 $\mu\text{g/mL}$), somatostatin (0.01 $\mu\text{g/mL}$), glycyl-L-

histidyl-L-lysine acetate (0.1 $\mu\text{g/mL}$), hydrocortisone (10 nM), and TSH (10 mU/mL). 5% FBS, 2 mM glutamine, 1% penicillin/streptomycin, 1% Non-Essential Amino Acids and 20 $\mu\text{L}/100\text{ mL}$ gentamicin were also added. For LC-MS experiments, cells were grown up to 80% of confluence, harvested using 0,05 % trypsin/EDTA, and centrifuged at 500 G for 5 min (ThermoScientific SL-8 centrifuge). Cell pellets were washed twice with PBS 0.1M and stored at -80°C until the day of analysis.

2.2.2. TSC/P-c and PT

Primary adult male rat thyrocytes (PT) at passage 4 (P4), and adult male rat TSC/P at passage 22 (P22) were isolated from the thyroids of 50-75 gr. (3-4 weeks old), Sprague Dawley® (SD®) male rats, as previously described [8-10]. TSC/P successfully cloned (TSC/P-c) and PT cells were cultured in DMEM-LG with 10% FBS and 5% FHS, 1% of Non-Essential Amino Acids, 1% glutamine, 1% penicillin-streptomycin and 20 $\mu\text{L}/100\text{ml}$ of gentamicin. For LC-MS experiments, cells were grown up to 80% of confluence, harvested using a cell scraper, and centrifuged at 272 G for 10 min (ThermoScientific SL-8 centrifuge). Cell pellets were washed twice with PBS 0.1M and stored at -80°C until the day of analysis.

2.2.3. TSC/P-T based 3D organoids and EDs treatment

TSC/P-c were differentiated to functionally thyrocytes for 7 days following this protocol:

TSC / P-c were seeded at either $300 \times 10^3\text{ cc} / \text{cm}^2$ in transwells using a sandwich technology (Matrigel 95% as a basis, Matrigel 50% as a cellular vector bearing the dispersed cell solution) and cultured in P24 multiwells surrounded by medium DMEM-LG, 10% FBS, 5% FSH, 1% of Non-Essential Amino Acids, 1% glutamine, 1% penicillin-streptomycin and 20 $\mu\text{L}/100\text{ml}$ of gentamicin for 3 days. On the fourth day the culture medium was changed and replaced with TM implemented with additional bovine TSH, 10 mU/ml / 24 hs until the seventh day.

For mass spectrometry analysis, the 3D organoids formed up to the seventh day of culture were then subjected to 48 hours of treatment with EDs. Table 1 summarizes the scheme of this *in vitro* assay expected to overcome current tests limitations, being more sensitive at low doses of chemical exposure, thus enabling prediction of EDs toxicity on human health in a biological sex-specific manner. The assay consists in using 3 different scalar concentrations (minimum, medium & maximum) of each of the 7 EDs chosen: BAP, BKF, BAA, DIDP, TPP, TDCPP, PCB153, as well as a reference molecule positive control molecule - PTU whose action is well known [35]. Untreated organoids (U) were used as negative controls (DMSO 0,1%). For each chemical, three biological

replicates were obtained leading to a total of 72 treated samples, and 8 controls, one for each molecule tested (80 total samples, 9 treatments).

At the end of the acute administration of EDs, the organoids were detached from the Matrigel culture and pelleted, using a well-established protocol kindly provided by Dr. Sabine Costagliola (University of Brussels). Finally, thyroid organoids cell pellets were resuspended in 8M urea lysis buffer in 50mM ABC and frozen at -80°C until use.

CLASS	NAME	ACRONYM	CONCENTRATION
Polycyclic aromatic hydrocarbons (PAHs)	Benzo[a]pyrene	BAP	minimum_250 nM
			medium_2.5 µM
			maximum_25 µM
	Benzo[k]fluoranthene	BKF	minimum_250 nM
			medium_2.5 µM
			maximum_25 µM
	Benz[a]anthracene	BAA	minimum_250 nM
			medium_2.5 µM
			maximum_25 µM
Phthalates	Diisodecyl phthalate	DIDP	minimum_1 µM
			medium_10 µM
			maximum_100 µM
Organophosphate flame retardants (OPFRs)	Triphenyl phosphate	TPP	minimum_150 nM
			medium_1.5 µM
			maximum_5 µM
	Tris(1,3-dichloro-2-propyl)phosphate	TDCPP	minimum_50 nM
			medium_500 nM
			maximum_5 µM
Polychlorinated biphenyls (PCBs)	PCB-153	/	minimum_17 nM
			medium_170 nM
			maximum_1.7 µM
Reference compounds	Propylthiouracil	PTU	minimum_12 nM
			medium_120 nM
			maximum_1.2 µM
	Controls (DMSO 0.1%)	U	/

Table 1: Scheme of the 3D rat thyroid organoids experiment: EDs and concentration used.

2.3. Samples and Experiments

As previously reported, in this work, two rat thyroid cell models were developed and characterized; the first using FRTL-5 cell line and the second using TSC/P-c differentiated to thyrocytes (TSC/P-T).

2.3.1. First rat thyroid cell model: FRTL-5 cells

The molecular characterizations (carried out by LC-MS / MS selected reaction monitoring (SRM) targeted analysis) of the first rat thyroid cell model developed, are shown in Table 1. A panel of thyroid protein biomarkers (Table S1_Supplementary Materials) was used for the assay, and then for the relative quantification of these proteins in FRTL-5 samples compared with those of PT. In FRTL-5 samples, also the intracellular TG (Table S2) and the thyroid hormones fT3 and fT4 (Table S3) were absolutely quantified. Table 2 also indicates the number of cells (counted by inverted light microscope) in the samples analyzed, and the numbers of biological and instrumental (technical) replicates used in the various experiments.

Analysis Aim	Rat samples	Cells in pellet (inverted light microscope)	N° of Biological Replicates	N° of Technical Replicates	SRM transitions list
Proteins relative quantification: FRTL-5 vs PT	FRTL-5	1,5 x 10 ⁶	1	2	Table S1
	PT	1 x 10 ⁶	1	2	
TG Absolute Quantification	FRTL-5	1,5 x 10 ⁶	2	3	Table S2
THs Absolute Quantification	FRTL-5	1,5 x 10 ⁶ ; 3 x 10 ⁶	2	2	Table S3

Table 2: Experiments performed for the first rat thyroid cell model: analysis aim, rat samples, number of cells, of biological and technical replicates, and tables list (Supplementary materials) for SRM transitions used for the assays.

2.3.2. Second rat thyroid cell model: TSC/P-T

The molecular characterizations (carried out by LC-MS / MS SRM targeted analysis) of the second rat thyroid cell model developed, are shown in Table 3. Initially, a panel of protein biomarkers (Table S4) was used for this assay in TSC/P-c samples, to compare the protein profile (relative quantification) with that of PT and thus eventually check their expected possibility to be differentiated, thanks to appropriate stimuli, into functional thyrocytes. Once the successful differentiation of TSC/P-c to functional thyrocytes (TSC/P-T) was verified, these cells were used for the development of 3D thyroid organoids (as described in paragraph 2.2.4) involved for *in vitro*

evaluation of the effect of the lowest but effective concentration of 7 EDs among those originally selected by the SCREENED Consortium. As indicated in Table 3 these samples were subjected to qualitative untargeted LC-MS analysis. In particular, to set the parameters of this last analysis, 3 of the 8 control samples (U) were used for a pilot study (data not reported); this explains the number of 5 biological replicates for U samples indicated in table 2, used for the final proteomics of our interest.

Analysis Aim	Rat samples	Cells in pellet (inverted light microscope)	N° of Biological Replicates	N° of Technical Replicates	SRM transitions list
Proteins relative quantification: TSC/P-c vs PT	TSC/P-c	1,8 x 10 ⁶	3	2	Table S4
	PT	1 x 10 ⁶	1	2	
Proteins qualitative analysis: Thyroid organoids	Thyroid organoids (TSC/P-T)	3 x 10 ⁵	3 (Treated samples); 5 (Untreated samples)	1	/

Table 3: Experiments performed for the second rat thyroid cell model: analysis aim, rat samples, number of cells, of biological and technical replicates and table list (Supplementary materials) of SRM transition used for the assay.

2.4. Morphological characterization

2.4.1. Scanning electron microscopy (SEM)

FRTL-5 cells and PT after 3 days of grown on 2D monolayer, were fixed in glutaraldehyde 2.5% in cacodylate sodium buffer 0.1 M for twenty minutes and rinsed in PBS (0.1 M, pH 7.4). Using a graded series of ethanol (50% to 100%), samples were dehydrated and then subjected to critical point drying (Balzers) in a 100% ethanol/liquid CO₂ exchange system. Dried samples were mounted on aluminum stubs, metallized for 90s (Balzers apparatus) with a 60 nm gold film, and observed with a Philips SEM 501.

2.4.2. Light Microscopy (LM)

TSC/P-T, after 7 days of culture (as described in paragraph 2.2.3) were observed using inverted LM (Leica DMi1). Subsequently, these samples, were also observed by LM (Zeiss Axiophot) after, hematoxylin-eosin (HE) staining. In particular, Matrigel drop was fixed with 4% paraformaldehyde-0.1M phosphate buffer for 30 minutes, washed twice with PBS 0.1M and dehydrated with graded series of ethanol (50% to 100%), and finally embedded in paraffin. After microtome cutting (5 µm slides), samples were stained HE, and analyzed by LM.

2.5. Protein extraction and sample preparation for mass spectrometry analysis

2.5.1. FRTL-5, TSC/P-c and PT cells

Total proteins were extracted from FRTL-5 or TSC/P-c or PT cell pellets respectively, using a primary buffer of 8M urea, SDS 0,5% (v/v), TX100 1% (v/v), DTT 50mM in ABC 50mM. Samples were subjected to orbital shaking (Orbital shaker IKA Werke Schuttler MTS 4 S2 Shaker) for 15 minutes, sonicated at 4°C for 20 minutes (Bransonic ultrasonic cleaner 2510E –DTH) and centrifuged at 4°C for 10 min at 15,000 rpm. The pellets obtained was subjected to a further extraction with 8M urea, SDS 0,5% (v/v), TX100 1% (v/v) in ABC 50mM; then samples were subjected to orbital shaking for 15 minutes, sonicated at 4°C for 20 minutes and centrifuged at 4°C for 10 min at 15,000 rpm. The supernatants obtained from the two buffers extraction were subjected to buffer exchange using 50 mM ABC in Amicon Ultra 0.5 mL centrifugal filters (Sigma-Aldrich) (Darmstadt, Germany) with a 3-kDa MWCO. Ensuing protein extracts, then, were assayed for protein dosage by using the Bradford method, based on adding 10 µL of each sample to 500 µL of a previously diluted (1:5) Bradford reagent solution (Absorbance = 595 nm). Calibration curve was achieved using BSA standard solution (1 mg/mL) diluted in the range of 25-400 µg / mL [FRTL-5 and PT: $y=0,0008(\pm 3,09E-05)x$, $R^2=0.997$; TSC/P-c and PT: $y=0,0012(\pm 4,57E-05)x$, $R^2=0.996$].

Subsequently, samples were reduced using 100 mM DTT (30 min at 37° C), alkylated by 200 mM IAA (30 min at room temperature-RT in the dark), the reaction was stopped by adding 100 mM DTT (15 min at RT). All samples were digested by incubation with trypsin (1:50 enzyme/substrate ratio) at 37 °C overnight and, the digestion stopped with 1 µL of FA added to each sample. Finally, samples were dried under nitrogen flow and stored at -20°C until use. The tryptic peptides were reconstituted with 150 µL of a water / acetonitrile / FA aqueous solution (49.95 / 49.95 / 0.1, v / v) before being analyzed with LC-MS/MS.

TG in FRTL-5 samples was also quantified using bovine thyroglobulin standards (concentration range: 0.5-10 µg/mL). Each bovine TG solution was treated as described above.

2.5.2. 3D Thyroid Organoids

3D thyroid organoids lysates were digested with trypsin using the S-Trap (Protifi) 96-well plate platform; thus, lysates were thawed from -80°C and proteins solubilized and denatured using a 10% SDS, 100 mM TEAB (pH 7.55) solution, added at a 1:1 ratio. Proteins were reduced and alkylated using concentrations of 200 mM DTT and 400 mM IAA, respectively. S-Trap binding buffer (9:1 ratio of methanol:TEAB) was added to each sample and loaded onto a 96-well S-Trap plate. After multiple wash steps, sequencing grade modified trypsin (Promega) was used to digest the proteins overnight (16 h) at 37°C. Following digestion, samples were dried down and reconstituted with 30

μL of 2% acetonitrile/0.1% TFA water solution. Finally, peptides in each sample were quantified on a DeNovix DS-11 spectrophotometer (Absorbance= 215 nm). All samples were stored at -80°C until mass spectrometry analysis.

2.6. FRTL-5 cells: Hormones biomarkers extraction and preparation for mass spectrometry analysis

Thyroid hormones (fT3 and fT4) were extracted from FRTL-5 cell pellets as previously described [26]. Homogenization buffer pH 7.4 (HB: 250 mM D-(+) Sucrose, 20 mM HEPES, 1 mM EDTA) was made and stored in aliquots at -20°C for single use. HB was mixed 1:1 with 0.1 N NaOH to produce lysis buffer 1 (LB1). Lysis buffer 2 (LB2) consisted of 30% v/v glacial acetic acid in HB. Both LB1 and LB2 were made freshly for each cell lysis. FRTL-5 cell pellets were lysed at RT by adding 300 μL LB1 with orbital shaking (600 rpm) for 5 min followed by addition of 150 μL LB2 with orbital shaking (600 rpm) for 5 min. Finally, 100 μL methanol were added to each sample and the resulting 550 μL cell lysates were transferred into 2 mL Eppendorf tubes and incubated in the dark at 37°C for 1 hour. 1 mL of 30% v/v 2-propanol /70 % v/v t-butyl methyl ether (TBME) was added to the lysate and the mixture was vortexed for 5 min. The phases were separated via centrifugation at 5000 rpm for 10 min, at room temperature. The upper (non-aqueous) phase was transferred to a new 2 ml Eppendorf tube, and the extraction process was repeated. The combined organic phases were dried under nitrogen flow and stored at -20°C until LC-MS/MS analysis. Samples were then reconstituted with 50 μL of methanol solution containing ¹³C₆-T₃ and ¹³C₆-T₄ both at a concentration of 1 μg / mL.

2.7. LC-MS/MS SRM analysis

2.7.1. Proteins Biomarkers: FRTL-5, TSC/P-c and PT

Protein biomarkers analysis was carried out by LC-MS/MS SRM targeted analysis. Each tryptic solution was eluted by using a Xbridge Peptide BEH C18 (250 mm x 2,1 mm, 5 μm particles) column (Waters, Milford, MA) equipped with a C18 pre-filtering column. An elution system based on a solvent gradient [solution A: 0.1% aqueous FA (v/v) / solution B: acetonitrile] was delivered at 0.2 mL/min. Gradient elution was as follows: solvent B was set at 5% for 2 min, then delivered by a linear gradient from 5% to 95% in 90 min. The mobile phase was delivered by the LC Agilent HP 1200 (Agilent Technologies, CA, USA) equipped with a 100-vial capacity sample tray. Injection volume was 10 μL. A QTRAP 4000 triple quadrupole mass spectrometer (ABSCIEX, Foster City, USA), equipped with a pneumatically assisted ESI interface was used. The sheath gas (nitrogen, 99.999% purity) and the auxiliary gas (nitrogen, 99.998% purity) were delivered at flow rates of 45 and 5 arbitrary units, respectively. Optimized conditions of the interface were as follows: ESI voltage

5.5 kV, declustering potential (DP) 80 V, entrance potential (EP) 10 V, collision energy (CE) 40 V, collision exit potential (CXP) 10 V, capillary temperature 350 °C. Experiments were performed under positive ion-SRM conditions using nitrogen as collision gas (pressure of $2,1 \times 10^{-3}$ mbar) and a 20 ms-dual time for each transition monitored. The SRM transitions monitored were reported in Table S1 (FRTL-5 vs PT), Table S2 (TG quantification in FRTL-5), Table S4 (TSC/P-c vs PT); the most intense SRM transition was monitored for each protein biomarker, whereas the other transitions reported were monitored for confirmatory purposes.

2.7.2. Hormones Biomarkers: FRTL-5

The identification and quantification of thyroid hormones was performed modifying a previously published method [31]. Samples were eluted by using a C18 Luna (50 mm $\times$ 2.1 mm, 3 μ m particles) column (Phenomenex, CA, USA) equipped with a C18 pre-filtering column. An elution system based on a solvent gradient [(A) aqueous FA 0.1% solution (v/v)/(B) methanol:acetonitrile (1:4)] was delivered at 0.2 mL/min. Gradient elution was as follows: solvent B was set at 5% for 4.5 min, then delivered by a linear gradient from 5% to 90% in 5 min and to 100% in 8 min. The analysis was carried out using the same instrumentation described in paragraph 2.7.1. Injection volume was 25 μ L. Analytical method performance was initially assessed using thyroid hormone standards in free form (fT₃ and fT₄) and their respective labelled standards (¹³C₆-T₃ and ¹³C₆-T₄). For both hormones, calibration curves were performed using fT₃ or fT₄ standards (serial dilutions: 0.001-0.005-0.01-0.05-0.1-0.5-1-5-10 μ g/mL) spiked with 1 μ g/mL of the respective labeled standard (¹³C₆-T₃ or ¹³C₆-T₄). Standard solutions were analyzed in triplicate (n=3 technical replicate). The limit of quantification for both hormones was 5 ng/mL so the calibration curves were performed in the concentration range 0.005-10 μ g/mL [fT₃: $y = 6.7335(\pm 0,074)x$, $R^2 = 0.999$; fT₄: $y = 7.4518(\pm 0,08)x$; $R^2 = 0.999$]. The SRM transitions monitored and the related parameters were reported in Table S3. For quantitative purposes, the most intense SRM transition was monitored for each hormone biomarker, whereas the other transitions reported in Table S3 were monitored for confirmatory purposes.

2.8. LC-MS qualitative analysis: 3D Thyroid organoids

Samples were prepared for discovery LC-MS using a Bruker timsTOF Pro coupled to an EvoSep One LC system. As reported above (paragraph 2.5.2), all samples were resuspended in 2% acetonitrile/0.1% TFA, and peptides were quantified using A215 on a DeNovix DS-11 spectrophotometer. Five hundred nanograms of peptide were loaded onto EvoSep EvoTips using the manufacturers' protocol. Samples were introduced onto a Bruker timsTOF in a pre-determined worklist run order; in particular, the worklist was flanked by commercially available HeLa human

cell line digests to monitor instrument performance over time (1 Blank, 3 HeLa samples, 1 Blank, 77 rat samples, 1 Blank, 3 HeLa, 1 Blank). For this experiment, the 30 samples per day EvoSep method (44-minute gradient) was used. The timsTOF Pro mass spectrometer was operated in positive ion polarity, with TIMS (Trapped Ion Mobility Spectrometry) and PASEF (Parallel Accumulation Serial Fragmentation) modes enabled. The accumulation and ramp times for the TIMS were both set to 100 ms, with an ion mobility ($1/k_0$) range from 0.62 to 1.60 Vs/cm. Spectra were recorded in the mass range from 100 to 1700 m/z. The precursor (MS) Intensity Threshold was set to 2500, and the precursor Target Intensity set to 20000. Each PASEF cycle consisted of one MS ramp for precursor detection, followed by 10 PASEF MS/MS ramps, with a total cycle time of 1.17 s.

2.9. Data analysis, bioinformatics, and quantitative evaluation of biomarkers

2.9.1. LC-MS/MS: Thyroid protein biomarkers relative quantification

Thyroid protein biomarkers from rat cells were considered for the assay. FASTA protein sequences were obtained from Uniprot *Rattus norvegicus* proteome database (Uniprot Taxon identifier 10116) and SRM transitions for each protein were simulated by Skyline (version 20.1, SCIEX) (Redwood City, CA, USA), setting trypsin as digestion mode with no missed cleavage and carbamidomethylation of cysteins as structural modification. Uniqueness of candidate peptide sequences was assessed by BLASTp tool (basic local alignment search tool; www.ncbi.nlm.nih.gov link NCBI BLAST) search (algorithm: blastp; ATRIX PA 30; GAP COASTS: existence 10, extension:1; DATABASE: non redundant protein sequences) from NCBI (National Center for Biotechnology Information) (Bethesda, MA, USA). LC-MS/MS SRM data were analyzed using the Analyst v 1.4 software and the peaks areas integrations were obtained by the MultiQuant program (version 2.1, ABSCIEX). Relative quantitation of thyroid biomarkers peptides in rat samples, was achieved through normalization of data with respect total proteins content obtained by Bradford assays and expressed as the value of the area (intensity of ionic current or cps / min) under each MS peak.

2.9.2. LC-MS/MS: TG quantification in FRTL-5 samples

Tryptic peptides derived from bovine (*Bos taurus*, Uniprot Taxon identifier 9913; obtained as reported in the previous paragraph) TG standards were used for TG quantification in FRTL-5 rat samples (*Rattus norvegicus*, Uniprot Taxon identifier 10116) (Table S2). For quantitative purposes the WEAQNSAGQALTPAELLMK biomarker m/z transition (1030->1040) from bovine TG and the VIFDASSPVIVK biomarker m/z transition (638->555) from rat thyroglobulin were selected as providing the most intense signal and a calibration curve was performed using the WEAQNSAGQALTPAELLMK form TGb standards [$y=5655,5(\pm 161.8)x$, $R^2=0.997$; concentration

range: 0.5-10 µg/mL]. Subsequently the FRTL-5 samples were spiked with a known concentration of TGB standard and the quantitation of thyroglobulin in FRTL-5 samples was achieved through proportion, by comparing the ionic intensity corresponding to a known concentration of the selected tryptic peptide of TGB standard (WEAQNSAGQALTPAELLMK) with that of the selected tryptic peptide of rat TG (VIFDASSPVIVK) in the volume of FRTL-5 sample with unknown concentration. Finally, thanks to normalization of data with respect total protein content obtained from Bradford assay, TG amount in FRTL-5 samples was expressed as µg of TG/ µg of total proteins (µg of total proteins=298).

2.9.3. LC-MS: Qualitative analysis of thyroid organoids

Raw MS data from Bruker timsTOF, were processed using MaxQuant (v 1.6.17.0)[36]; protein identification used the Andromeda search engine [37], against the *Homo sapiens* reviewed database for HeLa samples (*Homo sapiens*, Uniprot Taxon identifier 9606; 20361 proteins) and against the *Rattus norvegicus* reviewed database for rat samples (Uniprot: 8143 proteins). For databased searching, trypsin was chosen as a specific digestion mode with up to two missed cleavages; protein N-terminal acetylation and oxidation of methionine were set as variable modifications and carbamidomethylation of cysteine as a fixed modification. Minimum peptide length was fixed at 7, mass tolerances and false discovery rate for both peptides and proteins was set at 1%. The label free quantification (LFQ) parameter was set at 1 and match between runs parameter was used for protein identification. Subsequently, Perseus software (v 1.6.15.0) was used for MaxQuant analysis of rat data. Reverse proteins (matching with peptides deriving from the inverted part of the decoy database) and potential contaminants were deleted. Proteins LFQ intensity values were transformed to a log2 scale; this transformation generated a pool of NaN (Non Assigned Number) values that were sorted choosing 1 as a minimum number of valid values in at least one group (which means that a protein must be present in at least one group to be considered a valid value). After these processes, sample were considered and analyzed based on the treatment, thus 9 groups were obtained, and an initial assessment data quality was made: overlap and variability between biological replicates was reported as Venn diagrams and Pearson correlations. Finally, hierarchical clustering with Euclidean distances (considering the means of the biological replicates of each treatment) was performed in order to obtain 8 heat maps representation of ED treatment samples vs U samples.

3. Results and Discussion

3.1. First rat thyroid cell model: FRTL-5 cells characterization

The goal of the first part of this work was to culture and then characterize FRTL-5 cells in order to use them in the development of a rat thyroid cell model for *in vitro* screening of ED on the thyroid function. Therefore, initially, FRTL-5 cells were characterized and compared with PT. Figure 1 depicts the morphological characterization, by SEM analysis, of PT grown for 3 days in standard 2D monolayer, compared to FRTL-5 cells. Both PT and FRTL-5 cells display morphological features consistent with their origin as adult epithelial cells when grown in a standard 2D monolayer. Indeed, both cell types result of polygonal morphology, giving rise to a pavement characterized by cell groups with variable compactness. However, as opposed to primary mature thyrocytes, FRTL-5 do not release thyroid hormones (fT3 and/or fT4) in the first 4-7 days of a 2D culture, nor they give rise to polarized follicles when cultured in a suspension culture [38]. Nevertheless, literature data show that FRTL-5 may synthesize and intracellularly accumulate TG and forms of poorly-iodinated thyronines and have a functional TSHR (thyroid stimulating hormone receptor) and related intracellular signaling system [14,15,38,39]. This confirm that FRTL-5 cells have very strong functional similarities to mature thyrocytes. As such, they may be exploited as a reasonable rodent cell surrogate to preliminary analyze and predict the capacity of mature thyroid epithelial cells (like those forming thyroid follicles) to internalize magnetic particles, for their eventual guidance onto a 3D scaffold, and preserving their basic molecular milieu.

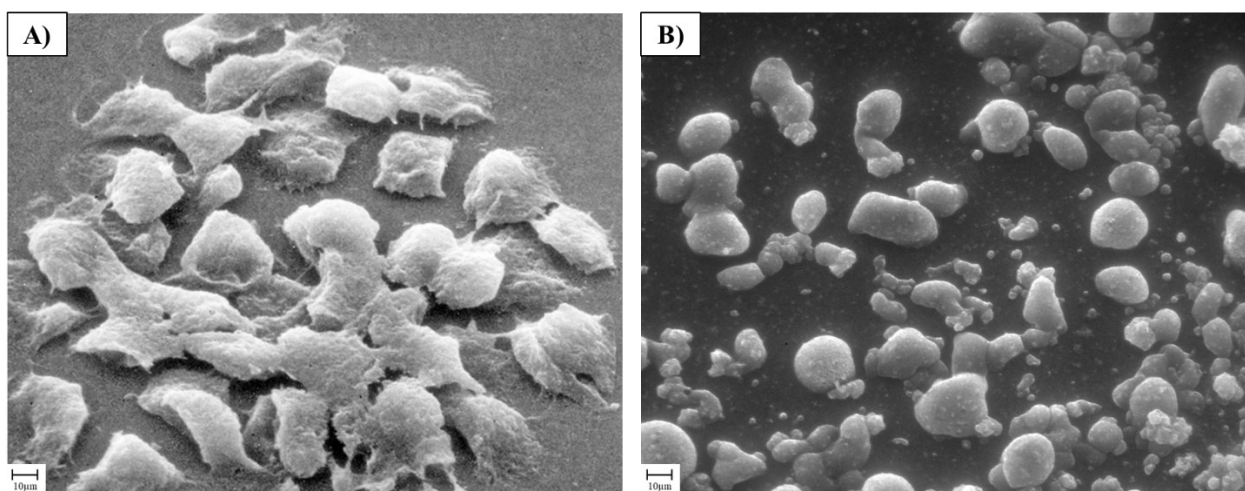


Figure 1: SEM (320 X; scale bar = 10 µm) of rat cells grown for 3 days in standard 2D monolayer: A) cytopinned PT; B) FRTL-5 cells.

To this aim, we started to develop an analytical approach (based on LC-MS/MS SRM analysis) sensitive, specific and flexible enough to be applied to rodent epithelial thyroid cells and, eventually, to thyroid follicular cells treated in the same way as FRTL-5.

In a first step, we moved to define a list of peptide markers known to be characteristic of differentiated thyroid epithelial cells, to compare their distribution in PT with respect to FRTL-5 cells (Table S1). This, with the aim to determine similarities and differences in the level of cell differentiation between these two cell types. Figure 2 collects the semiquantitative cumulated results of all proteins biomarkers found in PT vs FRTL-5 cells (as determined by LC-MS/MS SRM analysis), grouped according to their putative biological significance for thyroid epithelial cells. Fundamental markers of thyrocyte terminal differentiation and functionality [34,39-42] all are expressed to quite comparable level in both cell types including TG, TSHR, NIS, and TPO (Figure 2A). Even more, a preeminence of TG occurs in FRTL-5, in which we also quantified the intracellular TG ($\mu\text{gTG}/\mu\text{g}$ total proteins: $5,994 \times 10^{-3}$). Similarly, to previous molecules, biomarkers of thyroid-specific commitment [40,42-44] are equally expressed at remarkable level in both cells types, with even a prevalence in FRTL-5 cells (Figure 2B). Equally, proteins typical for cell polarization and follicular formation [40,45-50] are present in both cells types at quite comparable levels, with the only substantial difference of Crumbs Cell Polarity Complex supporting the current knowledge that FRTL-5 would not be able to give rise to functional thyroid follicles in suspension culture (Figure 2C). However, it should be kept in mind that also PT fail to provide functional 3D polarized follicles when cultivated in a standard 2D monolayer and, only when rapidly induced in suspension culture may give rise to polarized follicles. Thus, this point remains to be elucidated for FRTL-5 cells using appropriate 3D growth systems, and we are currently investigating it. Very interestingly, FRTL-5 cells depict a level of the multipotentiality marker ABCG2 (ATP-binding cassette super-family G member 2) substantially lower than that observed in lysates of primary thyroid cultures. The reason is likely due to the fact that primary thyroid culture in monolayer necessarily harbor a small amount of ABCG2, as previously observed by our research group [10]. Indeed, it is from these primary monolayer cultures that we have isolated and characterized adult male rat ABCG2-positive stem cells / progenitors, tested for their capacity to be stably differentiated to functional thyroid follicles. Thus, looking also at the other stem cells markers [40,51] analyzed (Figure 2D), it clearly emerges that FRTL-5 cells are a “purified” population of terminally-differentiated rat thyrocytes, selectively retaining the molecular features of mature adult rat thyrocytes even more than primary thyroid cells deriving from a standard monolayer culture. Consistently, markers of embryological origin are very similarly distributed in PT and FRTL-5 cells (Figure 2E), with the latter retaining a higher signature of their original derivation from the endoderm, as proven by the higher level of the OCT3 (Octamer 3) protein, a gene product strictly related to the development of epithelial cells from the primitive endoderm [40]. The expected enrichment of differentiated cells in the FRTL-5 population with respect to PT is confirmed by their selective conservation of the MCT8 (Monocarboxylate transporter

8) thyroid hormone transporter and Dual Oxidase 2, and the quite similar distribution of other receptor proteins (Figure 2F). Finally, proteins related to mechanical and functional stress like heat shock proteins result tendentially more expressed in FRTL-5 cells with respect to PT (Figure 2G). This phenomenon is well described in a number of commercial cells lines and is likely related to the original procedures of cell selection and spontaneous “immortalization” of these differentiated cells. However, the presence of these proteins ensures a mechanism of stabilization of their cellular functions, acting as “regulators” of metabolism through shuttling specific molecules (by a so-called chaperone activity) in and out the cells to favor a functionality consistent with their native phenotype [40,52].

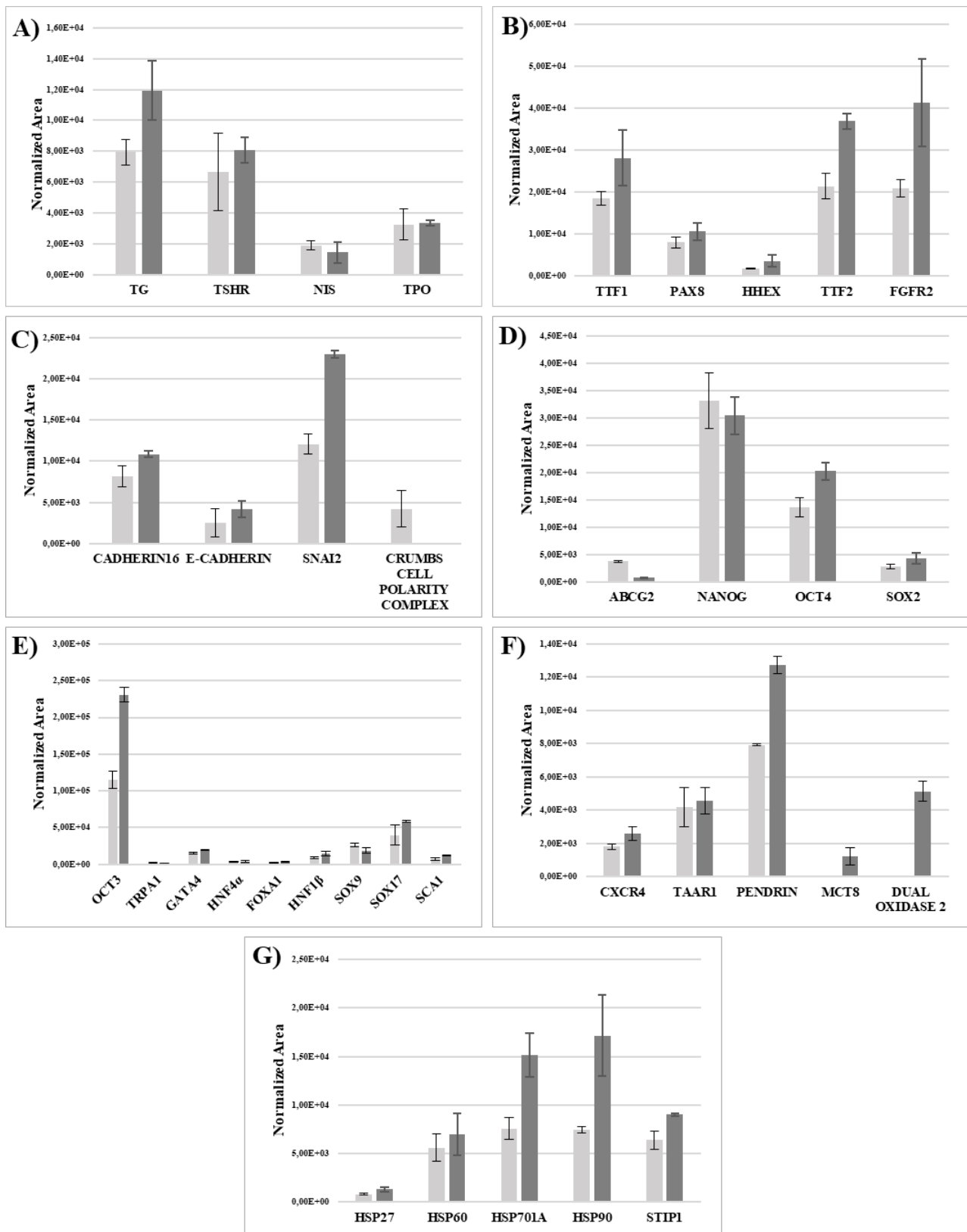


Figure 2: Thyroid protein biomarkers detected by LC-MS/MS SRM analysis in PT (light grey) and FRTL-5 cells (dark grey); results are shown as means $\pm$ standard deviation. A) Biomarkers of thyrocyte terminal differentiation; B) Biomarkers of thyroid-specific development; C) Biomarkers of cellular polarization and follicular self-assembly; D) Biomarkers of stemness; E) Biomarkers of

endodermal and meso-endodermal lineage; F) Receptors, transporters, and enzymes involved in thyrocyte function; G) Heat shock proteins.

In a second step, we tested the capacity of our system to quantify thyroid hormones, both as standards and native molecules in lysates of FRTL-5 grown in standard 2D monolayers. To this aim, we used labeled and unlabeled free form of thyroid hormone standards, fT3 and fT4, allowing for identification of the transitions reported in the Table S3. Considering a limit of technical detection of 5 ng/mL with our instrument, we confirmed literature data that fT3 and fT4 are not readily apparent in FRTL-5 lysates, at least above our concentration threshold. This evidence supports the general concept that, once in 2D monolayer, differentiated thyroid epithelial cells rapidly lose their capacity to release fT3 and fT4 in the culture supernatant [38]. However, previous evidence by our laboratory, showed that PT in standard 2D culture may release fT3 and/or fT4, but only in ranges of few picog/mL. Therefore, we cannot exclude that fT3 and/or fT4 are present in FRTL-5 cytoplasms in those concentration ranges. This, also in accordance with the evidence that the molecule of deposit for thyroid hormones, i.e. TG, can be detected in FRTL-5 cells (Figure 2A). Thus, 3D culture systems are now on the way to verify whether FRTL-5 cells might be able to synthesize thyroid hormone at levels detectable with MS in dependence on the 3D geometry of their growth environment.

3.2. Second rat thyroid cell model

3.2.1. TSC/P-c characterization

In an endeavor to pursue the SCREENED project's goal of obtaining functional, stable, and handle TSC/P to construct 3D assays for ED screening [1], TSC/P were cloned, characterized and compared with PT. Figure 3A grouped protein biomarkers detected in both cell types grouped according to their putative biological significance for thyroid epithelial cells. Of primary importance is the presence of TSHR, expressed in both cell types at comparable levels (Figure 3B) supporting the assumption that TSC/P-c were sensitivity to exogenous TSH and thus may undergo thyrocytic differentiation, as a result of the presence of a functional TSH receptor as in PT. These same cells also exhibited well-defined molecular markers of original thyrocyte lineage including TTF1 and PAX8, but also CADHERIN-16, a molecule that stimulates apical polarization, thus ensuring maintenance of their developmental profile consistent with diffuse presence of the TSHR [40]. Both cell types (Figure 3B) also, express the markers of multipotency-stem cell, as ABCG2, and meso-endodermal lineage (as OCT3, GATA4 etc.) leading to hypothesize their localization in both the parafollicular space and mixed with the follicular epithelium, partly co-localizing in the same cells; furthermore, these very markers indicates that TSC/P-c cells might equally be addressed to differentiate to other cell lineages including adipocytes and hepatocytes, fully confirming their molecular signature as multipotent stem

cells [10,53] . Finally, heat shock proteins were also expressed at comparable levels in both cell types (e.g., HSP27 in Figure 3B) indicating that clonogenesis did not excessively perturb TSC/P.

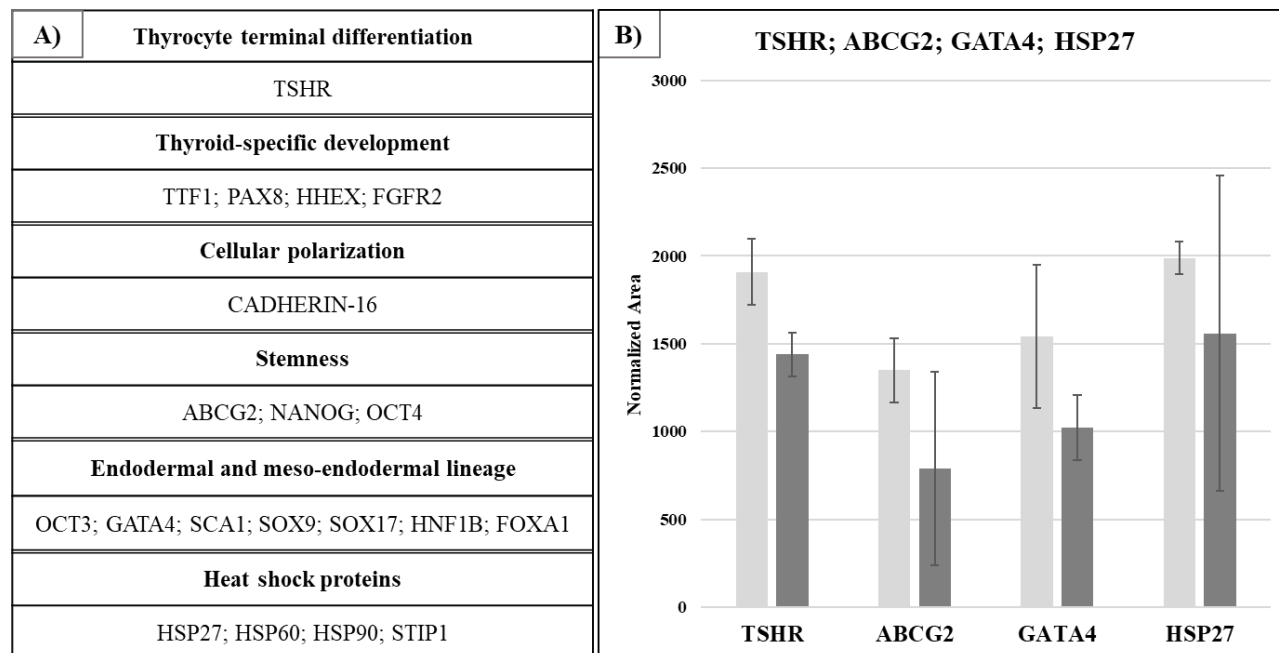


Figure 3: Protein biomarkers detected by LC-MS/MS SRM analysis in both PT and TSC/P-c; results are shown as means $\pm$ standard deviation. A) List of the protein biomarkers detected, grouped according to their putative biological significance for thyroid epithelial cells; B) Semiquantitative cumulated results of TSHR, ABCG2, GATA4, HSP27 in PT (light grey) and TSC/P-c (dark grey).

3.2.2. 3D thyroid organoids development

Subsequently, once assessment TSC/P-c potential (and so of TSC/P) to be differentiated to thyrocytes, and thyroid follicles, these cells were differentiated to obtain 3D functional thyroid organoids available for testing EDs. Thus, a specific cell culture system based on Matrigel and specific thyrogenic cocktail of differentiating factors including TSH, previously successfully tested on TSC/P, was used. In fact, these very cells differentiated into thyrocytes, already in 2D culture expressed the biomarkers TG, TPO, NIS and released fT3 in the culture medium [54]. Figure 4 illustrates inverted LM observations of naturally forming thyroid organoids obtained using TSC/P-T in “transwell sandwich-like” 3D Matrigel culture. Using inverted LM, follicular formation was clearly detected at day 7 (Figure 4A); these organoids appeared as lobulated structures (Figure 4B), enriched with cavities structurally compatible with those of classical thyroid follicles (Figure 4C). These data pointed to the possibility to give rise to self-organized 3D thyroid organoids, with structural features consistent with those of a developing rat thyroid gland, thus confirming efficiency of our methodology to isolate and handle TSC/P and drive them to differentiate thyrocytes and related

thyroid follicles. Therefore, rat thyroid cell model based on 3D thyroid organoids obtained with differentiated TSC/P-c were developed for the screening of EDs, as summarized in Table 1. Details on this assay for EDs screening have been submitted for registration to the European Joint Research Center (JRC). To date, evaluation of accuracy and efficacy of this innovative *in vitro* assay by EU JRC requirements yielded a score of 28.5 / 35.5 in Test Readiness Criteria, providing an A Grading Test consistent with a reliable tool for *in vitro* screening low doses EDs on thyroid cell activity.

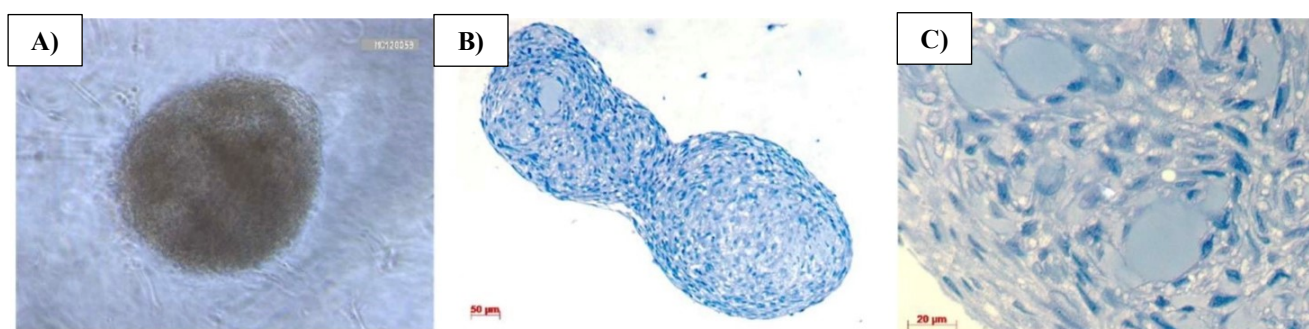


Figure 4: LM images of naturally forming thyroid organoids using TSC/P-T in 3D “sandwich-like transwell” Matrigel culture. A) Inverted LM image of follicular formation B) H&E staining and LM analysis of a bilobated thyroid organoid; C) H&E staining and LM analysis of follicular cavities filled with opaque material (colloid) as in a developing 3D rat thyroid gland.

3.2.3. 3D thyroid organoids based in vitro assays for EDs screening: Qualitative Proteomics

To assess the *in vitro* effect of the lowest but effective concentration of EDs on rat thyroid organoids used as cell models, we selected qualitative proteomic analysis as a powerful, sensitive and reliable analytical technology to obtain a reference molecular panel of EDs treated thyroid organoids. Specifically, we want to compare the protein expression profiles of thyroid organoids treated or not with EDs to identify possible differences in protein expression between treated and untreated samples. In total, 1341 proteins were found among all rat samples; specifically, 1056 proteins were identified in the samples of the BAP treated group, 1019 for BKF, 1030 for BAA, 1034 for DIDP, 1035 for TPP, 828 for TDCPP, 753 for PCB153, 690 for PTU and 757 for U samples respectively. Biological replicates of each treatment had good reproducibility (Pearson correlation coefficient > 0.7) and a good overlap, > 50%. The effect of EDs treatment on the protein profile of the organoids is visible from the heat map representations which initially allow us to appreciate how the treated and untreated samples are divided into two clusters, provided the first spectrum of molecular effects of each of the EDs tested against U. Figure 5 shows as an example, the initial evaluation of MS-discovery data, (performed for each EDs tested), for BAA-treated samples: the multi scatter plot shows the good correlation and reproducibility between biological replicates as shown by the Pearson coefficient > 0.7 (Figure 5A) whereas Venn diagrams show good overlap that is also comparable between the three

BAA concentrations (Figure 5B); finally from the heat map, it is possible to appreciate how the untreated samples cluster apart from the BAA treated ones and how, based on the growth condition, it is possible to identify some protein clusters that are highly expressed (red), poorly expressed (green) or not detected (gray), (Figure 5C). This first data elaboration provides us with an overview of the possible up-down regulatory effects on the protein profile of organoids treated or not with EDs at the various concentrations; further evaluations are underway, in collaboration with the Statistics group of the University of Bologna (Italy, Dr Matteo Farnè and Prof. Angela Montanari) to compare, using the Kruskal-Wallis non-parametric test (under the possible hypothesis of non-normal Gaussian distribution of the data), the various samples protein profiles, and therefore to understand whether the effect of the ED is to significantly increase or decrease the biosynthesis of some proteins and significantly increase or decrease the catabolism of others. The next step will be to investigate which signal transduction systems these proteins belong to, to understand what type of physiological alteration the ED at a given concentration produces in the cellular physiology of the thyroid organ with the ultimate goal of discovering new protein biomarkers, indices of a given treatment.

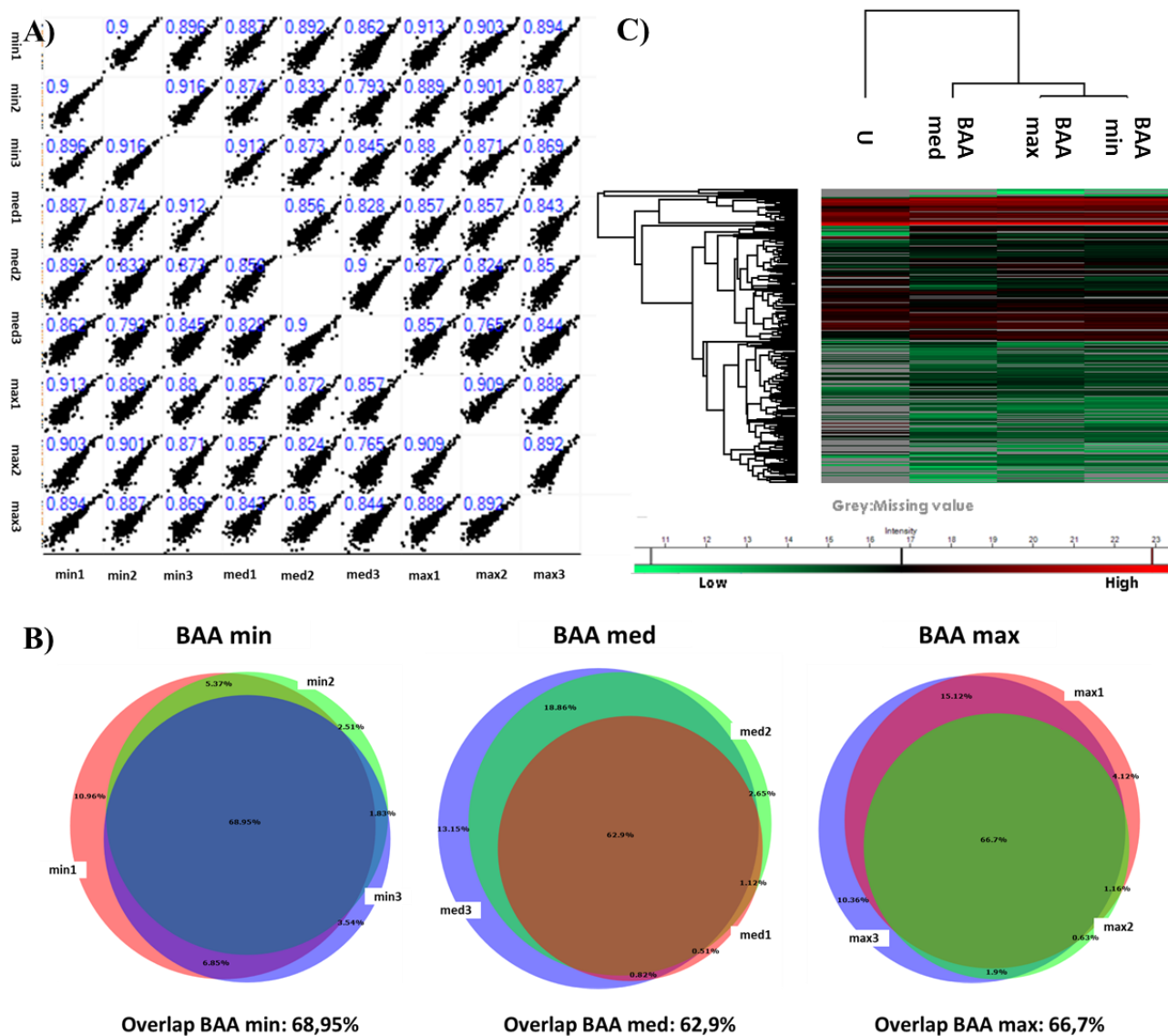


Figure 5: BAA treated 3D thyroid organoids data evaluation: A) Multi scatter plot and Pearson correlation of all BAA treated samples; B) Overlap between biological replicates (BAA min, BAA med, BAA max) expressed by Venn diagrams; C) Heat map of BAA treated samples vs U samples: each column represents a different sample (U, BAA min, BAA med, BAA max) and each row indicates a different protein; highly expressed proteins are in red, poorly expressed proteins are in green and not detected ones are in grey.

4. Conclusions

The Horizon 2020 European Project SCREENED focuses on the development of *in vitro* 3D assays to screen the effect of low dose of EDs on thyroid function. Here in this frame, two rat thyroid cell models, were successfully developed and characterized, overcoming current tests limitations, and

possibly avoiding sacrifice of additional experimental animals to obtain thyroid tissue for EDs *in vitro* tests, as recommended by current EU policies. The first model concerns FRTL-5 cells: these cells displayed a molecular panel highly comparable to that of native, primary adult rat thyrocytes as provided by targeted proteomics; due to their capacity to replicate, remain stable in standard 2D culture systems, and retain their thyroid-specific molecular signature through generations, they might be usefully exploited as a preliminary and robust cell model to test the effect of uptake of magnetized particles in thyroid epithelial cells. In fact, demonstration of their ability to be magnetized and guided onto a 3D collagen scaffold will represent a strong proof-of-concept for the eventual magnetization of stable and functional rodent thyroid follicles, to be used for colonization of either decellularized or engineered 3D matrices to be used as *in vitro* 3D assays.

The second cell model is based on 3D thyroid bioconstruct replicating the 3D organization of an adult, male rat thyroid lobe. To achieve this, adult male rat TSC induced to thyrocyte differentiation via an innovative and original Matrigel - transwell system, and a very specific rat thyroid differentiation medium, were adopted. This bioconstruct, submitted for registration to JRC, was used to set an innovative *in vitro* assay for screening the effect of low doses EDs on thyroid cell function. Proteomic profile of control and EDs-treated thyroid organoids was achieved by qualitative mass spectrometry obtaining the first spectrum of molecular effects of each of the tested EDs on male rat thyroid follicular cells, introducing groundwork for a new, rapid, and reproducible *in vitro* assay to test low doses EDs on thyroid cell function. Future statistical evaluation of the ensuing high dimensional data matrices will be performed with the final aim to find new protein biomarkers.

Authors' Contributions

G.R.: liquid-chromatography mass spectrometry, analytical studies and article writing. F.B. and G.C.: cell cultures, light and ultrastructural SEM investigations. R.T. and L.E.: research conceptualization, project administration, article supervision and revision.

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Supplementary Materials

Supplementary Table S1, Table S2, Table S3, Table S4

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Supplementary Materials

Table S1: Thyroid proteins SRM transitions with their m/z values selected for LC-MS/MS analysis of FRTL-5 vs PT. Sequences chosen for semiquantitative evaluation are in bold.

Protein	Peptide sequence	Precursor (m/z)	Product (m/z)
ABCG2	EILSDINGIMKPLNAILGPTGGGK	1233	1215
	EKPIIENLAEFYINSTIYGETK	1287	1289
	NDPTGMQNR	517	616
OCT3	GIALPETVEDVEK	700	355
	LGSSQLHQC[+57]GR	622	657
HNF4-A	EQLLVLEWAK	664	696
	SQVQVSLEDYINDR	833	871
	GQAATPETPQPSPPSGSGSESYK	1131	1280
GATA4	FSFPGTTGSLAAAAAAAAAAR	905	382
	MNGINRPLIKPQR	769	686
	LHGVPRPLAMR	624	407
TG	VIFDASSPVIVK	638	555
	AVSVPEDR	437	357
	NVFSPGPLIC[+57]NLESQR	916	448
SCA1	ALSAGLDYSPPSAPR	751	624
	SGLHLGKPGHR	580	790
	SEDEPPLTLPKPSLIPQEVK	1109	558
PAX8	LSIDSQSSSSGPR	661	905
	QHYPEAYASPSHTK	808	429
	ATLTPSNTPLGR	614	442
TSHR	FLGIFNTGLR	569	578
	ADLSYP SHC[+57]C[+57]AFK	778	1191
	HQAQAYQAQR	601	699
HHEX	TVNDYTHALLR	652	831

	QENPQSNK	473	372
	DELDSLDTSC[+57]DQGQDLPSEQNK	1248	702
FOXA1	DPSGPVNPSAESPIHR	830	357
	DPSGPVNPSAESPIHR	830	522
FOXA2	HSLSFNDFLK	604	870
SOX9	EAVSQVLK	437	614
	NGQAEAEATEQTHISPNAIFK	1193	689
	EGRPLAEGGR	521	343
HNF1-A	ELENLSPEEAHQA	798	686
	AVVESLLQEDPWR	771	970
NANOG	LSGPEADEGPEK	615	258
	TSNGLTQK	425	702
CXCR4	SSAQHALNSMSR	645	778
SOX17	LAQQNPDLHNAELSK	839	555
	VQHQMDHPNYK	699	739
	DGTEPNQPTTELLGEVDR	935	403
TTF1	FMGPASGMNMSGMGGLGSLGDVSK	1145	336
	NMAPLPAPR	527	527
	VAVPVLVK	413	270
CDH16	NVEELVGPGGPAATATVTILVER	1196	895
	ASVPALILASGPSR	718	749
	DLDQPADSVPLK	649	357
OCT4	VEPSPEESQDMK	688	413
	FEALQLSLK	525	789
	FEALQLSLK	525	701
HNF1-B	LSGDEGSEDGDDYDTPPILK	1062	1062
	EALVEEC[+57]NR	560	831
FGFR2	APYWTNTEK	555	733
	NQHWSLIMESVVP SDK	935	446
	LSSTADTPMLAGVSEYELPEDPK	1226	676
S10A6	DQEVNFQEYVAFLGALALIYNEALK	1430	1471
HSP27	EGVVEITGK	466	547
	AVTQSAEITIPVTFEAR	917	819

HSP60	TVIIEQSWGSPK	673	1032
	TVIIEQSWGSPK	673	771
	VGLQVVAVK	457	497
HSP70-1A	FELSGIPPAPR	592	744
	NALESYAFNMK	644	860
HSP90A	ADLINNLGTIAK	622	641
	TDTGEPMGR	482	460
TRPA1	FFHYFLSMQETR	803	864
	WNFVLNAVK	546	660
STIP1	LDPQNHVLYSNR	728	888
	HEANNLQLK	534	729
	HDSPEDVK	464	340
NIS	INLMDFDPDPR	667	484
	INLMDFDPDPR	667	734
TPO	STLLITER	467	631
	AEVQDAEQEPAYGSR	825	871
	AEVQDAEQEPAYGSR	825	650
PENDRIN	NLEEPEGVK	508	529
	NLEEPEGVK	508	486
	FSSPIFYGNVDGFK	789	322
DUOX2	EGSSSSPALR	496	456
	LMFTMYDLGNGFLSK	926	1066
	VELLPSTVYLQFQRPK	988	1060
TAAR1	ANLQVGLEGESR	637	846
	ANLQVGLEGESR	637	696
E-CADHERIN	SLGHHHR	491	586
	DTGVISVVTSLDR	710	934
	GLDARPEVIR	563	513
CRB3	QTEGTYRPSSEEQFSHAAAEAR	1227	836
	QTEGTYRPSSEEQFSHAAAEAR	1227	1237
SNAI	VSPLPSSDTSSK	603	783
	LSDPHAIEAEK	605	316
	HEESGC[+57]C[+57]VAH	593	860

TTF2	GKPPYSYIALIAMAIAHAPER	1135	401
	GNYWALDPNAEDMFESGSFLR	1210	1318
	HATAYPGAVDR	579	614
SOX2	SEASSSPVVTSSSHSR	851	549
	APCQAGDLR	494	585
MCT8	LVSGHISDSIPGLK	712	930
	LVSGHISDSIPGLK	712	896

Table S2: Rat and bovine thyroglobulin SRM transitions with their m/z values selected for LC-MS/MS analysis in FRTL-5 samples. Sequences chosen for thyroglobulin quantification in FRTL-5 sample are in bold.

Organism	Peptide sequence	Precursor (m/z)	Product (m/z)
Rat	VIFDASSPVIVK	638	555
	AVSVPEDR	437	357
	NVFSPGPLIC[+57]NLESQR	916	448
Bovine	WEAQNSAGQALTPAELLMK	1030	1043
	WEAQNSAGQALTPAELLMK	1030	1215
	EAAEIVTYSN SSR	743	913
	FLSGSDYAIR	565	637
	APEFAAPWPDFVPR	800	587
	APEFAAPWPDFVPR	800	916

Table S3: Thyroid hormones SRM transitions with their m/z values, declustering potential (DP) and collision energy (CE) selected for LC-MS/MS analysis in FRTL-5 samples. Sequences chosen for quantitative evaluation are in bold.

Analyte	Precursor (m/z)	Product (m/z)	DP (V)	CE (V)
T ₃	652	197	103	89
	652	479	103	47
	652	508	103	29
	652	606	103	29

T ₄	778	351	106	62
	778	605	106	51
	778	634	106	34
	778	732	106	33
¹³ C ₆ -T ₃	658	203	103	89
	658	485	103	47
	658	514	103	29
	658	612	103	29
¹³ C ₆ -T ₄	784	357	106	62
	784	611	106	51
	784	640	106	34
	784	738	106	33

Table S4: Proteins SRM transitions with their m/z values selected for LC-MS/MS analysis of TSC/P-c vs PT. Sequences chosen for semiquantitative evaluation are in bold.

Protein	Peptide sequence	Precursor (m/z)	Product (m/z)
ABCG2	TLAEGDVLSFHHITYR	930	1308
	EILSDINGIMKPGLNAILGPTGGGK	1233	516
	GLSGDVLINGAPQPANFK	899	576
	LMFHGPAQK	515	529
OCT3	GIALPETVEDVEK	700	355
	LGSSQLHQC[+57]GR	622	657
	TQVSTSNV	418	507
HNF4-A	EQLLVLEWAK	664	696
	ILDELVLFPQELQIDDNEYAC[+57]LK	1390	1411
	LLPGAITTIVKPPSAIPQPTITK	1179	1108
GATA4	FSFPGTTGSLAAAAAAAAAAR	905	995
	FSFPGTTGSLAAAAAAAAAAR	905	1428
	MNGINRPLIKPQR	769	686
SCA1	ATALPSDNHC[+57]VEGVAWLPSTPGSR	1262	357

	ALSAGLDYSPPSAPR	751	624
	ALSAGLDYSPPSAPR	751	527
	SVHPYESR	536	421
	GSVMVLPNSSSTPSADLETQQATHR	1264	587
	GSVMVLPNSSSTPSADLETQQATHR	1264	1074
	SGLHLGKPGHR	580	693
	SGLHLGKPGHR	580	790
	SGLHLGKPGHR	580	466
	GQPVPDASALLK	598	497
	GQPVPDASALLK	598	699
	IEPSKPTATR	550	555
	IEPSKPTATR	550	673
	IEPSKPTATR	550	545
PAX8	IVDLAHQGVRPC[+57]DISR	918	934
	LSIDSQSSSSGPR	661	731
	LSIDSQSSSSGPR	661	905
	LSIDSQSSSSGPR	661	805
	QHYPEAYASPSHTK	808	429
	ATLTPSNTPLGR	614	387
	ATLTPSNTPLGR	614	657
TSHR	TIPSLAFSSLPNISR	802	817
	LDAVYLNK	468	562
	ADLSYPSHC[+57]C[+57]AFK	778	550
	HQAQAYQAQR	601	699
	ISEEYTQTAL	578	696
HHEX	HDPLGKPLLWNPFLQRPLHK	1204	1272
	FSNDQTIELEK	662	693
	QENPQSNK	473	372
	DELDSLDTSC[+57]DQGQDLPSEQNK	1248	702
FOXA1	QPGAGGGSGGGGSK	537	612
	DPSGPVNPSAESPIHR	830	357
	DPSGPVNPSAESPIHR	830	667
	DPSGPVNPSAESPIHR	830	522
FOXA2	HLSFNDFLK	604	870

SOX9	EAAGAGSGGGK	431	544
	EAVSQVLK	437	614
	NGQAEAEATEQTHISPNAIFK	1193	689
	EGRPLAEGGR	521	343
HNF1-A	GESC[+57]GGTR	412	766
	ELENLSPEEAHQK	798	996
	AVVESLLQEDPWR	771	840
NANOG	LSGPEADEGPEK	615	258
	LSGPEADEGPEK	615	670
	LSGPEADEGPEK	615	745
	TVFSQAQLC[+57]ALK	683	803
	TSNGLTQK	425	489
CXCR4	YLAIVHATNSQRPR	813	984
	SSAQHALNSMSR	645	695
	SSAQHALNSMSR	645	809
SOX17	GEVVASSGAPAGTSSR	717	926
	LAQQNPDLHNAELSK	839	555
	DGTEPNQPTTELLGEVDR	935	403
TTF1	VGMEGGGLGAPLAAYR	760	926
	NSASGPGWYGANPDPR	823	484
	FMGPASGMNMSGMGGLGSLGDVSK	1145	336
	NMAPLPSAPR	527	527
CDH16	IHVTMELDR	557	582
	ESDSNHVQLR	593	670
	ESDSNHVQLR	593	766
	ASVPALILASGPSR	718	749
	NGPYSEFALGNPTVQR	859	600
	DLDQPADSVPLK	649	842
	DLDQPADSVPLK	649	357
OCT4	VEPSPEESQDMK	688	413
	FEALQLSLK	525	789
	WVEEADNNENLQEIC[+57]K	996	1201
	SETLVQAR	452	658
HNF1-B	LSGDEGSEDGDDYDTPPILK	1062	1062

	LSGDEGSEDGDDYDTPPILK	1062	567
	MLSEDPWR	517	576
	EALVEEC[+57]NR	560	671
FGFR2	APYWTNTEK	555	733
	APYWTNTEK	555	834
	YGPDGLPYLK	562	520
	LSSTADTPMLAGVSEYELPEDPK	1226	676
	DINNIDYYK	579	848
	MDKPTNC[+57]TNELYMMMR	1018	375
HSP27	VSLDVNHFapeeltvk	899	983
	EGVVEITGK	466	547
	QDEHGYISR	553	567
HSP60	TVIIQSWGSPK	673	684
	TVIIQSWGSPK	673	771
	GANPVEIR	428	243
	GYISPYFINTSK	695	421
	VGLQVVAVK	457	497
HSP70-1A	FGDPVVQSDMK	612	320
	FELSGIPPAPR	592	440
	NALESYAFNMK	644	860
HSP90A	ELHINLIPNK	596	358
	ADLINNLGTIAK	622	641
	TDTGEPMGR	482	601
	TDTGEPMGR	482	460
	ESDDKPEIEDVGSDEEEEEK	1154	1158
	NPDDITNEEYGEFYK	917	935
	LGIHEDSQNR	585	665
TRPA1	FLLSQGANPNLR	665	717
	SPLILATASASWNIVNLLLSK	1106	1199
	AVAMLLSYNADILLNK	875	1151
	INTLNSFPLK	574	790
STIP1	HEANNLQLK	534	729
	AAALEAMK	403	456
	HDSPEDVK	464	340

	LILEQMQK	502	597
	LMDVGLIAIR	551	585

CHAPTER III: Decellularization Detergents As Methodological Variables in Mass Spectrometry of Stromal Matrices

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Personal Contribution

G.R. contributed to mass spectrometry and analytical studies and article writing.

Abstract

Collagens, elastin, fibrillin, decorin, and laminin are key constituents of the extracellular matrix and basement membrane of mammalian organs. Thus, changes in their quantities may influence the mechanochemical regulation of resident cells. Since maintenance of a native stromal composition is a requirement for three-dimensional (3D) matrix-based recellularization techniques in tissue engineering, we studied the influence of the decellularization detergents on these proteins in porcine kidney, liver, pancreas, and skin. Using a quick thawing/quick microwave-assisted decellularization protocol and two different detergents, sodium dodecyl sulfate (SDS) vs Triton X-100 (TX100), at identical concentration, variations in matrix conservation of stromal proteins were detected by liquid chromatography–mass spectrometry coupled to light and scanning electron microscopies, in dependence on each detergent. In all organs tested except pancreas, collagens were retained to a statistically significant level using the TX100-based protocol. In contrast fibrillin, elastin (except in kidney), and decorin (only in liver) were better preserved with the SDS-dependent protocol. Irrespective of the detergent used, laminin always remained at an irrelevant level. Our results prompt attention to the type of detergent in organ decellularization, suggesting that its choice may influence morphoregulatory inputs peculiar to the type of 3D bioartificial mammalian organ to be reconstructed.

Keywords: mass spectrometry, collagen, elastin, fibrillin, decorin, laminin

Impact Statement

Simple change of the protocol's main detergent leads to a very substantial difference in the panel of the stromal proteins detected by qualitative and semiquantitative mass spectrometry in acellular porcine matrices. This remarkable methodological variable promises to yield proteomic reference panels in a number of different species-specific acellular matrices allowing for selective retainment of peculiar mechanochemical inputs, to differently address the development of the seeded cells in relation to the type of organ to be bioartificially reconstructed.

1. Introduction

In mammals fibrous and nonfibrous proteins, proteoglycans and glycosaminoglycans contribute to the formation of the interstitial and basement membrane (BM) extracellular matrix (ECM) or stromal matrix (SM) of their organs [1]. The SM plays a key role in regulating development, growth, and function of resident cells through mechanochemical interactions [2,3] whose those mediated by collagens, elastin, fibrillin, decorin, and laminin are of fundamental importance. Indeed, fibrillary collagens provide the primary three-dimensional (3D) ECM scaffold to each tissue/organ ensuring its 3D stability, resistance to deformation, and plasticity [4]. Similarly, elastin contributes to ECM resilience and elasticity using as a template the glycoprotein fibrillin, the latter acting as a sheath to the developing central core of amorphous elastin fibers [5,6]. In contrast, the proteoglycan decorin resides in the interstitial ECM where it functions as a bridge between the microfibrillar interstitial proteins and membrane receptors/adhesion molecules such as integrins to stabilize cells in their 3D matrix environment, and prevent their neoplastic transformation [7]. Similarly, laminin is a trimeric protein of the BM that anchors the SM to the cells, and controls cell differentiation including that of stem cells leaving their niche during regeneration processes [8]. Collectively, the ECM molecules give rise to a 3D microfibrillar network that provides an informational backbone for cells, an essential requirement for tissue engineering approaches based on decellularized stromal matrices eventually recellularized to give rise to functional bioartificial organs [3,9,10]. However, in the context of the decellularization methodologies, a very critical step resides in the chemical treatment used to remove cytoplasmic and nuclear material without adversely affecting composition, biological activity, and 3D macroscopic and microscopic architecture of the ECM [2,3,11-14]. A number of chemical treatments have been proposed^{1,15} among which the detergents sodium dodecyl sulfate (SDS) and Triton X-100 (TX100) have gained widespread acceptance despite their cytotoxicity. In fact efficient removal of chemicals from the SM becomes mandatory to favor adequate recellularization of the intact 3D stroma. Therefore, choice of washing steps like those based on microwave irradiation may account for reliable cleaning of the 3D SM within a limited procedural time [16]. Finally accurate quantification of collagens, elastin, fibrillin, decorin, and laminin of the SM is very desirable to gain insights into how different amounts of specific ECM proteins act as a guide to induce in vitro/ex situ 3D selfassembly of seeded cells during recellularization [17-18]. To this aim, a number of different liquid chromatography/mass spectrometry (LC-MS/MS) techniques have been successfully used to quantify ECM proteins [19-23]. Therefore, starting from the same thawing-based/ microwave-assisted protocol, we have studied the effect of an SDS-dependent treatment with respect to a TX100-dependent treatment on the LC-MS/MS detection of collagens, elastin, fibrillin, decorin, and laminin in the decellularized SM of porcine kidney, liver, pancreas, and skin.

2. Methods

2.1. Reagents

SDS was from Lisapharma S.p.A (Como, Italy). TX100, sodium hydroxide (NaOH), ethylene diamino tetraacetic acid (EDTA), 2-propanol and Tris-HCl, trypsin (p.n. T1426), pepstatin A, proteinase K (p.n. P2308), iodoacetamide (IAA), dithiotreitol (DTT), HEPES, trifluoroacetic acid (TFA), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were all purchased from Sigma-Aldrich (Darmstadt, Germany). Protein assay dye reagent concentrate was from Bio-Rad. LC-grade water, acetic acid, acetonitrile, analytical reagent grade formic acid (FA), urea, and ammonium bicarbonate (ABC) were purchased from Carlo Erba (Milan, Italy).

2.2. Porcine tissues

Fresh porcine tissues were taken from a slaughterhouse, cut into pieces of appropriate size, and stored at -20°C until use. Microwave-assisted decellularization Tissues were let to quickly thaw at room temperature (RT), sliced into 2 mm thick pieces, washed for 3 h in distilled water, and immersed in either Solution 1 (Sol 1) made with SDS (1%, w/v)—NaOH (0.05%, v/v) or Solution 2 (Sol 2) made with TX100 (1%, v/v) NaOH (0.05%, v/v). All solutions were kept at 37°C. Then, using a microwave oven (Sharp®), tissues were treated as given in Table 1. Before immersion in the decellularization solutions (Sol 1 or Sol 2), skin was pretreated by incubation in a 70% solution of 2-propanol and microwaved with 20 pulses of 5 s each pulse. Finally, all samples were rinsed in distilled water for 4 h, and subjected to 560 W microwave pulses (10 pulses, 5 s each; dwell time: 1 min) until complete detergent removal.

2.3. Light microscopy

Decellularized matrices and quickly RT-thawed control tissues were fixed by immersion in 4% paraformaldehyde– phosphate buffered saline (PBS) for 24 h at 4°C, paraffin embedded, cut at the microtome (8 µm sections), stained with hematoxylin-eosin (HE), and analyzed using a Zeiss Axiophot light microscope under differential interference contrast (Nomarsky optic), and an AxioVision 4.8 image analysis software.

2.4. Scanning electron microscopy

Decellularized matrices were fixed in glutaraldehyde 2.5%– cacodylate sodium buffer 0.1 M overnight at 4°C, and rinsed in PBS (0.1 M, pH 7.4). Two different though complementary scanning electron microscopy (SEM) modalities were used, in dependence on the type of decellularization (i.e., type of detergent used):

- with Sol 1, samples were dehydrated in graded series of ethanol, and subjected to critical point drying (Balzers) in a 100% ethanol/liquid CO₂ exchange system. Dried samples were mounted on aluminum stubs, metallized (Balzers apparatus) with a gold film, and observed with a Philips SEM 501.

- with Sol 2, samples were subjected to freeze drying for 24 h using a Christ Alpha 2-4 LSC plus freeze dryer. Dried samples were mounted on aluminum stubs, metallized (Balzers apparatus) with a gold film, and observed with a Zeiss Gemini SEM. For each sample, both the surface and cross-section were studied. Representative images were analyzed using an ImageJ software (NIH, Bethesda, MD), and random measurements of putative blood vessel and corpuscular annexa were taken.

Table 1. Scheme of the Number of Microwave Pulses Performed for Each Tissue

<i>Tissue</i>	<i>Sol 1—SDS</i>	<i>Sol 2—TX100</i>
	<i>No. of pulses^a</i>	
Kidney	100	190
Liver	200	200
Pancreas	150	130
Skin	180	220

Microwave power 560 W.

^aPulse duration: 5 s; dwell time: 1 min.

2.5. Analysis of the residual DNA content

Decellularized matrices and quickly RT-thawed control tissues were weighted and digested in 1 mL of lysis solution (LS) at 56°C for 48 h. The LS was composed of 1 mg/mL proteinase K in 50 mM Tris-HCl pH 7.6 and a protease inhibitor cocktail, made with 1 mM EDTA +1 mM IAA +10 µg/mL Pepstatin A diluted in DMSO. For DNA measurements, the CyQUANT® Cell Proliferation Assay kit (C7026; Invitrogen) was used. A calibration curve was prepared in a 96-multiwell plate using 200 µL serial dilutions (0, 62.5, 125, 250, 500, 1000 ng/mL) of the bacteriophage λ DNA in CyQUANT dye solution. The latter was prepared by mixing the working buffer with the CyQUANT GR dye 400 supplied with the kit. For analysis, 5 µL of each digested sample (intact control and decellularized tissues) was dissolved in 195 µL of CyQUANT dye solution, and three replicates were prepared for each sample. Analyses of the calibration curve and samples were performed using a Victor 3V Multilabel Plate Readers (Perkin Elmer) set at the Fluorescein protocol (485/ 535 nm of wavelength—l). The calibration curve was $y = 2296 (-94) x + 135,974 (-44,000)$ with an $R^2 = 0.993$. For each sample, the amount of DNA was normalized to the dilution factor and wet weight of the tissues and expressed as ng DNA/mg wet tissue.

2.6. Protein extraction and sample preparation for mass spectrometry analysis

One milligram wet weight of each decellularized sample was solubilized in a solution of 8 M urea–10 mM HEPES, immersed in an ultrasonic bath at 37°C for 3 h using a Branson ultrasonic cleaner 2510E-DTH, and sonicated at 42 kHz to break aggregates and extract proteins. Buffer exchange was performed using 50 mM ABC in Amicon Ultra 0.5 mL centrifugal filters (Sigma-Aldrich) with a 3-kDa molecular weight cut-off. Ensuing protein extracts, then, were assayed for protein dosage using the Bradford method, based on adding 10 µL of each sample to 500 µL of a previously diluted (1:5) Bradford reagent solution (absorbance = 595 nm). Calibration curve was achieved using a BSA standard (1 mg/mL) diluted to a range of 10–400 µg/mL ($y = 0.00108x$; $R^2 = 0.992$). Subsequently, proteins were reduced using 10 mM DTT (30 min at 37°C), alkylated by 25 mM IAA (30 min at RT in the dark), and the reaction was stopped by adding 10 mM DTT (15 min at RT). All samples were digested by incubation with trypsin (1:50 enzyme/substrate ratio) at 37°C overnight and, the digestion was stopped with 1 µL of FA added to each sample. Finally, samples were dried under nitrogen flow and reconstituted with 50 µL of a water–acetonitrile–FA aqueous solution (49.95/49.95/0.1, v/v). Each matrix sample was analyzed in triplicate.

2.7. LC-LIT-Orbitrap XL analysis

Tryptic separation of the digested proteins was carried out by using an XB C18 AERIS PEPTIDE (150 · 2.1 mm, 5 µm) column (Phenomenex, Torrance, CA) equipped with a prefiltering column. Eluting mixtures included solvents A (0.1% aqueous FA, v/v) and B (0.05% FA, 100% acetonitrile, v/v) delivered under gradient elution at the flow rate of 200 µL/ min. Gradient was set as follows: 0 min 2% solvent B, 4 min 2% solvent B, 150 min 90% solvent B, 155 min 90% solvent B, and 170 min 2% solvent B. The mobile phase was delivered by a Dionex Ultimate 3000 chromatographic system (Dionex Corporation, San Jose', CA) equipped with a 100-vial capacity sample tray. The volume of the injected samples was 5 µL. Qualitative protein analysis was obtained by mass spectrometry using an LTQ linear ion trap-Orbitrap XL instrument (ThermoScientific Corporation, San Jose', CA), equipped with ESI (ionization electrospray source) interface and controlled by Xcalibur software. Optimized conditions of the interface were as follows: ESI voltage 3 kV, capillary voltage 13 V, capillary temperature 275° C, tube lens 100 V, sheath gas flow, auxiliary gas flow, and sweep gas flow delivered at 40, 10, and 5 arbitrary units, respectively. In the first scan event (full scan), the m/z window was 200–2000 with a resolution of 30,000. The four highest m/z ratios over threshold of 1000 counts were selected for collision-induced dissociation in the ion trap, with normalized collision energy of 35% in the collision cell.

2.8. LC-MS/MS SRM analysis

Relative quantitative analysis of SM proteins was carried out by LC-selected reaction monitoring (SRM)-MS/MS targeted analysis. Each SM tryptic solution was eluted by using a C18 Gemini (250 · 2.00 mm, 3 µm) column (Phenomenex) equipped with a C18 prefiltering column. An elution system based on a solvent gradient (solution A: 0.1% aqueous FA [v/v]/solution B: 0.08% FA in acetonitrile [v/v]) was delivered at 0.2 mL/min. The gradient elution program was as that described in the previous section. The mobile phase was delivered by the Agilent HP 1200 chromatographic system (Agilent Technologies) equipped with a 100- vial capacity sample tray. Injection volume was 10 µL. A QTRAP 4000 triple quadrupole mass spectrometer (ABSCIEX, Foster City, CA), equipped with a pneumatically assisted ESI interface, was used. The sheath gas (nitrogen, 99.999% purity) and the auxiliary gas (nitrogen, 99.998% purity) were delivered at flow rates of 45 and 5 arbitrary units, respectively. Optimized conditions of the interface were as follows: ESI voltage 5.5 kV, capillary voltage 50 V, and capillary temperature 350°C. Experiments were performed under positive ion-SRM conditions using nitrogen as collision gas (pressure of $2.1 \cdot 10^{-3}$ mbar) and a 20 ms-dual time for each transition monitored.

2.9. Data analysis, bioinformatics, and quantitative evaluation of ECM peptides

LC-LIT-Orbitrap XL data were processed using PEAKS X+ software (Bioinformatics Solutions, Inc., Waterloo, Canada) and Uniprot as protein database (www.uniprot.org, protein sequence database; suscrofa) setting trypsin as digestion mode with three as maximum number of post-translational modifications per peptide, no limit to possible modifications and false discovery rate active. For SRM targeted analysis, in silico evaluation of protein sequences was performed as follows: FASTA protein sequences were obtained from Uniprot Porcine proteome database, and SRM transitions for each protein were simulated by Skyline (v. 20.1; SCIEX, Redwood City, CA), setting trypsin as digestion mode with no missed cleavage and carbamidomethylation of cysteines as structural modification. Uniqueness of candidate peptide sequences was assessed by BLASTp tool (basic local alignment search tool; www.ncbi.nlm.nih.gov NCBI BLAST) search (algorithm: blastp; ATRIX PA 30; GAP COASTS: existence 10, extension 1; DATABASE: nonredundant protein sequences) from NCBI (National Center for Biotechnology Information, Bethesda, MA). LC-MS/MS SRM data were analyzed using the Analyst v 1.4 software, and integrations of the peak areas were obtained by the MultiQuant program (version 2.1; ABSCIEX). All samples were processed with the same extraction protocol resulting in the final clean tryptic digests ready for LC-MS/MS analysis. Relative quantitation of SM peptides was achieved through normalization of data with respect to total protein content measured for each sample by Bradford analysis before enzymatic digestion and expressed as

the value of the area (intensity of ionic current or cps/min) under each MS peak. This unit of measurement was reported as “scientific notation” or E series.

2.10. Statistical analysis

Differences in ng DNA/mg wet weight between controls and decellularization protocols were calculated after an initial estimate of the sample dry weight, based on the assumption of a 60% decrease in the experimentally recorded weight, as currently accepted. Then using a 2 · 2 chi squared test, ng DNA/mg dry weight above or below a threshold recommended as ideal for decellularization¹⁵ was compared. In contrast, differences in the quantity of cumulated SM collagens and proteins, and selective SM elastin, fibrillin, decorin, and laminin sequences between decellularization protocols were determined using one-way analysis of variance. All results were considered significant if $p < 0.05$.

3. Experiment

Both control and treated tissue samples were subjected to the methods previously detailed. Figure 1 shows that irrespective of the decellularization protocol, all treated samples resulted devoid of visible cellular material as opposed to control tissues retaining the native parenchymal and vascular structure. In intact kidney, renal glomerula were surrounded by convoluted tubules as compared with the absence of any recognizable cellular structure in decellularized matrices, consisting of a fibrous stromal network. Similarly, in intact liver, blood vessels were surrounded by parenchymal trusses as compared with the absence of any recognizable cellular structure in the decellularized matrices, depicting the stromal scaffold of cellular cords and vessels. In control pancreas, cords of exocrine pancreatic cells were well preserved as compared with decellularized matrices, retaining the tissue stromal structure including that of blood vessels. Finally, as in the control skin, the dermal structures were readily recognized in decellularized tissues, that exhibited a dermal fibrous structure and walls of blood vessels. Figure 2 shows the ultrastructural appearance of decellularized tissue matrices. Irrespective of the decellularization protocol applied and SEM technology used, all decellularized tissues depicted a well-preserved 3D stromal architecture devoid of any visible cellular residue, while retaining stromal septa and spaces of presumptive vascular conduits and corpuscular annexa. Indeed, in all types of matrix, random linear measurements revealed open lumina (30– 200 μm in diameter), anatomically compatible with either small blood vessels and their curling up (kidney, liver, pancreas) or cavities of annexa like hair follicles (skin). Figure 3 shows that the majority of decellularized tissues reached a statistically significant reduction in residual DNA with respect to the already low level of DNA in control tissues, the latter as an expected result of the quick defrost of the frozen

samples. Indeed, the thawing procedure was per se able to favor abundant detachment of cells by the SM, up to reduce tissue DNA below the ideal threshold of 50 ng DNA/mg dry weight (corresponding to 41.5 ng DNA/mg wet weight) in control kidney and pancreas. However, using the SDS-based protocol (Sol 1), a statistically significant reduction of matrix DNA was achieved in kidney, liver, and skin with respect to controls. In contrast, using the TX100-based protocol (Sol 2), a statistically significant reduction of matrix DNA was achieved in pancreas and skin. Supplementary Table S1 shows that using LC-LIT Orbitrap XL analysis, collagens types I–VI, XII, XIV and laminin subunits alpha 5, beta 1, and beta 2 were readily identified. Among different SM, a peculiar distribution of these molecules was observed, variable in dependence on the organ analyzed and within the same type of organ related to the detergent used for decellularization. However, irrespective of the decellularization detergent, sharing of identical molecules between organs accounted for only 3.7% of the total detected proteins, highlighting conservation of the structural uniqueness of each SM with respect to its native tissue. Supplementary Table S2 highlights in bold the peptide sequences tested using LC-MS/MS SRM analysis. Among the numerous available, only those having the best characterized chromatographic peaks were selected for quantification and comparison between decellularization procedures. Figure 4 shows that in all matrices except pancreas, a higher statistically significant collagen retention was observed with Sol 2 as opposed to Sol 1. In contrast, Figure 5 shows the semiquantitative cumulated results of all subunit/ isoforms of the stromal (SM) proteins, in all matrices. In particular, a statistically significant higher amount of these SM proteins was retained using Sol 1 as opposed to Sol 2. Finally, Figure 6 shows that in all organ matrices, a statistically significant higher amount of fibrillin was retained using Sol 1; similarly in liver, pancreas, and skin, also elastin (isoforms X1–X26) was equally preserved at a higher statistically significant level using Sol 1. However, only in liver, decorin remained at a higher statistically significant level using Sol 1. At variance, laminin (subunit alpha 5 isoforms X1 and X2) was never retained to either a statistically significant level or even to a simply detectable level in any of the tested matrices and irrespective of the protocol used.

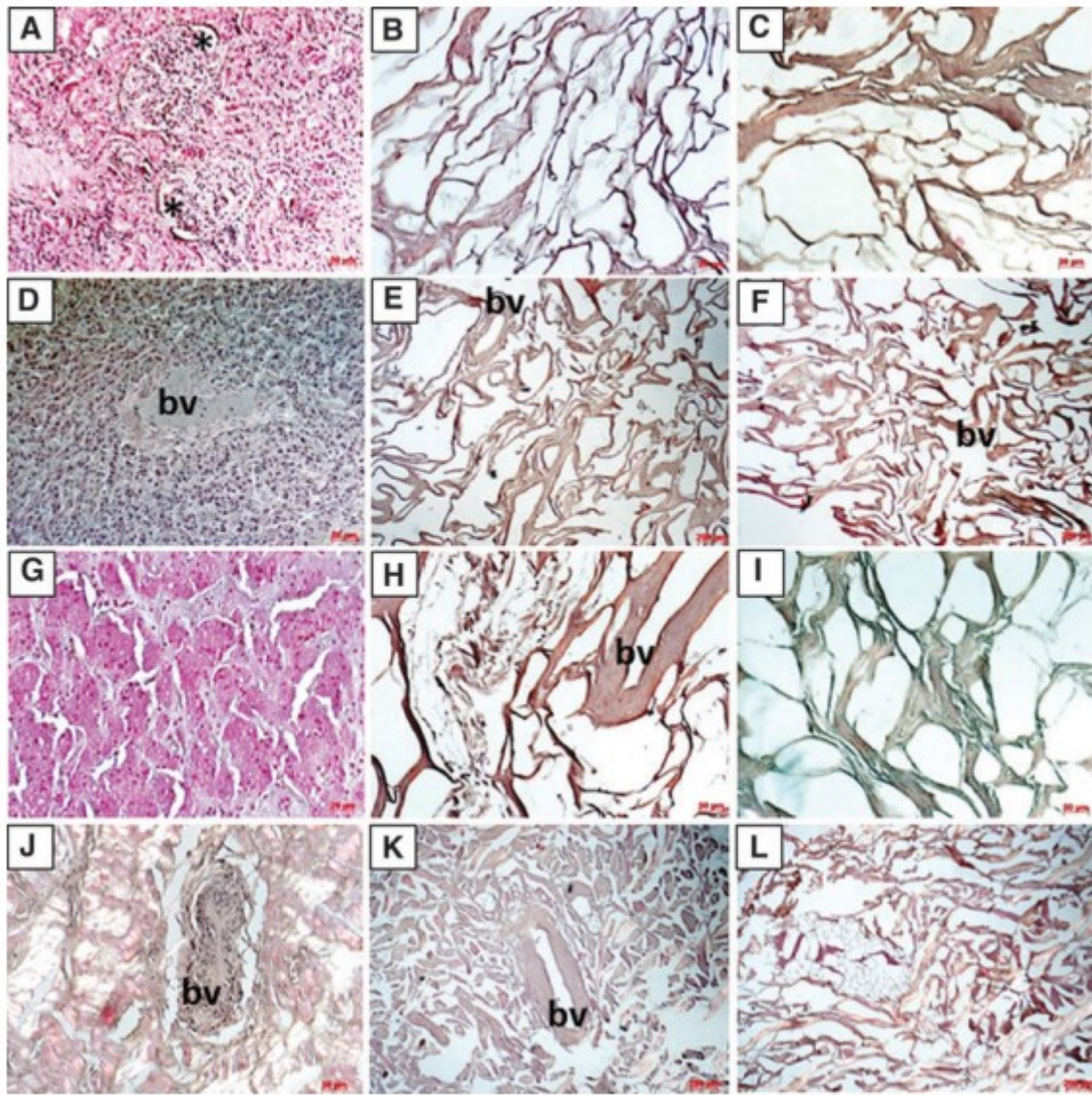


FIG. 1. Light microscopic evidence for removal of cellular debris in decellularized matrices: (A) control kidney (asterisk shows a glomerulus), (B, C) decellularized kidney treated with Sol 1 (B) and Sol 2 (C). (D) Control liver, (E, F) decellularized liver treated with Sol 1 (E) and Sol 2 (F); (G) control pancreas, (H, I) decellularized pancreas treated with Sol 1 (H) and Sol 2 (I); (J) control skin, (K, L) decellularized skin treated with Sol 1 (K) and Sol 2 (L). Red size markers: (A, C, D, H, I, J) = 150 μm ; (B, G) = 50 μm ; (E, F, K, L) = 400 μm . Each image refers to a prototypical tissue sample. bv, blood vessels; Sol 1, Solution 1; Sol 2, Solution 2.

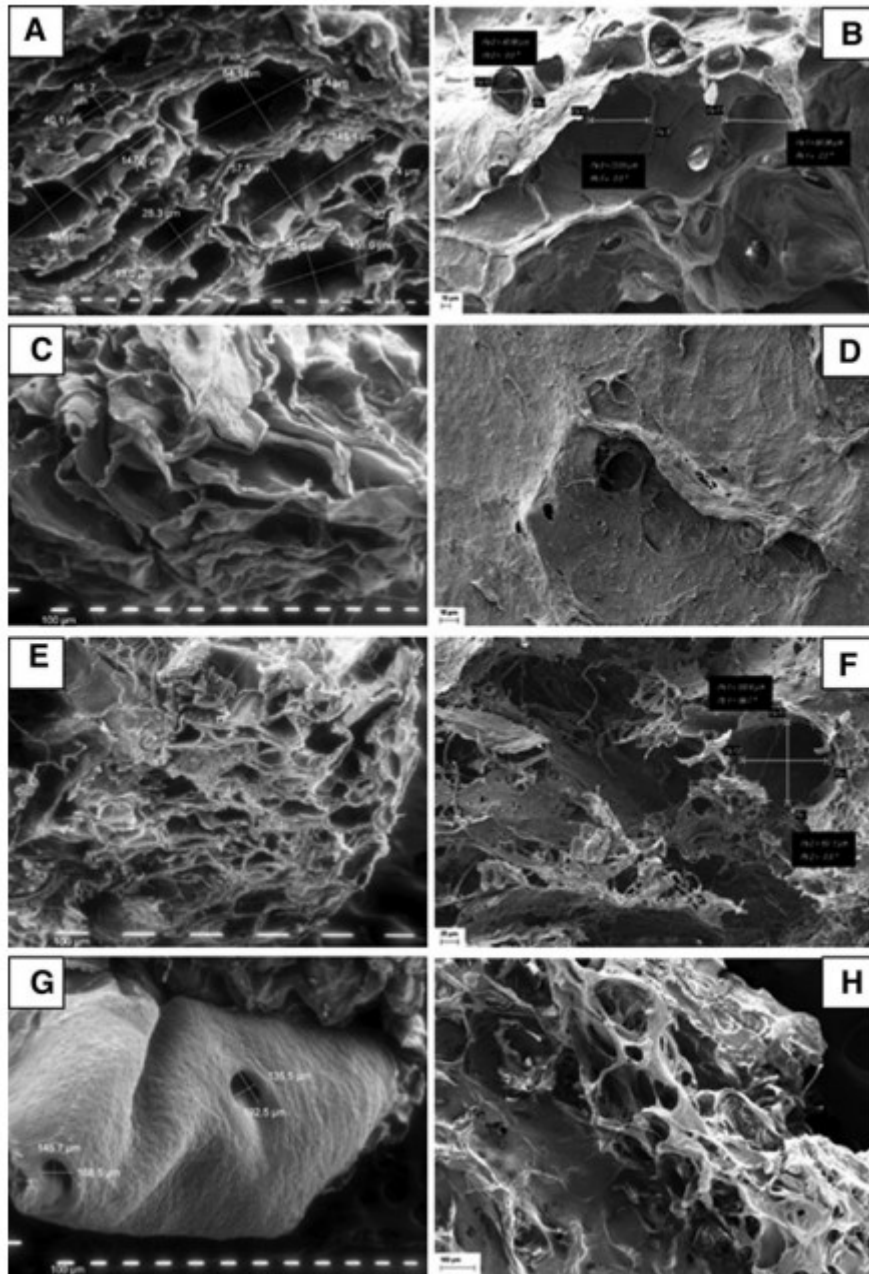


FIG. 2. SEM evidence for removal of cellular debris in decellularized matrices: (A) kidney cross section treated with Solution 1—Sol 1; (B) kidney cross section treated with Sol 2; (C) liver cross section treated with Sol 1; (D) liver surface treated with Sol 2; (E) pancreas cross section treated with Sol 1; (F) pancreas cross section treated with Sol 2; (G) skin surface treated with Sol 1; (H) skin cross section treated with Sol 2. Size markers: (A, C, E, G) = 100 μm ; (B) = 25 μm ; (D) = 15 μm ; (F) = 25 μm ; (H) = 100 μm . Each image refers to a prototypical tissue sample. SEM, scanning electron microscopy.

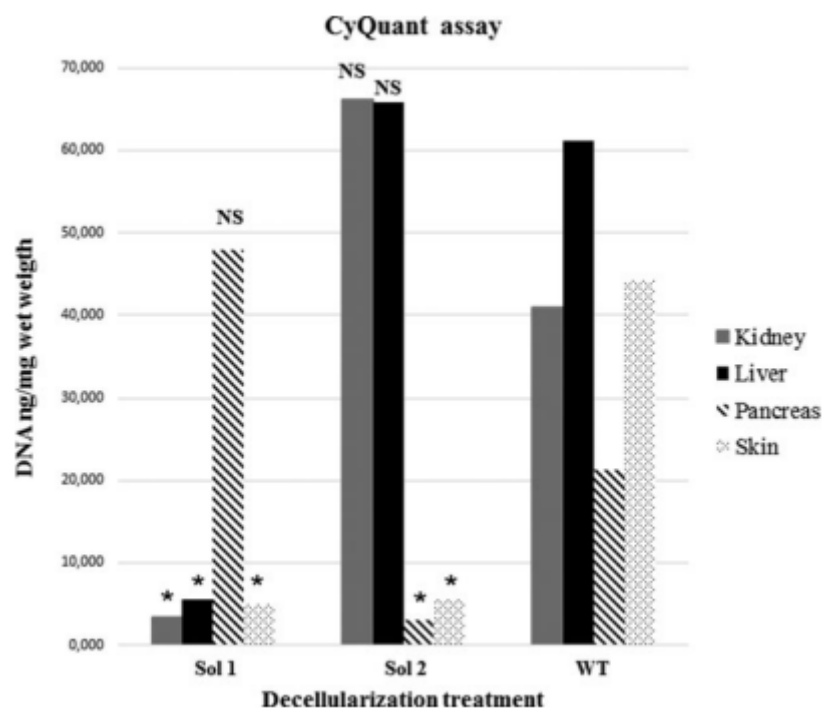


FIG. 3. Distribution of residual nuclear material (DNA) in decellularized matrices as compared with quickly RT-thawed control tissues. Each bar represents a single representative tissue sample, thus no error bar is depicted. * $p < 0.05$; NS, not statistically significant; RT, room temperature.

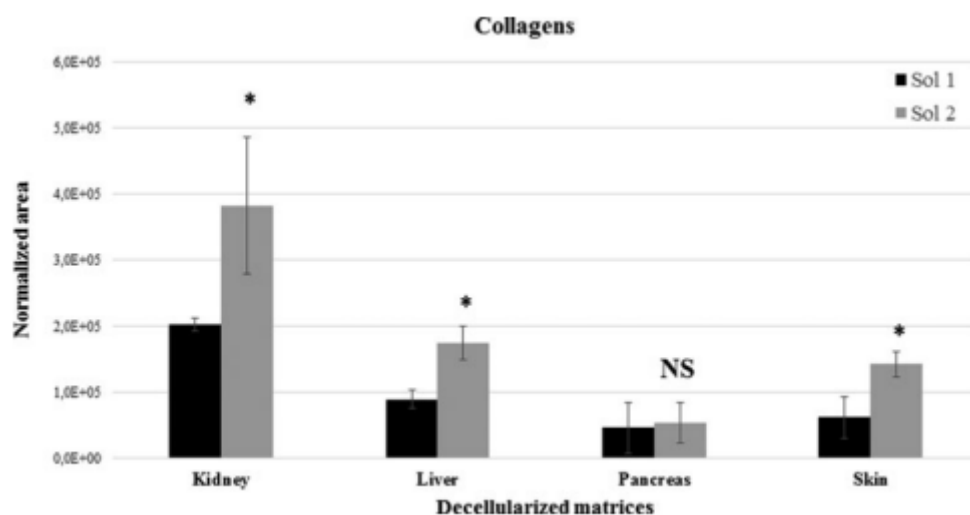


FIG. 4. Cumulated distribution of collagens types I–VI (including their isoforms) in each decellularized tissue using Sol 1 (SDS-based protocol) versus Sol 2 (TX100-based protocol), as determined by LC-MS/MS SRM analysis. * $p < 0.05$; data are the mean – SD of three independent samples. LC-MS/MS, liquid chromatography/mass spectrometry; SD, standard deviation; SDS, sodium dodecyl sulfate; TX100, Triton X-100.

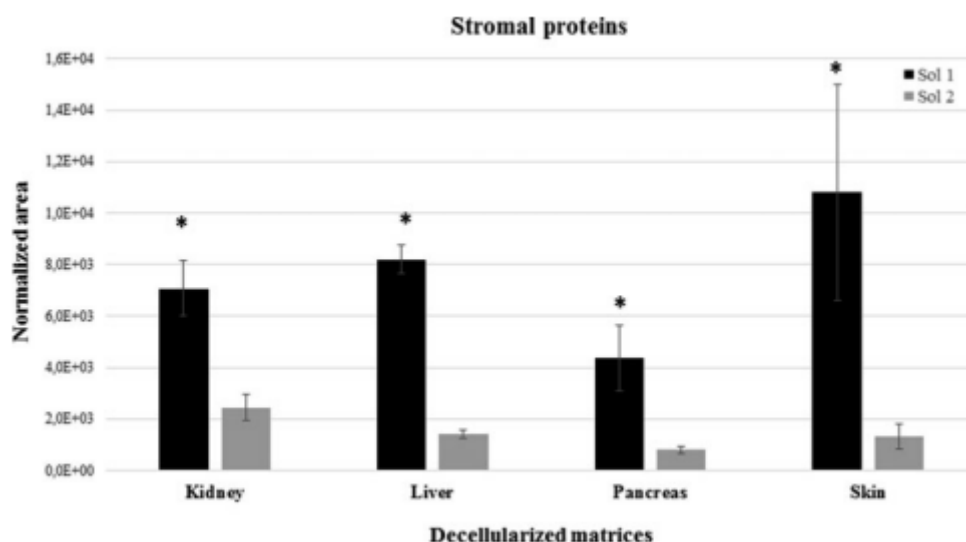


FIG. 5. Cumulated distribution of stromal proteins (including their subunits/isoforms) elastin, fibrillin, decorin, and laminin in each decellularized tissue using Sol 1 (SDS-based protocol) versus Sol 2 (TX100-based protocol), as determined by LC-MS/MS SRM analysis. * $p < 0.05$; data are the mean – SD of three independent samples.

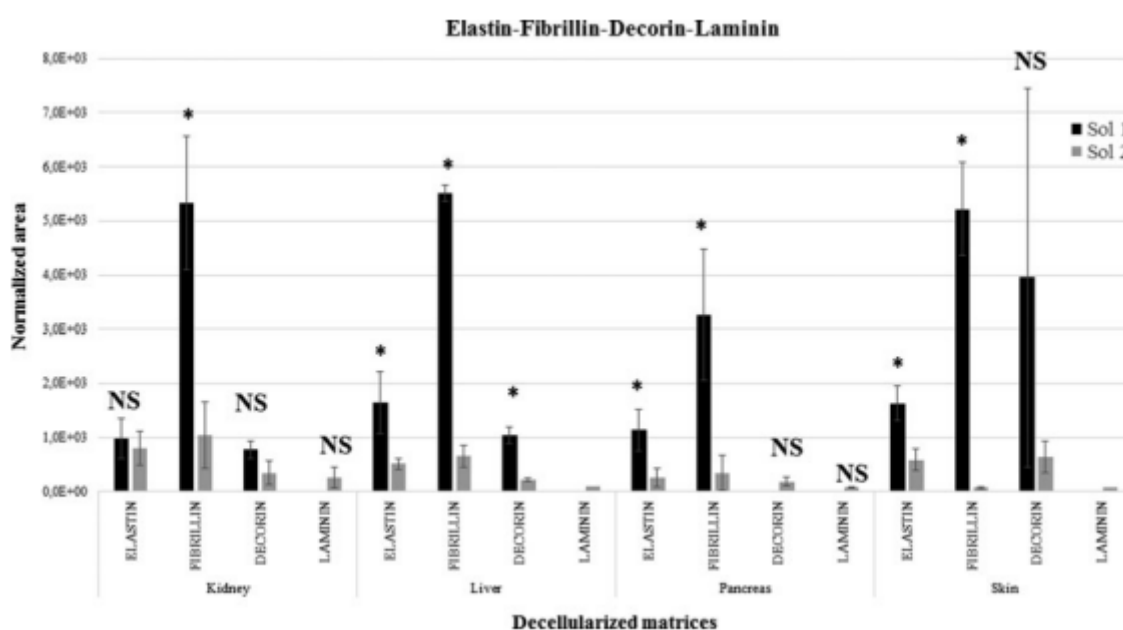


FIG. 6. Selective distribution of the stromal proteins elastin (isoforms X1–X26), fibrillin, decorin, and laminin (subunit alpha 5 isoforms X1 and X2) in each decellularized tissue using Sol 1 (SDS-based protocol) versus Sol 2 (TX100-based protocol), as determined by LC-MS/MS SRM analysis. * $p < 0.05$; data are the mean – SD of three independent samples, except for laminin in liver and skin where it was found only in one single sample.

4. Discussion

Collagens and proteins/proteoglycans such as elastin, fibrillin, decorin, and laminin represent key ECM molecules for 3D organ reconstruction in tissue engineering based on recellularization of decellularized organ's matrices with stem cells/progenitors [24]. Depending on the molecular composition of the decellularized SM, different patterns of cell self-assembly can be expected during ex situ organ reconstruction [25,26]. In addition, the availability and quantity of SM collagens depend on the decellularization procedure and primarily by the chemical detergents used for cell detachment/removal, as we have recently shown in decellularized rat thyroid matrices [14]. Therefore, based on a common and standard physical approach for quick breaking of cell–matrix fibrous bonds and cleaning of the SM from cellular debris, we have focused on the role played by two different and widely used chemical detergents (SDS and Triton-X) in influencing availability and quantities of SM proteins in decellularized 3D matrices of pig. This, with the intent to provide SM models suitable for 3D reconstruction of human organs for transplantation replacement [10] and to initiate a standardization of the decellularization method for eventual human applications. To this aim, we decided to use identical concentrations and times of application of the detergent solutions. In this manner, it has been possible to readily verify the effects of the detergents on a number of structural parameters of the decellularized matrices, potentially relevant for their recellularization. Remarkably, both SDS- and TX100-based preparations led to a light microscopic and ultrastructural evidence of comparable and adequate preservation of the 3D SM fibrous structures, and apparent removal of all cell nuclear material. However, more detailed analysis of the residual DNA content revealed that only the skin matrix reached a satisfactory DNA removal, that is, below the internationally accepted threshold of 50 ng DNA/dry weight [15] with either SDS or TX100. Specifically, the ionic detergent SDS is ideal for protein denaturation as opposed to the nonionic detergent TX100 whose properties favor rupture of lipid–protein and lipid–lipid interactions [27]. Since pancreas has the highest lipid content with respect to liver and kidney in both pig and man, [28,29] it is presumed that the highest efficiency of TX100 on the pancreatic matrix ensued from its more striking lipolytic action as opposed to SDS, which proved to be more effective in tissues such as kidney and liver harboring an abundant protein concentration [30].

In addition, since nuclear lipids give rise to a “morphofunctional shelter” to nucleic acids instrumental to DNA translation and gene transcription,³¹ inadequate removal of lipids from the nuclear compartment may have contributed to an insufficient denaturation of the DNA helix, which remained entrapped inside its natural lipid scaffold [32]. At a tissue level, these assumptions are confirmed by the ability of both detergent solutions to yield a satisfactory DNA removal from the skin matrix whose very rich lipid layer was detached by pretreatment with a lipid solvent (2-propanol

70%). The key role played by the detergents in the preparation of each organ's matrix was further confirmed by mass spectrometry analysis. In particular using TX100, collagen types I–VI were better retained in liver, kidney, and skin, whereas no differences were observed in pancreas as compared with SDS. Collagen types I, II, III, and V play a key role in ensuring tensile strength and 3D stability of the SM including their 3D assembly with fibril-associated collagen types XII and XIV [4]. Differently, collagen type IV is a network-forming collagen residing in the BM where it forms a reticular network to promote cell attachment and connection to other local proteins [33–35]. Finally, collagen type VI is particularly abundant in parenchymal organs such as the kidney glomerulus, and involved in both mechanical and cell growth-regulating activities [17]. Therefore, we suggest that availability of these collagens may play a critical role during 3D ex situ recellularization of matrices requiring a specific 3D geometry for organ's strength. This is the case of in vivo liver, kidney, and skin dealing with anatomical constraints and/or dynamic insults as opposed to pancreas, which is physically protected in the abdomen by the surrounding viscera. As a consequence, a TX100-based decellularization protocol would ideally fit with the preparation of liver, kidney, and skin 3D matrices. In the liver, then, this approach additionally favored high conservation of the interstitial proteoglycan decorin, able to regulate endothelial cell autophagy and cell proliferation [7], two functional properties shared with collagen type IV [33] to ensure structural stability of the wide hepatic vascular field, as confirmed in recent studies on mammalian liver and kidney [36–38]. Differently, using SDS, a better conservation occurred to all the identified elastin isoforms (except for kidney) and fibrillin in all matrices tested. Elastin and its associated protein fibrillin regulate elastic module and deformability of the SM through mechanochemical interactions with a number of extracellular glycoproteins to ensure adequate tissue remodeling during organ's regeneration [5,6]. Therefore, our results suggest that an SDS-based protocol would ideally fit for the 3D ex situ recellularization of matrices requiring an high deformability for in vivo organ's activity such as skin, but also viscera, enriched with arteries including liver, kidney, and pancreas might equally benefit of it. Finally, none of the two protocols tested was able to retain the glycoprotein laminin (subunit alpha 5 isoforms X1 and X2) in any of the matrices studied. Laminin anchors the BM to the SM, and promotes homing and survival of stem cells in their local tissue niche,⁸ indeed, it has been found to in vitro stimulate morphogenesis of pancreatic endocrine islets from mouse pancreatic ductal cells in vitro,³⁹ suggesting that its loss in decellularized matrices might hamper growth and differentiation of precursor cells during recellularization. Thus, different and/or less aggressive decellularization approaches should ideally be developed to sufficiently preserve laminin in all these matrix types, particularly if recellularization is planned to occur using stem cells/progenitors. In conclusion, our study has enlightened the very selective action of two widely used chemical detergents in tissue

decellularization, SDS and TX100. Starting from a common tissue-breaking and similar washing steps through microwave irradiation, we showed that relevant quantitative differences occurred to key fibrous and nonfibrous extracellular and/or basal membrane proteins of porcine liver, kidney, pancreas, and skin in dependence on the detergent used. Since pig matrices may find application in transplantation therapy as natural scaffolds for engineering of human organs, and each detergent allows for saving selective morphoregulatory and mechanochemical ECM patterns, hints have been provided for optimized preparation of these 3D matrices eventually to be used as tools for recellularization of 3D bioartificial human organs.

Authors' Contributions G.R. contributed to mass spectrometry and analytical studies and article writing. F.B. and G.C. were involved in DNA analysis and light and ultrastructural SEM investigations. G.T. carried out SEM studies. C.B. was in charge of mass spectrometry/analytical investigations. R.T. was involved in article supervision including English language. L.E. carried out research conceptualization, project administration, methodology, data evaluation, and article revision.

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Supplementary Materials Supplementary Table S1 Supplementary Table S2

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Supplementary Materials

Table S1. LC-LIT-Orbitrap XL results for different decellularized matrices in relation to the detergent used (SDS-based protocol = Sol 1; TX100-based protocol = Sol 2). Proteins are classified with their respective Uniprot IDs, names, percentage of the protein sequence covered by identified peptides (coverage), and abundance (sample area).

Kidney in Sol 1

PROTEIN ID	PROTEIN NAME	COVERAGE (%)	-10lgP	SAMPLE AREA
A0A480EQ15	Collagen alpha-3(VI) chain isoform 1	22	397.40	3,04E+09
A0A481ABN5	Collagen alpha-3(VI) chain isoform 4	24	384.99	1,00E+09
A0A481DB47	Collagen alpha-5(VI) chain isoform 1	14	354.23	5,05E+09
A0A480W6C8	Collagen alpha-2(VI) chain isoform 2C2	20	320.02	5,83E+10
A0A481CZN2	Collagen alpha-2(I) chain	37	312.92	2,88E+11
A0A480S0U9	Collagen alpha-1(VI) chain	17	302.04	3,76E+10
A0A480JME9	Collagen alpha-1(I) chain isoform X1	14	268.04	1,13E+10
A0A481CFH7	Collagen alpha-1(I) chain preproprotein	14	268.04	1,13E+10
A0A480EEJ3	Collagen alpha-1(II) chain isoform 1	13	268.04	1,13E+10

A0A480K6F4	Collagen alpha-1(VI) chain	9	238.30	5,74E+09
H0V0L8	Collagen type I alpha 1 chain	9	236.89	1,14E+08
A0A480V9U6	Collagen alpha-1(IV) chain isoform 1 preproprotein	12	213.18	3,88E+10
A0A480TWI2	Collagen alpha-1(IV) chain	10	213.18	2,46E+10
A0A481CFH1	Collagen alpha-1(IV) chain isoform X1	8	213.18	2,46E+10
A0A480QYS5	Collagen alpha-1(XII) chain long isoform	11	210.80	3,85E+09
A0A480LF68	Collagen alpha-1(XII) chain isoform X3	7	210.80	3,85E+09
A0A480EMK3	Collagen alpha-1(XII) chain isoform X1	5	210.80	3,85E+09
A0A480E8L6	Laminin subunit alpha-5 isoform X1 (Fragment)	4	184.23	2,97E+08
A0A480YSA6	Laminin subunit alpha-5 isoform X1	4	184.23	2,97E+08
A0A480YRT3	Laminin subunit alpha-5	4	184.23	2,97E+08
A0A480RIQ0	Laminin subunit alpha-5 isoform X3	5	184.23	2,97E+08
A0A480V9U0	Collagen alpha-2(IV) chain preproprotein	4	140.71	7,46E+09
A0A480EU58	Collagen alpha-2(IV) chain preproprotein (Fragment)	3	140.71	7,46E+09
A0A480VFZ6	Laminin subunit beta-1	3	88.93	2,84E+08
A0A480KUV6	Laminin subunit beta-1 isoform X1	3	88.93	2,84E+08
A0A480QI40	Collagen alpha-3(IV) chain	3	76.23	1,67E+09
A0A481BAY5	Collagen alpha-3(IV) chain isoform X3	2	76.23	3.12E4
Q29032	Collagen alpha-3 type (IV) Fragment	17	76.23	3.12E4
A0A481BYE8	Collagen alpha-1(XIV) chain	1	61.14	3,82E+07
A0A480KJV3	Collagen alpha-1(XIV) chain isoform X2	1	61.14	3,82E+07
A0A480W167	Collagen alpha-2(V) chain preproprotein	2	51.89	1,06E+09
A0A480JJN3	Collagen alpha-2(V) chain	2	51.89	1,06E+09

Kidney in Sol 2

PROTEIN ID	PROTEIN NAME	COVERAGE (%)	-10lgP	SAMPLE AREA
A0A480S0U9	Collagen alpha-1(VI) chain	24	525,52	6,86E+10
I3LQ84	Collagen type VI alpha 2 chain	21	430,78	6,24E+10

A0A480W6C8	Collagen alpha-2(VI) chain isoform 2C2	19	430,78	6,24E+10
A0A480XYY0	Collagen alpha-3(VI) chain isoform X4	24	379,26	4,66E+10
A0A480VL24	Collagen alpha-3(VI) chain isoform X3	24	379,26	4,66E+10
A0A480KGD0	Collagen alpha-3(VI) chain isoform 4	20	379,26	4,82E+10
A0A480VLV7	Collagen alpha-3(VI) chain isoform 5	21	379,26	4,66E+10
A0A480WGA5	Collagen alpha-3(VI) chain isoform 4 (Fragment)	16	379,26	4,82E+10
A0A480KJ79	Collagen alpha-5(VI) chain isoform 1	11	343,48	2,10E+10
A0A480VXN1	Laminin subunit alpha-5	15	333,04	1,52E+09
A0A480USJ4	Laminin subunit alpha-5 isoform X3	10	333,04	1,52E+09
A0A481D8A1	Laminin subunit alpha-5 isoform X1	8	333,04	1,52E+09
A0A480EEJ3	Collagen alpha-1(II) chain isoform 1	6	263	1,42E+11
A0A480L718	Collagen alpha-1(I) chain preproprotein	6	263	1,42E+11
A0A480L1U0	Collagen alpha-1(I) chain isoform X1	6	263	1,42E+11
A0A480JL2	Collagen alpha-2(I) chain	5	129,72	7,60E+10
A0A480XXW6	Collagen alpha-2(I) chain (Fragment)	3	129,72	7,20E+10
A0A481BAY5	Collagen alpha-3(IV) chain isoform X3	0	114,86	8,86E+08
A0A480QI40	Collagen alpha-3(IV) chain	0	114,86	8,86E+08
A0A480SKT7	Collagen alpha-2(IV) chain preproprotein	1	106,08	4,56E+09
A0A480UTM1	Collagen alpha-1(IV) chain isoform 1 preproprotein	1	88,62	1,59E+09
A0A480P9A8	Laminin subunit beta-2	2	84,02	5,58E+08

Liver in Sol 1

PROTEIN ID	PROTEIN NAME	COVERAGE (%)	-10lgP	SAMPLE AREA
A0A481BTG6	Collagen alpha-1(I) chain preproprotein	37	369.41	2,82E+11
A0A480JME9	Collagen alpha-1(I) chain isoform X1	32	369.41	2,92E+11
A0A480EEJ3	Collagen alpha-1(II) chain isoform 1	31	369.41	2,92E+11
A0A480JL2	Collagen alpha-2(I) chain	23	332.97	9,10E+11
A0A480XXW6	Collagen alpha-2(I) chain (Fragment)	23	332.97	9,10E+11

A0A480LY74	Collagen alpha-3(VI) chain isoform 4	9	215.11	3,50E+09
A0A480KMZ0	Collagen alpha-3(VI) chain isoform X4	9	215.11	3,50E+09
A0A480WQC6	Collagen alpha-3(VI) chain isoform 4 (Fragment)	10	215.11	3,50E+09
A0A480YTP0	Collagen alpha-3(VI) chain isoform X3	9	215.11	3,50E+09
A0A480VLV7	Collagen alpha-3(VI) chain isoform 5	13	215.11	3,50E+09
A0A480VZL2	Collagen alpha-1(III) chain preproprotein (Fragment)	5	76.76	2,25E+10
A0A480NPP4	Collagen alpha-1(III) chain preproprotein	3	76.76	2,25E+10
A0A480X7G2	Collagen alpha-2(VI) chain isoform 2C2	2	58.00	0
A0A480XSP6	Collagen alpha-2(VI) chain isoform 2C2a (Fragment)	1	58.00	0
A0A480JHJ0	Collagen alpha-2(VI) chain	1	58.00	0

Liver in Sol 2

PROTEIN ID	PROTEIN NAME	COVERAGE (%)	-10lgP	SAMPLE AREA
A0A480K3X6	Collagen alpha-3(VI) chain isoform X4	22	476,88	3,36E+10
A0A480LDS1	Collagen alpha-3(VI) chain isoform 4	24	476,88	3,36E+10
A0A480VL24	Collagen alpha-3(VI) chain isoform X3	26	476,88	3,36E+10
A0A480WGA5	Collagen alpha-3(VI) chain isoform 4 (Fragment)	17	476,88	3,36E+10
A0A480VLV7	Collagen alpha-3(VI) chain isoform 5	23	476,88	3,36E+10
A0A480EEJ3	Collagen alpha-1(II) chain isoform 1	10	463,6	1,81E+11
A0A480L718	Collagen alpha-1(I) chain preproprotein	10	463,6	1,81E+11
A0A1S7J210	Collagen alpha1 (I) chain	11	463,6	1,81E+11
A0A480L1U0	Collagen alpha-1(I) chain isoform X1	12	463,6	1,81E+11
A0A480S0U9	Collagen alpha-1(VI) chain	16	386,9	8,21E+08
I3LQ84	Collagen type VI alpha 2 chain	13	324,44	1,41E+10
A0A480W6C8	Collagen alpha-2(VI) chain isoform 2C2	12	324,44	1,41E+10

Pancreas in Sol 1

PROTEIN ID	PROTEIN NAME	COVERAGE (%)	-10lgP	SAMPLE AREA
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A0A481CZN2	Collagen alpha-2(I) chain	17	274.18	8,75E+10
A0A480XWZ1	Collagen alpha-2(I) chain (Fragment)	15	274.18	8,75E+10
A0A481A293	Collagen alpha-1(I) chain preproprotein	18	261.37	3,34E+11
A0A480EEJ3	Collagen alpha-1(II) chain isoform 1	14	261.37	3,33E+11

Pancreas in Sol 2

PROTEIN ID	PROTEIN NAME	COVERAGE (%)	-10lgP	SAMPLE AREA
A0A480L1U0	Collagen alpha-1(I) chain isoform X1	5	244,8	1,20E+10

Skin in Sol 1

PROTEIN ID	PROTEIN NAME	COVERAGE (%)	-10lgP	SAMPLE AREA
A0A480EQ15	Collagen alpha-3(VI) chain isoform 1	24	512.38	1,26E+11
A0A481CZN2	Collagen alpha-2(I) chain	22	362.74	3,34E+11
A0A481BTG6	Collagen alpha-1(I) chain preproprotein	37	353.51	4,14E+10
A0A480JME9	Collagen alpha-1(I) chain isoform X1	30	353.51	4,14E+10
A0A480EEJ3	Collagen alpha-1(II) chain isoform 1	29	353.51	4,14E+10
A0A480QX91	Collagen alpha-1(VI) chain	15	343.54	7,29E+09
H0V0L8	Collagen type I alpha 1 chain	16	297.12	1,76E+10
I3LQ84	Collagen type VI alpha 2 chain	19	211.99	2,15E+09
A0A480W6C8	Collagen alpha-2(VI) chain isoform 2C2	17	211.99	2,15E+09
A0A481CHD4	Collagen alpha-1(III) chain preproprotein (Fragment)	1	72.81	1,35E+09
A0A480U8S2	Collagen alpha-1(III) chain preproprotein	1	72.81	1,35E+09

Skin in Sol 2

PROTEIN ID	PROTEIN NAME	COVERAGE (%)	-10lgP	SAMPLE AREA
A0A480L042	Collagen alpha-1(I) chain isoform X1	16	478,38	8,52E+11
A0A481CG05	Collagen alpha-1(I) chain isoform X1 (Fragment)	16	478,38	8,52E+11
A0A480NHP8	Collagen alpha-1(I) chain preproprotein	18	478,38	8,55E+11
A0A1S7J210	Collagen alpha1 (I) chain	15	478,38	8,55E+11

A0A480EEJ3	Collagen alpha-1(II) chain isoform 1	14	478,38	8,55E+11
A0A480XXA2	Collagen alpha-2(I) chain	15	382,82	1,29E+11
A0A480XXW6	Collagen alpha-2(I) chain (Fragment)	14	382,82	1,29E+11
A0A480KTB4	Collagen alpha-3(VI) chain isoform X4	7	240,5	9,80E+09
A0A480XMU7	Collagen alpha-3(VI) chain isoform 4	7	240,5	9,80E+09
A0A481D218	Collagen alpha-3(VI) chain isoform X3	6	240,5	9,80E+09
A0A480WQC6	Collagen alpha-3(VI) chain isoform 4 (Fragment)	8	240,5	9,80E+09

Table S2. SRM transitions with their m/z values selected for LC-MS/MS analysis of porcine stromal collagens (types I to VI), elastin (isoforms X1- X26), fibrillin, decorin, and laminin (subunit alpha 5 isoforms X1 and X2). Sequences chosen for semiquantitative evaluation are in bold.

PROTEIN	PEPTIDE SEQUENCE	PRECURSOR (m/z)	PRODUCT (m/z)
Collagen I	SAGISVPGPMGPSGR	733,9	952,5
Collagen I	GEPGDAGAK	401,2	456,2
Collagen I	VFC[+57]NMETGETC[+57]VYPTQPSVPQK	1286,6	655,4
Collagen II	AGSC[+57]VQDGQR	539,2	603,3
Collagen II	DVWKPEPC[+57]R	593,8	432,2
Collagen II	QGAPGASGDR	458,2	257,1
Collagen III	DGESGRPGRPGER	685,3	602,3
Collagen III	INTDEIMTSLK	632,8	686,3
Collagen III	LMGSNEGEFK	556,3	632,3
Collagen IV	GGC[+57]AGSGC[+57]GK	455,7	490,2
Collagen IV	GQTGPTGDK	430,7	441,2
Collagen IV	ILYHGYSLLYVQGNER	963,0	1322,7
Collagen V	AAQPADLLK	463,8	271,1
Collagen V	AAQPADLLK	463,8	559,3
Collagen V	GPMVSAQESQAQAILQQAR	1007,0	1086,5
Collagen V	TVLEIDTPK	508,3	556,3
Collagen VI	DAEEVISQTIHTIADMIK	1007,5	1142,6
Collagen VI	QVNEPHIR	496,8	651,4
Collagen VI	QVNEPHIR	496,8	522,3
Elastin	FPGVGVLPGVPTGTGVK	791,5	912,5
Elastin	AGYPTGTGVGTQAAAAAAAK	903,0	292,1
Elastin	YGAAGLGGVLGVTRPFLGGR	1008,1	743,4
Fibrillin	SIQHC[+57]NIR	514,3	853,4

Fibrillin	C[+57]MNGGSC[+57]SDDHC[+57]LC[+57]QK	964,8	969,3
Fibrillin	ELNQLEDK	494,8	504,3
Decorin	VVQC[+57]SDLGLDK	617,3	689,3
Decorin	VVQC[+57]SDLGLDK	617,3	747,4
Decorin	VHENEITK	485,3	609,3
Decorin	SAIQLGNYK	497,3	513,3
Laminin	GLEYNENVTLDLGQVFHVAYVLIK	1417,3	1460,7
Laminin	GLEYNENVTLDLGQVFHVAYVLIK	1417,3	1486,9
Laminin	C[+57]RPNVAGR	465,2	528,2
Laminin	C[+57]RPNVAGR	465,2	516,3
Laminin	QVLLQADDGAGEFSVLVTRPK	1122,1	1232,7

CHAPTER IV: Development and single laboratory validation of a targeted liquid chromatography-triple quadrupole mass spectrometry-based method for the determination of insulin like growth factor-1 in different types of milk samples

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Personal Contribution

Giulia Remaggi: Writing – original draft, Investigation

Abstract

A simple and reliable targeted liquid chromatography-electrospray-tandem mass spectrometry (LC-MS/MS) method was developed and validated through the selection of two biomarker peptides for the identification and determination of bovine insulin like growth factor-1 (IGF-1) in milk samples. Two urea-based sample extraction procedures were tested. The validation results provided detection limits at the 1–5 ng IGF-1/mL level as a function of the milk matrix, precision ranged from 3 to 8% and the method accuracy in the different milk matrices was assured. Finally, IGF-1 was measured in milk samples obtained by treatment with eleven different technological processes: IGF-1 concentrations were spread over a wide range from 11.2 ± 0.3 ng/mL to 346 ± 8 ng/mL with a median of 57.0 ± 0.2 ng/mL. The highest amount of IGF-1 was found in fresh whole milk samples and no significant correlation was found between the total milk protein content and the IGF-1 concentration level.

Keywords: Insulin like growth factor-1 distribution Cow milk samples Method validation Urea-based extraction

1. Introduction

Insulin like growth factor-1 (IGF-1) is the growth hormone mediator, a strong mitogenic factor capable of stimulating various cellular responses, including cell growth, proliferation and differentiation by activation of IGF-1 receptor (IGF1R) and the subsequent upregulation of phosphoinositol-3-kinase-protein kinase B signaling cascade (Fig. 1), (Guidi, Laricchia-Robbio, Gianfaldoni, Revoltella, & Del Bono, 2001; Melnik, 2009). IGF-1 receptors are present in almost every cell in the human body (Melnik, 2009) and IGF-1 acting as hormone, circulates in serum with more than 90% of molecules that forms a ternary complex bound to its principal carrier/binding proteins, IGF-binding protein-3 and acid labile subunit (Ketha & Singh, 2015). IGF-1 synthesis is subject to other hormones, nutrition, age, sex and genetic variability (Ketha et al., 2015). Researches have shown that a diet rich in milk and dairy products increases serum IGF-1 levels; milk exerts its signaling mechanisms by inducing long-lasting increase in serum IGF-1 levels and postprandial fast upregulation of insulin secretion (Clatici, Voicu, Voaides, Roseanu, Icriverzi, & Jurcoane, 2018; Melnik, John, & Schmitz, 2011). It is also interesting to note that in milk, regardless the processed treatment (pasteurization, homogenization, and digestion), there are high concentrations of growth-stimulating hormones such as IGF-1 (Clatici et al., 2018). Mammals produce structurally highly similar IGF-1 and the bovine and human molecules have the same amino acid sequence (Juskevich & Guyer, 1990), therefore bovine IGF-1 can bind to the human IGF receptor (Francis, Upton, Ballard, McNeil, & Wallace, 1988). In addition, IGF-1 remains active in serum after milk consumption because IGF-1 digestion in the gut is being protected by milk's proteins (Melnik, 2009). Interestingly, it's the hepatic IGF-1 production stimulation via amino acid transfer induced by milk that increase the consumer's serum IGF-1 level (Melnik, John, & Schmitz, 2013). Although benefits related to milk consumption are known such as increased bone mineral content, reduced risk of protein-deficiency malnutrition and rickets and protects against dental caries and fractures, milk consumption has also been associated with numerous diseases due to the presence of growth factors such as IGF-1 (Clatici et al., 2018; Furstenberger & Senn, 2002). Circulating IGF-1 can penetrate the blood–brain barrier suggesting the possibility that reduced IGF1 signaling in the brain can led to an extended mammalian life span (Taguchi & White, 2008). IGF-1 is also a key element required for differentiation of pre-adipocytes into adipocytes (Blüher, Kratzsch, & Kiess, 2005) so milk intake may also be a risk factor for obesity (Olsen et al., 2007). In addition, IGF-1 is involved in the pathogenesis of acne, a chronic inflammatory disease that affect almost 9.4% of the global population (Rodighiero et al. 2020). In terms of food-safety and nutrition, little is known about the so-called threshold doses, i.e. the minimum amount of an active ingredient which is able to cause a reaction. Concerns about the potential health risk of IGF-1 have drawn increasing attention to the development

of rapid, sensitive and accurate IGF-1 detection assay (Guidi et al., 2001) in order to improve food labeling directives and to increase consumer protection. Numerous assays have been developed over the years for the identification and quantification of IGF-1, especially in plasma: ELISA assay (Ginjala & Pakkanen, 1998), electrochemiluminescent assay (McGrath, Bogosian, Fabellar, Staub, Vicini, & Widger, 2008), automated surface plasmon resonance-based biosensor system (BIA-technology) (Guidi et al., 2001), immunometric assays (Anderson, Tamayose, & Garcia, 2018), radioimmunoassay (RIA) (Mann et al., 2020a; Mann et al., 2020b). RIA methodology is still used today thanks to its high specificity and sensitivity but there are several difficulties in the automation of this very expensive assay such as specialized structures for the storage and handling of radioactive material, the high cost of waste disposal, long analyzes and IGFBPs should be dissociated from IGF-1 as immunoassay interferents (Ketha and Singh, 2015; Salameh, Redor-Goldman, Clarke, Mathur, Azziz, & Reitz, 2014). A study published by Chanson et al., (2016) compared the analytical performances of six different immunoassays for the detection of IGF-1 in the serum of the same large population of healthy subjects. The data obtained evidenced that the reference intervals differed somewhat from one assay to another, and agreement between assays was moderate to good. Moreover, the concordances between the manufacturers' reference intervals and those obtained in the study were generally poor (Chanson et al., 2016). An alternative to immunoassays is liquid chromatography-tandem mass spectrometry (LC-MS/MS), already used for IGF-1 quantification mainly in serum (Ketha and Singh, 2015; Cox et al., 2014; Such-Sanmartín, Bache, Callesen, Rogowska Wrzesinska, & Jensen, 2015; Lopes, Cowan, Thevis, Thomas, & Parkin, 2014; Bronsema, Klont, Schalk, Bischoff, Kema, & van de Merbel, 2018; Thevis, Thomas, & Schanzer, 2011; Cox, Hughes, & Eichner, 2014; Mongongu et al 2021; Seo et al. 2021; Coppieters et al 2020). In particular, Bronsema et al. (2018) developed and validated an LC-MS/MS targeted method for the detection of IGF-1 in serum in the 10–1000 ng/mL concentration range by selecting two signature peptides; an urea-based sample preparation procedure was proposed and no analyte enrichment step was required. Tanna, Lame and Wrona (2020) developed and validated a sensitive (LOQ 5 ng/mL) and reliable (mean accuracy of 101.76%) ultra-pressure (UP)LC-MS/MS targeted method for the detection of intact IGF-1 in clinical samples. Pratt, van Faassen, Remmelts, Bischoff and Kema (2021) recently published an antibody free LC-MS/MS method for the detection of intact IGF-1 and IGF-2 in human plasma with LOQ values ranging between 5.9 and 8.4 ng/mL. The performances of the LC-MS/MS method were compared to the results obtained by immunoassay for the detection of these two molecules in human plasma samples. Even if a good data correlation was observed ($R^2 = 0.97$) between the two techniques, a statistically significant overestimation of the concentration levels detected by immunoassay was observed (Pratt et al., 2021). In the last years, increasing emphasis has

been put on the development of confirmatory and screening LC–ESI-MS/MS based-methods for the identification and determination of active compounds in foods (Miranda, Bianchi, Krupova, Trossat, & Martin, 2020). Welsh et al. (2019) used LC-MS/MS for detection of contaminants (pesticides, antibiotics and hormones) in organic and conventionally produced milk samples sold in the USA and they detected IGF-1 in milk via LC–high-resolution linear ion trap (LTQ Orbitrap). By considering that the direct application of immunoassay kits to the detection of IGF-1 in different types of milks can fail to provide reliable quantitative data due to antibody-antigen binding variability in complex matrices and most of the LC-MS/MS methods were developed for plasma analysis, here we propose a developed and validated LC-MS/MS assay for IGF-1 identification and quantification in cow's milk of different brands and subjected to different technological treatments. By using a shotgun proteomic approach, IGF-1 was enzymatically degraded to peptides and three selected biomarker peptides were then analyzed under SRM mode in a triple quadrupole (QqQ) mass analyzer. The quantitation of IGF-1 was carried out by selecting one quantifier biomarker peptide and evaluated in terms of sensitivity, selectivity, accuracy, recovery, limits of detection and quantitation. Moreover, the IGF-1 extraction procedure from milk must be reproducible and throughput in order to identify and quantify this very low abundant peptide hormone in such different matrices. Here, we tested two urea-based extraction protocols and after validation, the LC-MS/MS method was applied to the detection of IGF-1 in commercially available bovine milk samples and the results were discussed.

2. Materials and methods

2.1. Chemicals

Ammonium bicarbonate (ABC), acetonitrile (HPLC purity), formic acid (analytical reagent grade), urea were purchased from Carlo Erba (Milan, Italy). Bovine insulin growth factor-1 (white powder, purity >95%), protein assay dye reagent concentrate were from Bio-Rad (Hercules, California, USA). Isotopically labeled IGF-1 peptide (LEMYCAPLKPA{K*} {K*} = Lys [13C6, 15 N2] isotopic enrichment: >99%) was from Pepscan (Presto AB Lelystad, NL). Bovine serum albumin (BSA), Tris-HCL, trypsin from bovine pancreas, iodoacetamide (IAA), dithiotreitol (DTT) were from Sigma-Aldrich® (Darmstadt, Germany).

2.2. IGF-1 extraction and sample preparation

Eleven cow milk types coming from different providers (fresh whole milk, UHT whole milk, fresh semi-skimmed milk, UHT semi-skimmed milk, fresh whole microfiltered milk, fresh semi-skimmed microfiltered milk, UHT skimmed milk, UHT semi-skimmed microfiltered milk, UHT lactose free milk, UHT lactose free low-fat milk, UHT low-fat milk, detailed in the Results section) for a total of

25 different samples were obtained from local food stores, aliquoted in 10 mL Falcon tubes and stored at -20°C until processing. All milk pH were measured and ranged from 6.61 to 6.74 pH unit (average pH 6.68 ± 0.04). Initially, two different extraction methods were tested on a fresh whole milk sample. In the first case, IGF-1 extraction was performed by adding 1 mL of a 8 M urea-Tris-HCl buffer (pH = 8) solution to 1 mL of milk. In the second case, IGF-1 extraction was performed by dissolving directly urea in 700 μL of each milk sample in order to obtain a final concentration of 8 M urea in each sample. Subsequently, for both methods, the samples were immersed in an ultrasonic bath (Bransonic ultrasonic cleaner 2510E –DTH) at 4°C for 10 min at 42 kHz and centrifuged (15000 rpm $\times$ 10 min); after centrifugation, it is necessary to remove the fat layer formed on each sample surface, and transfer the supernatants to new eppendorf (Eppendorf Srl, Milan, Italy). The proteins contained in each supernatant were assayed according to Bradford's method. The Bradford assay was performed by adding 10 μL of each diluted sample / standard to 500 μL of previously diluted (1: 5) Bradford reagent (Absorbance = 595 nm). Calibration was achieved using the BSA protein standard with a final concentration of 1 mg/mL from which serial dilutions were performed to cover the 25–400 $\mu\text{g}/\text{mL}$ range. By comparing the total milk protein content obtained from the two protocols, the second method (approx. 24 mg/mL) was more effective with respect to the first one (approx. 8 mg/mL) and it has been selected to perform all milk sample extraction. Subsequently, the supernatant was diluted with 50 mM ammonium bicarbonate buffer (pH 8.0) to decrease urea concentration under 2 M; the proteins were reduced and alkylated using 100 mM DTT (30 min at 37°C) followed by 200 mM IAA (30 min at room temperature in the dark), the reaction was stopped with 100 mM DTT (15 min at room temperature). Finally, all samples were digested by incubation with trypsin (1 $\mu\text{g}/\mu\text{L}$) at 37°C (protein:enzyme ratio 50:1) overnight. To stop the digestion, 1 μL of formic acid was added to each sample. Finally, samples were dried under nitrogen flow/lyophilized and reconstituted with 50 μL of a water / acetonitrile / formic acid aqueous solution (49.95 / 49.95 / 0.1, v/v/v).

2.3. *Liquid chromatography–triple quadrupole mass spectrometry*

LC separation was carried out on a C18 Luna (150 mm $\times$ 2.1 mm, 5 μm particles) (Phenomenex, CA, USA) column thermostated at 25°C using a gradient solvent elution system [(A) aqueous formic acid 0.1% solution (v/v)/(B) 0.08% (v/v) formic acid in acetonitrile]. Gradient elution was as follows: solvent B was set at 5% for 2 min, then delivered by a linear gradient from 5% to 80% in 60 min. Solvent B was maintained at 80% for 1 min before column re-equilibration (5 min). The flow-rate was 200 $\mu\text{L}/\text{min}$. The mobile phase was delivered by the Agilent HP 1200 chromatographic system (Agilent Technologies, Germany) equipped with a 100-vial capacity sample tray. Injection volume was 10 μL . A QTRAP 4000 instrument (ABSCIEX, CA, USA) equipped with a pneumatically

assisted ESI interface was used. The system was controlled by the Analyst software. The sheath gas (nitrogen, 99.999% purity) and the auxiliary gas (nitrogen, 99.998% purity) were delivered at flow-rates of 45 and 5 arbitrary unit, respectively. Optimized conditions of the interface were as follows: ESI voltage 5.5 kV, capillary voltage 20 V, capillary temperature 350 °C. MS/MS experiments were performed under both product-ion and SRM conditions with a collision gas (nitrogen) pressure of 2.1×10^{-3} mbar in the collision cell. The SRM transitions monitored were reported in Table 1. For quantitative purposes, the most intense SRM transition was monitored for IGF-1, whereas the other transitions reported in Table 1 were monitored for confirmatory purposes.

Table 1: List of IGF-1 peptides and SRM transitions monitored with their m/z values and fragment ions. Sequences chosen respective as qualifier transition and quantifier biomarker are in bold.

Peptide sequence	Precursor ion [M + 2H] ²⁺ m/z	Product ion m/z	Fragment ion
GFYFNKPTGYGSSSR	834.4	1153.6	y11
GFYFNKPTGYGSSSR (selected qualifier transition)	834.4	1039.5	y10
GFYFNKPTGYGSSSR	834.4	911.4	y9
GFYFNKPTGYGSSSR	834.4	757.4	b6
GFYFNKPTGYGSSSR	834.4	854.4	b7
GFYFNKPTGYGSSSR	834.4	955.5	b8
GFYFNKPTGYGSSSR	834.4	1012.5	b9
APQTGIVDEC[+57]C[+57]FR	776.8	1481.6	y12
APQTGIVDEC[+57]C[+57]FR	776.8	1098.5	y8
APQTGIVDEC[+57]C[+57]FR	776.8	985.8	y7
APQTGIVDEC[+57]C[+57]FR	776.8	886.3	y6
APQTGIVDEC[+57]C[+57]FR	776.8	782.4	b8
APQTGIVDEC[+57]C[+57]FR	776.8	911.4	b9
APQTGIVDEC[+57]C[+57]FR	776.8	1071.5	b10
LEMYC[+57]APLKPAP	710.9	1047.6	y9
LEMYC[+57]APLKPAP	710.9	884.5	y8
LEMYC[+57]APLKPAP	710.9	724.5	y7
LEMYC[+57]APLKPAP	710.9	653.4	y6
LEMYC[+57]APLKPAP (selected quantifier biomarker)	710.9	315.2	y3
LEMYC[+57]APLKPAP	710.9	768.3	b6
LEMYC[+57]APLKPAP	710.9	865.4	b7
LEMYC[+57]APLKPAP	710.9	978.4	b8
LEMYC[+57]APLKPAP	710.9	1106.5	b9

2.4. Method validation

Validation of the whole analytical procedure was performed under MS/MS mode on fortified milk samples according to Eurachem guidelines (Eurachem, 1998). For this purpose, milk samples were fortified with different IGF-1 amounts and measurements were carried out by monitoring the most abundant MS/MS transition (Table 1). The detection limits (LOD) and the quantitation limits (LOQ) were calculated from the calibration curve as:

$$\text{LOD} = 3s/\text{slope}$$

$$\text{LOQ} = 10s/\text{slope}$$

where s is the standard deviation of the blank signal obtained by performing 10 independent blank measurements. Linearity was assessed over one order of magnitude in a suitable ng IGF-1/mL matrix range [50–500 ng/mL], starting from LOQ values. Six equispaced concentration levels were tested and three replicated analysis were performed for each level. The Levene test was used to check homoscedasticity. The Mandel test was performed to check linearity. The first order calibration curve ($y = b_0 + b_1x$) was compared with the second order calibration curve ($y_2 = b_0 + b_1x + b_2x^2$) by using the following equations:

$$\Delta S^2 = (N-2)s^2_{y1} - (N-3)s^2_{y2}$$

$$F = \Delta S^2 / s^2_{y2}$$

where N is the total number of observations and s_y is the residual standard deviation for each equation. The significant of the intercept was tested by using the two-tailed Student's t -test (95% significant level). Precision was evaluated as relative standard deviation (RSD) in terms of intra-day repeatability and intermediate precision (inter-day repeatability). For this purpose, the within day repeatability was evaluated by performing three independent extractions of the matrix fortified with 10 and 50 ng IGF-1/mL matrix and three LC–MS/MS injections for each extract in the same day and the inter-day repeatability was calculated on five days by performing five independent extractions of the fortified matrix and three LC–MS/MS injections for each extract. The matrix effect was assessed by using the recovery function. The matrix matched calibration curve were obtained by analyzing the matrix extracts fortified with IGF-1 at six concentration levels [50–500 ng/mL] and treated applying the whole analytical procedure. Each level was analyzed three times. The recovery of the protein extraction procedure was evaluated by performing the extraction on the matrix fortified at two different concentration levels (10 and 50 ng IGF-1/mL). The total protein content was determined by using a Bradford assay. Relative quantitation of IGF-1 peptides in the milk samples was achieved through normalization of data with respect total protein content. Absolute quantitation was obtained by using the isotopically-labeled internal standard (IS) method, by adding IS (final concentration 30 ng/mL) to each extract before LC-MS/MS analysis. Differences in ng/mL between milk groups were calculated using a one-way ANOVA (95% significant level). All results were considered significant if $p < 0.05$.

2.5. Bioinformatic analysis and mass spectrometry (MS) data processing

IGF-1 protein from cow milk was considered for the assay. FASTA protein sequence was obtained from Uniprot Bovine proteome database and SRM transitions were simulated by Skyline (version 20.1, SCIEX) (Redwood City, CA, USA), setting trypsin as digestion mode with no missed cleavage and carbamidomethylation of cysteins as structural modification (Table 1). Uniqueness of candidate

peptide sequences was assessed by BLASTp tool (basic local alignment search tool; www.ncbi.nlm.nih.gov link NCBI BLAST) search (algorithm: blastp; ATRIX PA 30; GAP COASTS: existence 10, extension:1; DATABASE: non redundant protein sequences) from NCBI (National Center for Biotechnology Information) (Bethesda, MA, USA). LC-MS/MS SRM data were analyzed using the Analyst v 1.4 software and the peaks areas were obtained by the MultiQuant program (version 2.1, ABSCIEX).

3. Results

3.1. *In silico* selection of biomarker peptides

Bovine IGF-1 is a small protein composed by 154 amino acid residues (MW 17066 Da) with three disulfide bonds (55 ↔ 97; 67 ↔ 110; 66 ↔ 101) as post translational modifications (PTMs) (Fig. 1) and a 95.56% homology with the human IGF-1. In this work the peptides targeting the bovine IGF-1 protein were selected on the basis of the published sequences present in the protein database (<http://www.uniprot.org/>). As for biomarker peptide selection, different criteria, such as absence of missed cleavages, good ESI sensitivity, no PTMs and sequence specificity, were considered. Thereby, by analyzing a tryptic digest extract from raw milk a subset of peptides was directly identified by the mass spectrometer in a data-dependent acquisition mode to characterize IGF-1 protein with a sequence coverage average of ~65%. Those peptides providing good ESI-MS responses and unequivocally identifying the target protein were selected (Table 1). To develop a reliable qualitative and quantitative method, three peptides present in all the eight (1–8) bovin IGF-1 isoforms were selected and monitored (Table 1). It should be noted that these three peptides are all located in the second half of the aminoacid sequence close to C-term, have 100% homology with the human IGF-1 sequences and are present in all its four (1–4) human isoforms. These findings indicate that they could be successful candidates for the detection of bovin and human IGF-1 in different matrices. In a further step, product-ion MS/MS measurements were carried out on the $[M + 2H]^{2+}$ peptide signals, as the most abundant, by varying collision energy (from 20 to 45 eV) to select specific SRM transitions for each biomarker. The MS/MS spectra exhibited several fragments of the y- and b-series to cover and confirm their sequences (Table 1). All SRM transition tested for the two peptides GFYFNKPTGYGSSSR and LEMYC [+57]APLKPAK were detected in all kind of milk samples with excellent signal intensity and stability. On the other hand, only one transition of the APQTGIVDEC[+57]C[+57]FR peptide (m/z 776.8/911.4, b9 fragment) was detected in all milk samples (Table 1). Since this peptide contain a disulfide bridge, considered as PTM, even if reduction and alkylation step are carried out during the sample treatment protocol, it was no more considered a suitable IGF-1 biomarker because of its poor signal stability. Attention was thus paid to the selection

of those fragment ions that provide optimal signal intensity and that could discriminate the targeted peptides from other species present in the sample. The assay was constituted of a series of transitions (precursor/fragment ion pairs) in combination with the retention time for each targeted peptide as reported in Table 1.

3.2. *Chromatographic separation and LC–MS/MS method validation*

Milk is a complex matrix rich in proteins and taking into account that the chromatographic separation plays a fundamental role in the MS analysis of complex peptide mixtures, suitable separation methods have to be developed to improve resolution, sensitivity, analysis time and reduce matrix effects. By analyzing a IGF-1 protein aqueous solution a LC-MS/MS elution profile (within 8 min) of the peptides was obtained (Fig. 2). The chromatographic profile of the two peptides on the C18 column showed excellent peak shape (in-run-peak width (FWHM, average on the peptides) = 15.80 ± 0.07 s) and retention time stability (RSD < 1.0%). The primary goal of this work was to validate a sensitive and robust LC–MS/MS method for the analysis of IGF-1 at trace levels in foods. As for MS acquisition mode, the non-scanning nature of selected reaction monitoring (SRM) analysis, usually performed by a triple quadrupole mass spectrometer allows to obtain excellent sensitivity and enables the detection of low-abundance proteins in highly complex mixtures. For this purpose, studies on linearity, trueness, precision, selectivity and recovery were performed and the LEMYCAPLKPAK $[M + 2H]^{2+}/y3$ biomarker m/z transition was selected for quantitative purposes as providing the most intense signal. Even if little is known about the minimum concentration level able to provide reaction, detection limits between 1 and 5 ng of IGF-1 per mL of sample were determined from the matrix-matched calibration curves. LOQ values were found in the 3–12 ng protein/mL of milk sample. Generally, the LC–ESI-MS/MS linear range was explored over one order of magnitude of concentration [$y = 36.9(\pm 0.3)x$, $r^2 = 0.999$; concentration range: 50–500 ng/mL]. Homogeneity of variance of replicate measurements at different concentration levels was proved at the 95% confidence level ($p > 0.18$). After testing significance of the intercept (p value lower than 0.05 at 95% confidence level), linearity was mathematically verified by applying the Mandel fitting test. A p value of 0.781 demonstrated that the best data fit could be obtained using a first order regression model. The method accuracy was then tested both in terms of precision and trueness. Excellent precision in terms of intra-day repeatability was calculated providing RSD% in the 3–8% ($n = 9$) range. The intermediate precision results were found not exceed 15% ($n = 15$), confirming good method precision. As for trueness, a calculation of the recovery function was performed to ascertain the influence of the matrix for the determination of the IGF-1 biomarker peptide. For this purpose, an aqueous tryptic digest and different milk sample (fresh whole milk, fresh semiskimmed microfiltrated

milk, UHT semi-skimmed milk and UHT skimmed milk; $n = 3$, n = number of independent milk type) fortified with the tryptic digest (at six concentration levels $n = 6$) were analyzed. The slope and the intercept of the recovery functions calculated for the analytes were compared respectively with 1 and 0 by means of a t-test. The t-test performed on the intercept provided a p value at the 95% confidence level higher than 0.05 ($p > 0.05$) demonstrating that all the calibration equations were in the $y = b_1 \times$ form and thus the absence of constant systematic errors. In the case of the slopes, since the t-calculated resulted to be higher than the t-tabulated at the 95% confidence level (1.86), it can be inferred that the calibration curves obtained by spiking samples are significantly different from that obtained using standard solution as a function of the different milk matrices. In all the cases a signal suppression for the selected biomarker between 85% and 89%, was observed. These findings suggest that a matrix effect able to cause significant systematic error in the IGF-1 quantitation is present, but even that the percentage of matrix effect is very similar among different type of milks, independently from the technological treatment applied. To overcome matrix effect isotopically labeled internal standard was used to build up the calibration curve as quantification method. As for recovery, fresh whole milk sample, selected as the most complex matrix, was enriched with a isotopically labeled IGF-1 and values ranging from 89 % (SD = 4%) to 98 % (SD = 3%) were obtained.

3.3. Sample analysis

In the last step of the work the LC–MS/MS method was applied to a variety of commercial milk products to evaluate the presence of the IGF-1 under investigation. It must be considered that milk is a complex matrix whose proteome is made of very abundant proteins such as casein and medium to low abundance whey proteins such as β -lactoglobulin, lactoferrin, immunoglobulins, glycoproteins, enzymes and peptide hormones (Vincent et al., 2016). The urea-based protocol 2 was applied and no sample purification or enrichment was required. All samples analyzed, under MS/MS mode, exhibited naturally occurring IGF-1 levels (Table 2). As for confirmatory purposes, these samples were run under MS/MS SRM mode in order to acquire all biomarker peptides that univocally verified the presence of IGF-1. No isobaric interferents and carry-over effects were detected. The quantitative results are shown in Fig. 3 and Table 2.

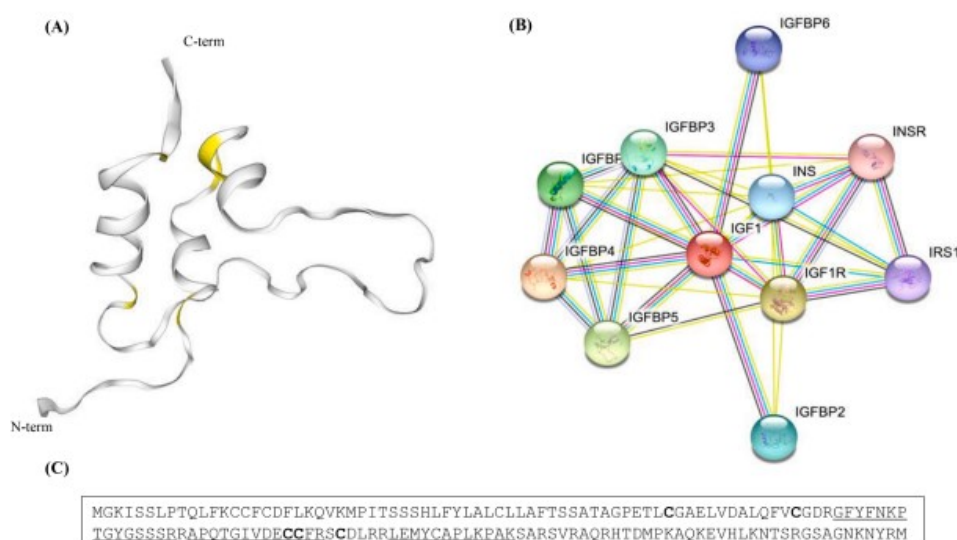


Fig. 1. Bovine IGF-1 protein. (A) 3D structure of bovine IGF-1 protein. (B) IGF-1 experimentally determined interaction networks mainly involving IGF-binding proteins (BP), IGF-receptor (R) (www.uniprot database; accession number: P07455. STRING interaction network analysis). (C) IGF-1 primary sequence (in bold the cysteine involved in the disulfide bonds); the three biomarker peptides selected for the LC-MS/MS analysis are underlined.

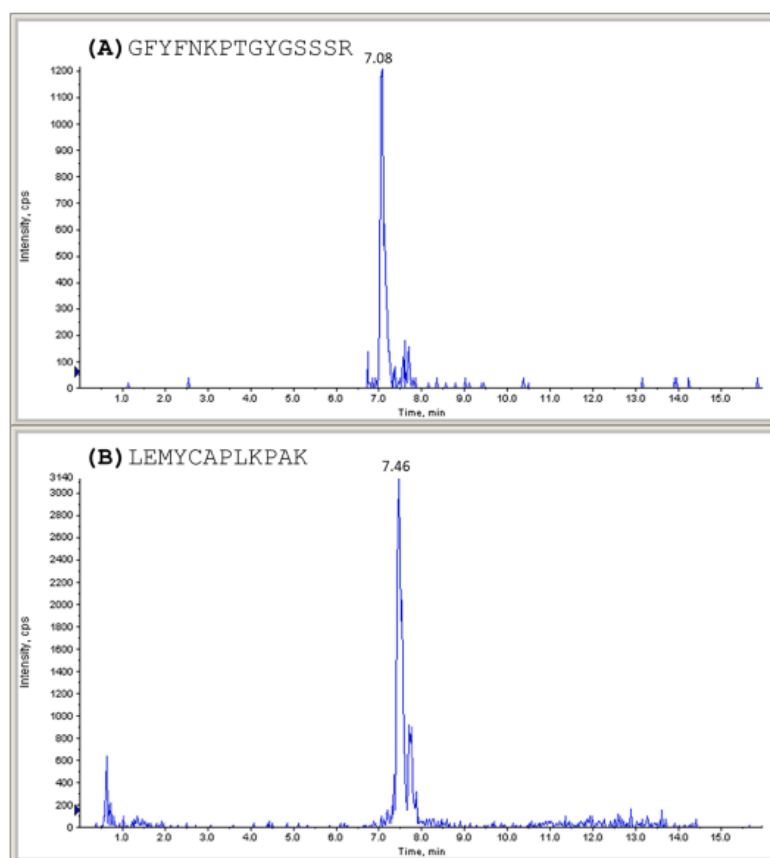


Fig. 2. Liquid chromatography separation. LC-ESI-MS/MS SRM profile of the two IGF-I biomarkers (A) GFYFNKPTGYGSSSR and (B) LEMYCAPLKPAK identified in a IGF-1 protein aqueous solution (IGF-1 conc. 50 ng/mL) respectively with retention time of 7.08 and 7.46 min.

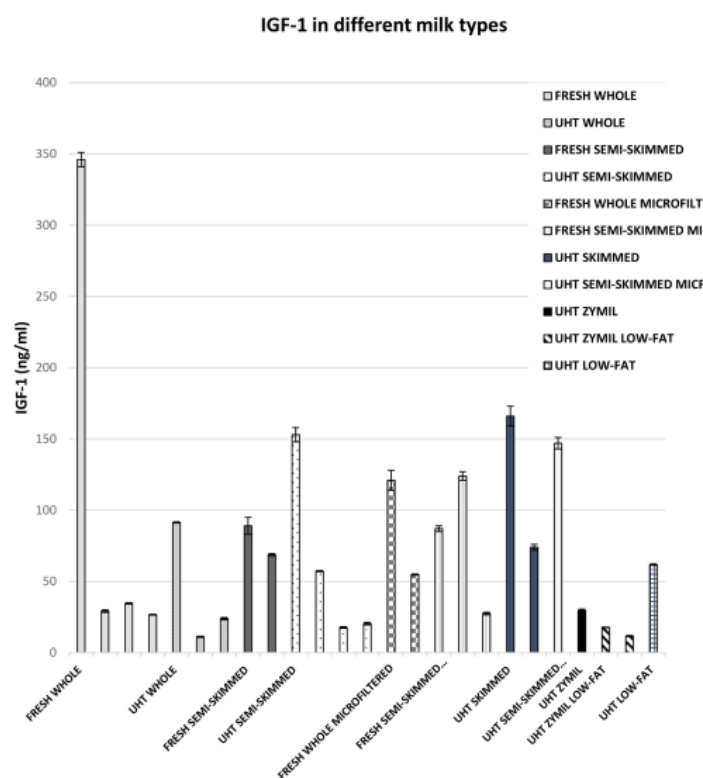


Fig. 3. IGF-1 concentration (ng/mL) in different milk types. Selective distribution of IGF-1 in 25 milk samples of 11 different types: fresh whole, UHT whole, fresh semi-skimmed, UHT semi-skimmed, UHT skimmed, fresh whole microfiltered, fresh semi-skimmed microfiltered, UHT semi-skimmed microfiltered, UHT lactose free, UHT lactose free low-fat, UHT low-fat. Data are expressed as mean $\pm$ standard deviation ($\pm$ SD).

Table 2: LC-ESI-MS/MS SRM determination of IGF-1 in different milk samples. Total protein content (mg/mL) and IGF-1 concentration $\pm$ standard deviation ($\pm$ SD) (ng/mL) are reported for the investigated milk samples.

Sample	Milk Types	Milk Brand	Total protein content (mg/mL)	IGF-1 concentration ($\pm$ SD) (ng/mL)
1	Fresh whole	Conad	10.6	346 $\pm$ 5*
2		Esselunga	32.1	29.2 $\pm$ 0.9
3		Granarolo	25.8	34.6 $\pm$ 0.5
4		Granarolo	21.1	26.7 $\pm$ 0.3
5	UHT whole	Coop	17.4	91.4 $\pm$ 0.4
6		Esselunga	23.4	11.1 $\pm$ 0.4**
7		Parmalat	21.7	23.9 $\pm$ 0.8
8	Fresh semi-skimmed	Conad	24.4	89 $\pm$ 6
9		Granarolo	23.6	68.8 $\pm$ 0.8
10	UHT semi-skimmed	Coop	19.3	153 $\pm$ 5
11		Granarolo	23.5	57.2 $\pm$ 0.4
12		Parmalat	37.9	17.7 $\pm$ 0.6
13	Fresh whole microfiltered	Esselunga	29.7	20.4 $\pm$ 0.9
14		Conad	30.3	121 $\pm$ 7
15	Fresh semi-skimmed microfiltered	Esselunga	27.3	54.8 $\pm$ 0.4
16		Conad	29.2	87 $\pm$ 2
17	UHT skimmed	Esselunga	27.4	124 $\pm$ 3
18		Parmalat	24.0	27.5 $\pm$ 0.9
19	UHT semi-skimmed microfiltered	Coop	18.5	166 $\pm$ 7
20		Esselunga	23.6	74 $\pm$ 2
21	UHT lactose free	Coop	20.5	147 $\pm$ 4
22		Parmalat	28.8	29.7 $\pm$ 0.8
23	UHT lactose free low-fat	Parmalat	27.8	17.8 $\pm$ 0.2
24		Granarolo	31.2	11.7 $\pm$ 0.4**
25	UHT low-fat	Granarolo	22.9	61.8 $\pm$ 0.5

*Highest IGF-1 concentration. ** Lowest IGF-1 concentration.

4. Discussion

Daily cow milk consumption is a diffused aspect of human diet and generally recommended by different nutritional guidelines as important supplier of calcium, magnesium, iodine, vitamin B12, proteins and other important nutrients (Givens, 2020). However, the study of the health benefits and risks associate to milk intake is still a hot topic under investigation, involving different scientific disciplines such as microbiology, anatomy, endocrinology, cardiology, dermatology and biochemistry. Among these studies, several papers deal with the regulation level of the hormone like protein IGF-1 in milk and its correlation between milk consumption and health effects (Guidi et al., 2001; Clatici et al., 2018; Melnik et al., 2011). IGF-1 is usually secreted from bovine mammary gland at high levels during the last 2 weeks ante partum followed by a rapid decrease during the first milkings post-partum. IGF-1 appeared mainly in the bound form (91%) at days 40–2 ante partum, whereas free IGF-1 preponderated in the first milkings post-partum (73%) and changed again to about 85% in the bound form after day 4 post- partum (Einspanier & Schams, 1991). Before to reach the consumers, raw milk usually undergoes different heat treatments commonly used as processing technologies in the dairy industry. In order to better understand the effects of milk intake on the human health the development and validation of reliable analytical methods for sample characterization plays a pivotal role. Therefore, IGF-1 determination is usually based on a common and standard radioimmunoassay approach for quick and reliable assay. RIA analysis presents various advantages (i.e. simplicity, sensitivity) but, remarkably, even several disadvantages (use of antibodies, use of radioactive compounds, false positive results). These reasons stimulated the development of alternative analytical approaches such as the use of LC-MS/MS as highly consolidated, sensitive and selective technique for the qualitative and quantitative analysis of analytes in complex matrices even at the trace levels. Here, we developed and validated in house an SRM targeted LC-ESI-MS/MS method for the unambiguous identification and determination of total IGF-1 content in milk, as reliable alternative to immunoassay. Welsh et al. (2019) reported the use of LC-MS/MS technique for the identification of IGF-1 in milk samples, but no complete validation was reported and an external standard method was used for quantitative analysis. Other methods (Kirsch et al., 2007) proposed the GFYFNKPTGYGSSSR peptide as biomarker for human IGF-1, because it was the only one that did not contain a cysteine residue. In our case both the GFYFNKPTGYGSSSR and LEMYC[+57]APLKPAK peptides were monitored and the last one exhibited the most intense (>70% relative intensity over all monitored SRM transitions) and stable signal (chromatographic peak area $n = 6$; RSD% 0.05) among technological treatments. Moreover, different average amounts of IGF-1 were found in the milk samples having different milk fat content (average value in the whole milks: 82 ng/mL; average value in the semi-skimmed milks: 74 ng/mL; average value in the skimmed

and low-fat milks: 66 ng/mL). However even in this case the high data variability (between 66 and 128%) did not allow to statistically ($p > 0.05$) confirm a significant trend.

5. Conclusions

As the demand to accurately measure active compounds in food is increasing, here a new effective LC-ESI-MS/MS method for the detection of IGF-1 in milk with different technological treatments was developed and successfully validated. By operating under SRM conditions, the validation procedure demonstrate the fit-to-purpose of the method to identify and detect IGF-1 through biomarker peptides with LODs from 1 and 5 ng IGF-1/mL matrix, precision from 3 to 8%, high accuracy and recovery in different types of milk. Our study has enlightened the very selective detection of IGF-1 in all kinds of milk samples at the levels of interest without any sample purification, preconcentration treatment or use of antibodies, in order to provide an accurate detection method useful for food control analysis. This method can be easily transferred to other laboratories equipped with a LC-triple quadrupole mass analyzer for routine quantitative analysis and does not require expensive high resolution MS instruments. Moreover, after methodical evaluation of the sample treatment procedure, this approach can be extended to the simultaneous and specific detection of IGF-1 at the trace level in other milk-based products and, as a consequence, it might represent a very flexible tool able to gain insights into the study of milk composition.

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CHAPTER V: PCSK9 ablation attenuates A β pathology, neuroinflammation and cognitive dysfunctions in 5XFAD mice

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Highlights

- The treatment of human astrocytes *in vitro* with exogenous human recombinant PCSK9 exacerbates the A β -mediated inflammatory response, thus increasing mRNA levels of the pro-inflammatory cytokines IL-6 and IL-1 β as well as the secretion MCP-1 chemokine).
- Genetic deletion of PCSK9 improves cognitive performance in Morris water maze test in 5XFAD mice, a severe mouse model of AD.
- At histological level, the ablation of PCSK9 induces the reduction in both plaque deposition and microglia activation, suggesting reduced neuroinflammation.

Abstract

Background: Increasing evidence highlights the importance of novel players in Alzheimer's disease (AD) pathophysiology, including alterations of lipid metabolism and neuroinflammation. Among them, a potential involvement in AD of Proprotein convertase subtilisin/kexin type 9 (PCSK9) has been recently proposed. Here, we have first investigated *in vitro* the effects of PCSK9 on neuroinflammation. Then, we examined the impact of a genetic ablation of PCSK9 on cognitive performances in a severe mouse model of AD. In addition, we evaluated the effect of PCSK9 loss on A β pathology, neuroinflammation, and brain lipids.

Methods: For *in vitro* studies, U373 human astrocytoma cells were treated with A β fibrils and human recombinant PCSK9. mRNA expression of the proinflammatory cytokines and inflammasome-related genes were evaluated by q-PCR, while MCP-1 secretion was measured by ELISA. For *in vivo* studies, the cognitive performances of a newly generated mouse line - obtained by crossing 5XFAD^{Het} with PCSK9^{KO} mice – was tested by the Morris water maze test. After sacrifice, immunohistochemical analyses were performed to evaluate A β plaques deposition, distribution and composition, BACE1 and synaptophysin immunoreactivity, as well as microglia and astrocyte reactivity. Cholesterol and hydroxysterols in mouse brains were quantified by fluorimetric and LC-MS/MS analysis, respectively. Statistical comparisons were performed according to one- or two-way ANOVA, two-way repeated measure ANOVA and Chi square test.

Results: *In vitro*, PCSK9 significantly increases IL-6, IL-1 β and TNF- α mRNA levels in A β fibrils-treated U373 cells without influencing inflammasome gene expression. *In vivo*, PCSK9 ablation significantly improves cognitive performances and ameliorates spatially directed strategies in 5XFAD mice; these changes are accompanied with a reduced corticohippocampal A β burden without affecting neither spatial/regional distribution and composition nor global BACE1 expression. Furthermore, PCSK9 loss in 5XFAD mice induces a decreased microgliosis and astrocytes reactivity,

indexes of attenuated neuroinflammation, in several brain regions. Finally, knocking out PCSK9 has minimal impact on brain cholesterol and hydroxysterols.

Conclusions: These data indicate a protective role of PCSK9 ablation against cognitive decline, through improved A β pathology and attenuation of neuroinflammation in a severe mouse model of AD. PCSK9 may thus be considered a novel pharmacological target.

Keywords: PCSK9; microglia; amyloidosis; cognitive dysfunction; 5XFAD mice

1. Background

Alzheimer's disease (AD) is a neurodegenerative condition representing the most frequent form of dementia in the elderly. Clinically, AD is characterized by a progressive cognitive decline, accompanied by worsening of executive abilities [1]. AD causes are still largely unknown, a fact that contributes to the lack of effective interventions to prevent, slow down or reverse the disease progression. Indeed, the available drugs are targeting symptoms, with transient effects, and novel treatments have mostly failed in clinical studies. The most investigated pathophysiological processes underlying the disease are the increased extracellular deposition of amyloid- β peptide (A β) aggregates and the enhanced intracellular content of hyperphosphorylated tau, forming the senile plaques and neurofibrillary tangles, respectively [2, 3]. However, a growing number of evidence highlights the importance of additional players concurring to the complex AD pathophysiology, including among the others alterations of lipid metabolism [4] and neuroinflammation [5]. The association between lipids and AD is supported by several genetic studies, in which loci and genes involved in cholesterol metabolism are risk factors for AD onset and development [6-8]. The most striking example is the evidence that the gene for ϵ 4 isoform of the apolipoprotein E (*APOE*), the main lipid transporter in the central nervous system (CNS), is one of the most powerful predictive factors for AD onset [9, 10]. Among brain lipids, cholesterol play a critical role in the maintenance of neuronal functions [11] and several reports suggest that defect in its homeostasis in CSN are associated to AD [12, 13]. The Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9), a protein primarily expressed in the liver, plays an important regulatory role on plasma cholesterol levels modulation [14]. Actually, PCSK9 is also expressed in the brain, where it may be involved in several relevant processes, including neuronal development and apoptosis [15, 16]. Most recent evidence suggests a potential implication of PCSK9 in the pathogenesis of AD [17]. For instance, we and others previously observed increased levels of PCSK9 in the cerebrospinal fluid (CSF) of AD patients compared to control subjects [18, 19], with the highest concentrations detected in *APOE* ϵ 4 carriers [19]. Consistently, it was observed an increased PCSK9 expression in post-mortem AD patients compared to controls, specifically in the frontal cortex, a brain region greatly affected by the pathology [20]. The mechanisms underlying PCSK9 involvement in AD may relate to the ability to degrade the neuronal APOE-interacting receptors (APOER), as occurs for low density lipoprotein receptor (LDLR) in the liver [21, 22], thus interfering with neuronal cholesterol uptake, the sole process warranting cholesterol supplying in adult neurons, for the maintenance of physiological functions [23]. Other possible mechanisms may rely on PCSK9 reported pro-apoptotic effect [22, 24]. In support of these hypotheses, we recently demonstrated an *in vitro* interference by PCSK9 on astrocyte and neuronal cholesterol metabolism, leading to a neuronal cholesterol depletion, due to degradation of the APOERs LDLR and ApoER2.

Moreover, PCSK9 worsened the neurotoxic effect induced by A β fibrils [25]. In addition, PCSK9, by targeting also the APOER low-density lipoprotein receptor-related protein 1 (LRP1), may also reduce the clearance of A β peptide through the blood-brain barrier (BBB), thus favoring plaques deposition in the brain [26]. Finally, PCSK9 may additionally exert a pro-inflammatory effect on cerebral cells, as previously reported in macrophages [27-30]. In this regard, the neuroinflammatory effect of PCSK9 has not been investigated yet. All these findings point to PCSK9 as a potential pharmacological target for the treatment of AD. However, despite this set of evidence, the relationship between PCSK9 and AD is still largely unknown and not fully supported by genetic studies, that did not provide convincing indications on the association between PCSK9 variants and disease risk [20, 31-33]. Thus, in order to shed more light into PCSK9 pathophysiological role in AD, in the present study, we have first investigated *in vitro* the effects of PCSK9 on neuroinflammation. Then we evaluated the impact of a genetic ablation of PCSK9 *in vivo* by evaluating cognitive performances of 5XFAD mice, a severe mouse model of AD. In addition, to add more mechanistic insights, we investigated the impact of PCSK9 loss on A β plaques deposition, distribution and composition. Finally, we explored *ex vivo* PCSK9 influence on microglia and astrocytes reactivity, as neuroinflammatory indexes, and measured the levels of brain cholesterol and its main oxidative metabolites.

2. Methods

2.1. *In vitro* inflammation studies

For *in vitro* inflammation studies, U373-MG human astrocytoma cell line were used. Cells were kindly donated by Prof. Bussolati from the Department of Medicine and Surgery of the University of Parma (Italy). Before use, the cell line underwent authentication by the Cell Line Authentication (CLA) service (Eurofins Genomic, Ebersberg, Germany). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) High Glucose supplemented by 10% [Fetal Bovine Serum](#) (FBS), 1% penicillin/streptomycin solution (10.000 U/mL and 10 mg/mL, respectively), 1% L-glutamine 200 mM and 1% non-essential amino acids 100X solution. Cell culture reagents were from EuroClone (Milan, Italy). A β was purchased from Bachem (cat. n. 4014447.1000) and recombinant human PCSK9 was purchased from Cayman Laboratories (cat. n. 20631).

To evaluate the influence of PCSK9 on inflammation, cells were treated for 24 hours (h) with human recombinant PCSK9 [5 μ g/ml], then incubated for the following 24h with A β fibrils [1 μ M], prepared from monomers as previously described [25]. At the end of the incubation, total mRNA was extracted by means of iScriptTM RNA extraction kit Bio-Rad and 1 μ l of total extract was used for the one-

step qPCR reaction (one-step qPCR SYBR mix, A&A Biotechnology) using specific primer mix combinations (**Table 1**). *18S* was used as loading control in each experiment.

For the evaluation of MCP1 secretion, after treatment of cells as described above, medium was collected and ELISA assay was performed, using the human MCP1 ELISA kit (R&D System, cat. n. DCP00; Intra- and inter-assay precisions are 5%). All data are shown as mean $\pm$ standard deviation (SD) and statistical comparisons were performed using the one-way ANOVA followed by the Tukey's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

2.2. Animals and procedure

A transgenic mouse line was generated by multiple crossings between 5XFAD^{+/+} (5XFAD^{Het}) mice and PCSK9^{-/-} (PCSK9^{KO}) mice. The 5XFAD transgenic mouse model of AD co-overexpresses a triple-mutant human amyloid precursor protein (APP) (Swedish mutation: K670N, M671L; Florida mutation: I716V; London mutation: V717I) and a double-mutant human presenilin 1 (PS1) (M146L and L286V mutations) under the transcriptional control of the neuron-specific Thy-1 promotor. Progenitors with no retinal degeneration allele *Pde6brd1* were purchased from Jackson Laboratories, Bar Harbor, and first generation progenies were obtained by crossing 5XFAD^{Het} mice with PCSK9^{KO} breeders. 5XFAD^{Het}PCSK9^{Het} mice were selected and crossed with PCSK9^{KO} mice to obtain 5XFAD^{Het}PCSK9^{KO} mice and related controls (PCSK9^{Het} and PCSK9^{KO}, hereinafter mentioned as Ctrl). Mice were kept in conditioned rooms with stable temperature (21 °C) and humidity (60%), on a light/dark cycle of 12 h; food and water were available *ad libitum*. All animal procedures were approved by the Committee on Animal Health and Care of the University of Modena and Reggio Emilia (protocol number: n° 511/2019-PR) and conducted in accordance with National Institutes of Health guidelines.

2.3. Behavioral screening and analysis

After reaching 7 months (mo) of age, both male and female mice were subjected to Morris water maze (MWM) procedure to assess learning and memory as previously described [34]. Briefly, mice were placed in a circular white pool with a diameter of 120 cm filled with water at $22 \pm 1^\circ\text{C}$ (made opaque by adding white non-toxic paint), and allowed to swim for 90 seconds (s) or until they found the location of a hidden circular platform with 11 cm diameter; during this learning phase mice were trained with 4 trials per day (starting from a different quadrant with different visual cues at each trial) for 7 days with 30 minutes (min) inter-trial interval and the platform was located in the South (S) quadrant of the pool. For each trial, the time to reach the platform and number of entries in the platform areas were analyzed (learning curve Day1-Day7, D1-D7). On the 8th day the platform was

moved to East (E) quadrant and two cues were removed; mice underwent a second learning phase consisting in 4 trials per day with a 60 min inter-trial interval between days D8-D13. To assess memory retention, the platform was removed on D14 and the animals were allowed to swim for 60 sec (probe test), and the latency to reach the platform area, total entries in the platform area and the % of time spent in target quadrant were evaluated. The analysis of target search strategy was carried out according to current literature [35]. Briefly, spatial accurate strategy and non-spatial strategy were distinguished considering individual swim paths during probe test.

Behavioural test was performed by an operator unaware of the treatment to avoid bias. Animal behaviour was conducted in a sound-proof room, recorded and automatically analyzed with ANY-maze Video Tracking system (Stoelting). Target search strategy was manually scored, independently, by two operators unaware of the treatment.

All data are shown as mean $\pm$ standard error of the mean (SEM) and were analysed by one-way analysis of variance (ANOVA), two-way repeated measures ANOVA followed by the Bonferroni's correction, and Fisher's test as appropriate using the statistical package SPSS (version 26). Differences were considered significant with $p < 0.05$ as the level for a significant difference and $0.10 > p > 0.05$ as trend for a significant difference.

2.4. Immunohistochemical analysis

A group of mice was anesthetized with isoflurane and sacrificed by intracardiac perfusion with cold 4% paraformaldehyde and fixed brains were cut at the cryostat (20 μ m) and processed according to established protocols to test cell-specific AD alterations by using specific antibodies against amyloid plaques and microglial cells through immunohistochemical (IHC) analysis [36, 37].

The following antibodies (Abs) were used to test specific AD-linked alterations at synaptic and cellular levels: mouse anti-human A β /APP 6E10 (epitope human A β 1-16, 1:500, Signet, cat. n. 9300-10), mouse anti-human A β 11A1 (epitope synthetic peptide of E22P-A β 10-35 part, 1:500, Tecan, cat. n. 10379), rabbit anti-ionized calcium-binding adapter molecule 1 (IBA1, 1:1000, Wako, cat. n. 019-19741), rabbit anti-glial fibrillary acidic protein (GFAP, 1:2000, Dako, cat. n. Z0334) and rabbit anti-synaptophysin (SYN, 1:1000, SYSY, cat. n. 101004), rabbit anti-beta-site APP-cleaving enzyme 1 (BACE1, 1:1000, Cell Signaling, cat. n. 5606). A pre-treatment with formic acid was performed for anti-A β Abs. Vectastain ABC-HRP (cat. n. PK-4002; cat. n. PK-4001) kit was used for peroxidase diaminobenzidine (DAB) stainings; slices were placed on gelatinized glass slides, dehydrated and mounted with Eukitt® mounting medium. Thioflavin-S (ThS, Sigma, cat. n. 1892) and Congo Red (Sigma, cat. n. 6277) stainings were performed according to established protocols [36].

2.5. Image and statistical analyses

Immunolabelled brain slices were visualized and photographed on a Nikon Eclipse CiL microscope (10x and 40x objective) through a Nikon DS-Fi3 camera under constant light conditions. Shading error correction was performed before image acquisition to correct for irregularities in illumination of the microscopic field. All evaluations were performed on coded slides by at least two experimenters.

ThS- or A β -immunoreactive plaque analysis was performed on images obtained with a 10x objective. Plaques were manually selected within the neocortex (Nctx) and the hippocampus (Hip) (Bregma between -1.46 and -1.58 mm from Paxinos and Franklin) and morphometric parameters such as plaque number as well as single plaque areas were measured through a Nikon NIS-Elements D software or ImageJ.

For SYN and BACE1 immunoreactivity (ir) analysis, three somatosensory cortex (sCtx) (exclusively for SYN) and CA3 field of hippocampus slices/mouse (Bregma between -1.46 and -1.58 mm from Paxinos and Franklin 2019) were acquired with 10x objective before counterstaining with Nissl stain to better delineate the hippocampal subregion boundaries. The semi-quantitative densitometric and morphometric analyses (area, length and thickness) were performed following established protocols [38, 39] using routines from the Nikon NIS-Elements D (5.21.00v) and ImageJ Fiji software. First, images were manually edited to remove edge artifacts, folds, and blood vessels, then subjectively thresholded.

For densitometric analysis, acquired images were converted to 32 bit grayscale and specific optical density values were obtained by subtracting the optical density of the sampled region from the optical density of non-specific staining, i.e., pyramidal layer for CA3 analysis.

For GFAP and IBA1 analysis, at least three slices/mouse from corpus callosum-cingulate cortex (CC-Cg) (Bregma between -0.5 and -0.9) and Hip (Bregma between -1.46 and -1.58 mm from Paxinos and Franklin 2019) were acquired with 10x objective. Acquired images were converted to 32 bit grayscale and the following thresholding procedure was applied: for GFAP staining -35, -20, -40, -5 grayscale units from the peak mean grey value for hippocampal CA1 field, CA3 field, dentate gyrus (DG) hilus and CC-Cg; for IBA1 staining -10 grayscale units for CC-Cg and DG hilus; then, % positive area, i.e., the area covered by pixels with gray value above the threshold, was automatically recorded by means of *analyse particles* function of ImageJ software. As specifically concerns microglial analysis, IBA1+ cell count was performed manually, within the area of interest by using a 10x objective.

Values are shown as mean $\pm$ standard error of the mean (SEM) and 95% confidence interval as appropriate. Group differences were analysed by means of one- or two-way ANOVA as appropriate.

Proportions were analysed by means of the Chi square test. Correlations were analysed by means of the Spearman test. Statistical analyses were performed through SPSS software, with $p < 0.05$ as the level for a significant difference and $0.10 > p > 0.05$ as trend for a significant difference. Detailed statistical reports are shown in **Suppl.Table 7**.

2.6. Total cholesterol (TC) quantification in mouse brain and serum

After the sacrifice, the right cerebral hemisphere of mice was weighted and mechanically homogenated; lipids were extracted by extracting homogenates for 24h with the Folch's Solution (Chloroform:Methanol [2:1] v/v). The lipophilic phase was then evaporated under nitrogen stream and re-suspended in Reaction Buffer (0.5 M potassium phosphate, 0.25 M NaCl, 25 mM cholic acid and 0.5% Triton X-100). The fluorometric Amplex® Red Cholesterol assay (Thermo-Fisher Scientific, MA, USA) was used to quantify cholesterol content in lipids extracted from brain following the manufacturer's instructions. To quantify TC levels in serum, peripheral blood was collected after sacrifice in BD VACUTAINER CAT tube and after 1 h incubation at room temperature (RT), serum was isolated by centrifugation at 2500 rpm for 10 min; then the fluorometric Amplex® Red Cholesterol assay (Thermo Fisher Scientific, MA, USA) was used to quantify TC following the manufacturer's instructions. All data are shown as mean $\pm$ SEM and statistical comparisons among the four genotypes were performed using the two-way ANOVA, considering a $p\text{-value} \leq 0.05$ as statistically significant and $0.10 > p > 0.05$ as a trend for a significant difference. Statistical comparison between wild type (Wt) B6SJL and 5XFAD mice were performed by unpaired t-test. A value of $p < 0.05$ was considered statistically significant.

2.7. Hydroxysterols quantification in mouse brain and serum

The three main hydroxysterols (24-OHcholesterol, 25-OHcholesterol and 27-OHcholesterol, 24-OHC, 25-OHC and 27-OHC, respectively) quantification in mouse brain and sera was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [40]. Specifically, the lipophilic phase extracted through the Folch's solution from the left cerebral hemisphere, as well as from 0.2 ml of serum, were supplemented with Butylhydroxytoluene (BHT) [0.1 $\mu\text{g}/\mu\text{l}$] and underwent alkaline hydrolysis in 1ml of 1M NaOH in 90% Ethanol for 2 h at 60°C. One ml of saline was then added and sterols were extracted with 2 ml of Petroleum Ether 40°- 60°. Dried lipid extracts were then resuspended in 2 ml of deionized H₂O supplemented with 5% v/v of Methanol and oxysterols were separated by Solid Phase Extraction (SPE) Cartridges C18 (Waters, MA, USA), as previously described [41].

LC-MS/MS analysis was then carried out by adding to each dried sample 100 µl of Methanol enriched with 1 µg/µl of 24-, 25- and 27-deuterated OHC (Avanti Polar Lipids, AL, USA), used as internal standards. Samples were subsequently eluted through a C18 Synergi Hydro-RP column (150 mm x 3mm, 80A, 4 µm particles; Phenomenex, CA, USA), equipped with a C18 pre-filtering column. The mobile phase was made of Methanol : acetonitrile : H₂O [14:1:0.6], added with 0.1% v/v formic acid. The elution was isocratic at a flow rate of 200 µl/min for 50 min, the injection volume was 20 µl and each sample was injected in duplicate. The analysis was carried out using an Agilent HP 1200 chromatographic system (Agilent Technologies, CA, USA), with a 100-vial capacity sample tray and coupled with a QTRAP 4000 triple quadrupole mass spectrophotometer (ABSCIEX, CA, USA), equipped with a pneumatically assisted ESI interface. Nitrogen (99.99% of purity) was used as a sheath gas and the auxiliary gas was delivered at flow rates of 45 and 5 arbitrary units, respectively. Optimized conditions of the interface were set as follows: ESI voltage 5.5 kV; declustering potential (DP) 35V; entrance potential (EP) 10V; collision exit potential (CXP) 10V and capillary temperature 350°C. Experiments were performed under positive ion-selected reaction monitoring conditions, using nitrogen as a collision gas (pressure of 2.1×10^{-3} mbar). The analytical method performed was initially set up by using 24-, 25- and 27-OHC spiked with 1 µg/µl of the respective deuterated internal standard. All data are shown as mean $\pm$ SEM and statistical comparisons were performed as described above.

2.8. Total RNA extraction, reverse transcription, and real time polymerase chain reaction (*ex vivo*)

A separate group of mice was sacrificed under deep anesthesia through intracardiac injection of cold PBS, the brain was dissected out and cerebral cortex (Ctx) was lysed by mechanical disruption in Trizol reagent (Qiagen), homogenized following the procedure provided by the manufacturer (RNeasy Plus Mini Kit, Qiagen) and processed for qPCR as described [42]. Isolated mRNA was reverse transcribed to cDNA using random hexamers and M-MLV Reverse Transcriptase (Promega Corporation) following the instructions provided by the manufacturer. Samples were heated at 70 °C for 5 min to eliminate any secondary structures, then incubated at 23 °C for 10 min, 1 h at 37 °C, and 5 min at 95 °C before being chilled at 4 °C using a thermocycler T Gradient (SimpliAmp, Applied Biosystem). The amount of cDNA was quantified with iTaq Universal SYBR Green Supermix (Bio-Rad) using a Bio-Rad RT-PCR iCycler. Each PCR reaction was performed in triplicate using 300 nM of each primer (**Table 1**), 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad), cDNA and nuclease-free water with the following cycling parameters: 2 min at 95 °C and 40 cycles of 5 min at 95 °C and 30 sec at 60 °C, followed by 5 sec at 95 °C and 65° to 95 ° melting curve analysis.

Murine primers for *ex-vivo* analysis

Function	Transcript	NCBI Ref Seq	Primer	sequence (5' - 3')
Housekeeping genes	<i>Gapdh</i>	NM_008084.3	Fw	CAAGGTCATCCATGACAACTTTG
			Rv	GGGCCATCCACAGTCTTCTG
	<i>CypA</i>	NM_008907.2	Fw	AGCATACAGGTCCTGGCATC
			Rv	TTCACCTTCCCAAAGACCAC
	<i>Rer1</i>	NM_026395.2	Fw	CCACCTAAACCTTTTCATTGCG
			Rv	TTGTAGCTGCGTGCCAAAAT
Lipid-related genes	<i>ApoE</i>	NM_001305843.1	Fw	TGTGGGCCGTGCTGTTGGTC
			Rv	GCCTGCTCCCAGGGTTGGTTG
	<i>Lpl</i>	NM_008509.2	Fw	GGATGGACGGTAACGGAAT
			Rv	GGCCACATCATTTCCCACCA
	<i>Lrp1</i>	NM_008512.2	Fw	AAGGATGACTGTGGAGAC
			Rv	ACTACAGCCACCATTATTC
	<i>Lrp5</i>	NM_008513.3	Fw	CAGTGTGCCCCTCTATGACC
			Rv	CGAATGACGTAGGGCCTGTA
	<i>CD36</i>	NM_001159558.1	Fw	GGAGCAACTGGTGGATGGTT
			Rv	TACGTGGCCCGGTTCTACTAA
Microglial factors	<i>Stmn1</i>	NM_019641.4	Fw	AGGTGCTCCAGAAAGCCATC
			Rv	TCCACGTGCTTGTCTTCTC
	<i>P2y12r</i>	NM_001357010.1	Fw	CATTGCTGTACACCGTCCTG
			Rv	AACTTGGCACACCAAGGTTT
	<i>Ifitm3</i>	NM_025378.2	Fw	CCGTGAAGTCTAGGGATCGGA
			Rv	GTGTGAAGGTTTTGAGCGTT
	<i>CD11b</i>	NM_001082960.1	Fw	TACCGTCTACTACCCATCTGGC
			Rv	TTGGTGAGCGGGTTCTGG
	<i>Cx3cr1</i>	NM_009987.4	Fw	ACGGAGACAGTGGCGTTCAG
			Rv	CGGCCAGGCACTTCCTATAC

	<i>Cst7</i>	NM_009977.3	Fw	CAGGAAGACCATGCATCACCA
			Rv	ATAGAGTCCGCTTCAAGGCAG
	<i>Il1β</i>	NM_008361.4	Fw	ATCTTTGAAGAAGAGCCCATCC
			Rv	TGTAGTGCAGTTGTCTAATGGG
Inflammatory mediators	<i>Ccl3</i>	NM_011337.2	Fw	CATATGGAGCTGACACCCCG
			Rv	TCTTCCGGCTGTAGGAGAAGC
	<i>Ccl6</i>	NM_009139.3	Fw	CTGGCCTCATACAAGAAATGGA
			Rv	TTGGAGGGTTATAGCGACGAT
Human primers for <i>in vitro</i> analysis				
Housekeeping gene	<i>18S</i>	X03205.1	Fw	CGGCTACCACATCCACGGAA
			Rv	CCTGAATTGTTATTTTTCGTCCTACC
	<i>PYRIN</i>	NM_000243	Fw	GTACTTCTCAGAAACCCTGCG
			Rv	AGATGAGGTTGGGGTAAGCG
Immune cell factors	<i>NLRP1</i>	NM_033004	Fw	TTCTACGTTGGCCACTTGGG
			Rv	GTGAAGGTACGGCTATGCGG
	<i>NLRP3</i>	NM_001243133	Fw	AAGTGGGGTTCAGATAATGCACG
			Rv	CACTGGAATCTGCTTCTCACG
	<i>NLRC4</i>	NM_001199138	Fw	CCTACAGAATCAACGGCTGC
			Rv	TCAGGCCTTCAGCTAGTTTTATAGC
	<i>TNFα</i>	NM_000594	Fw	ACTTTGGAGTGATCGGCC
			Rv	GCTTGAGGGTTTGCTACAAC
Inflammatory mediators	<i>IL6</i>	NM_000600	Fw	GGTACATCCTCGACGGCATCT
			Rv	GTGCCTCTTTGCTGCTTTCAC
	<i>IL1-β</i>	NM_000576	Fw	ATGCACCTGTACGATCACTG
			Rv	ACAAAGGACATGGAGAACACC

Table 1: List of primer used for Real-Time PCR analyses. For each transcript analyzed in *ex-vivo* or *in vitro* experiments, forward (Fw) and reverse (Rv) primer sequences are indicated alongside with general functional classification and NCBI reference.

2.9. PCR product and statistical analyses (*ex vivo*)

For gene expression analysis, mRNA levels of each experimental gene target were normalized to the average of three housekeeping genes. Glyceraldehydes-3-phosphate dehydrogenase (*Gapdh*), retention in endoplasmic reticulum sorting receptor 1 (*Rer1*) and cyclophilin A (*CypA*) reference genes were used as endogenous controls. For quantitative evaluation of changes, the comparative $\Delta\Delta C_t$ method was performed, using as calibrator the average levels of expression of PCSK9^{Het} mice. Statistical comparisons were performed using the two-way ANOVA, considering a value of $p \leq 0.05$ as statistically significant and $\# 0.10 > p > 0.05$ as a trend for a significant difference.

3. Results

3.1. *In vitro* analysis of inflammation

To define the potential involvement of PCSK9 on A β -induced inflammatory response, we performed *in vitro* studies by using the U373 astrocytoma cell line [43]. We first defined the inflammatory response of U373 to A β fibrils, used *in vitro* to simulate an AD-like condition [44]. As shown in **Fig.1**, A β fibrils (1 μ M) slightly but significantly upregulated the mRNA expression of *IL1- β* and *IL-6* without inducing significant changes in *TNF α* . Importantly, the co-incubation of A β fibrils and recombinant PCSK9 [5 μ g/ml] further induced the levels of all the three cytokines, suggesting a strengthening proinflammatory action of PCSK9 on basal A β fibrils activity. The A β -induced release of proinflammatory cytokines by astrocytes depends on the expression of several receptors implicated in cell activation such as the CD36 receptor. In addition, the binding of A β to the CD36 receptor triggers the assembly of a heterotrimeric complex with the Toll-like receptors (TLRs) 4 and 6, essential step for host defense against pathogens. We thus evaluated the impact of PCSK9 on these receptors [45]. We found that PCSK9 was not able to modulate CD36 or TLR-4 and 6 gene expression (not shown).

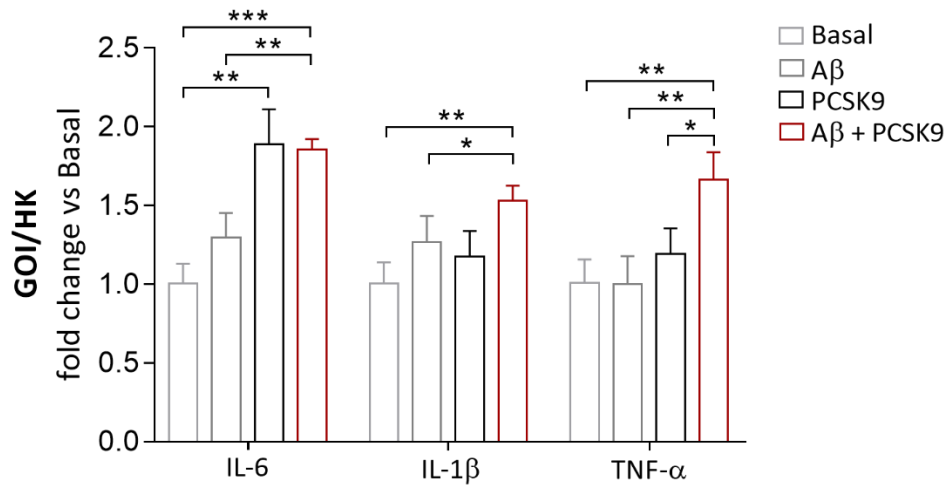


Figure 1: Effect of Aβ fibrils and PCSK9 on cytokines mRNA expression in U373 astrocytoma cell line. U373 cells were treated for 24 h with human recombinant PCSK9 [5 µg/ml], then incubated for the following 24 h with Aβ fibrils [1 µM]. At the end of the incubation total RNA was extracted and real time qPCR was performed. Data were performed in triplicate and are expressed as mean ± SD. Statistical analyses were performed using the one-way ANOVA with a Tukey's multiple comparison test; * p < 0.05; ** p < 0.01; *** p < 0.001.

To extend the data observed on mRNA levels of proinflammatory cytokines, we performed an additional analysis by measuring the release of Monocyte Chemoattractant Protein-1 (MCP-1), by ELISA into the conditioned media. This analysis revealed that Aβ fibrils significantly increased the release of this chemokine from U373 cell line, and this effect was further enhanced by the co-incubation with PCSK9 (**Fig.2**).

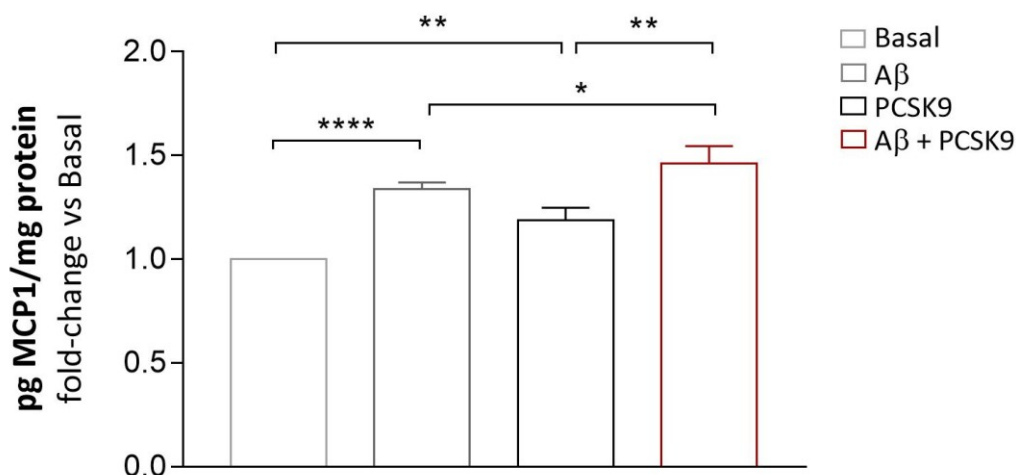


Figure 2: Effect of Aβ fibrils and PCSK9 on MCP1 secretion from U373 astrocytoma cell line cytokines. U373 cells were treated for 24h with human recombinant PCSK9 [5 µg/ml], then incubated for the following 24 h with Aβ [1 µM]. At the end of the incubation the conditioned media were collected, and ELISA was performed. Data were performed in triplicate and are expressed as mean ± SD. Statistical analyses were performed using the one-way ANOVA with a Tukey's multiple comparison test; * p < 0.05; ** p < 0.01; **** p < 0.0001.

The effect of A β and PCSK9 on cytokine mRNA levels, prompted us to investigate the expression levels of inflammasome genes, such as *Pyrin*, *NLRP1*, *NLRC4* and *NLRP3*, the complex that induces the activation and release of IL1- β [46]. As expected, A β significantly upregulated *NLRP1*, *NLRC4* and *NLRP3* and the co-incubation with PCSK9 had an additive effect on mRNA levels of *Pyrin*, and *NLRC4*, although not statistically significant (**Fig.3**). These data further support the contribution of PCSK9 on inflammatory response in U373 astrocytoma cell line already exposed to A β fibrils.

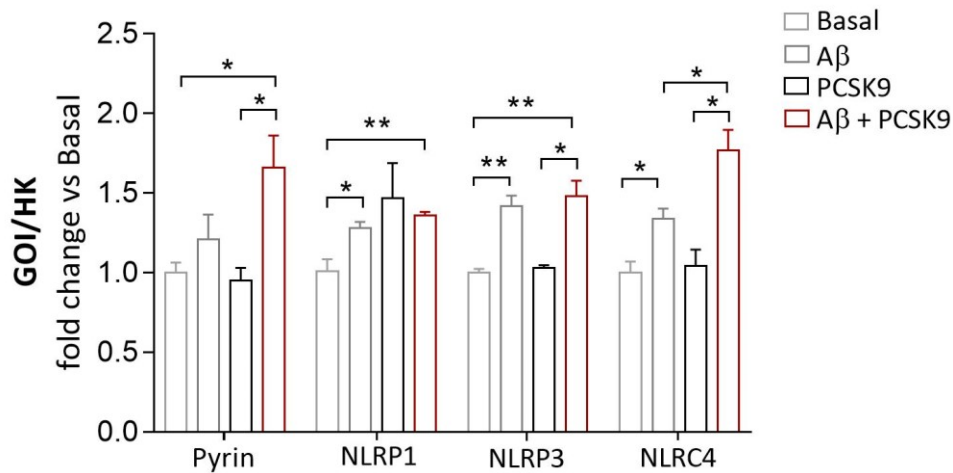


Figure 3: Effect of A β fibrils and PCSK9 on inflammasome mRNA expression in U373 astrocytoma cell line. U373 cells were treated for 24 h with human recombinant PCSK9 [5 μ g/ml], then incubated for the following 24 h with A β fibrils [1 μ M]. At the end of the incubation the conditioned media were collected, and ELISA was performed. Data were performed in triplicate and are expressed as mean $\pm$ SD. Statistical analyses were performed using the one-way ANOVA with a Tukey's multiple comparison test; * $p < 0.05$; ** $p < 0.01$.

3.2. Behavioral screening

In order to assess whether the absence of PCSK9 influenced cognitive decline in our *in vivo* model, learning abilities and spatial memory were tested in 10 mo 5XFAD-PCSK9^{KO} mice and related controls (Ctrls).

The performance of B6SJL and 5XFAD mice was preliminarily compared in MWM test, confirming a significant decrease in spatial learning abilities and memory in 10 mo 5XFAD mice (**Suppl.Figure1 A,B**). Subsequently, the MWM test was performed comparing PCSK9^{Het}, PCSK9^{KO}, 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO} mice. Swim speed of all tested groups did not differ throughout the experiment (not shown). The learning curve analysis showed a progressive decrease in escape latency to reach the hidden platform that was delayed in 5XFAD-PCSK9^{Het} mice and significantly ameliorated by PCSK9 loss, reaching values similar to those of Ctrls mice (**Fig.4 A**). Twenty-four hours after the last training session, mice underwent a 60 sec probe test to measure the task performance regardless of the absolute/precise location of the hidden platform relative to the intrapool

cues; the analysis of the performance was performed by measuring the % time spent in the target quadrant (E quadrant) and demonstrated a significant decrease in 5XFAD-PCSK9^{Het} mice that was only slightly counteracted by PCSK9 loss (Fig.4 B). Occupancy plots for grouped data showing the development of a quadrant- and platform position-specific preference are reported in Fig.4 C. To assess the qualitative aspects of learning the MWM task we further analyzed the search strategies displayed by the mice as a measure of the accuracy of swim trajectories used to find the platform location during the probe. The search strategy analysis demonstrated a statistically significant increase in spatially directed strategies and a decrease in non-targeted search strategies (random and non-cognitive) in 5XFAD-PCSK9^{KO} mice; furthermore, these changes were also accompanied with a minor prevalence of swimming close to the wall of the maze and a decreased preference for the adjacent sectors (N and S quadrants) compared to 5XFAD-PCSK9^{Het} mice (Fig.4 C-D). All together these results indicate a protective effect of PCSK9 loss in AD mice through an improvement of learning and memory accompanied with an ameliorated search strategy in locate platform position. Detailed statistical report is summarized in Suppl.Table 7.

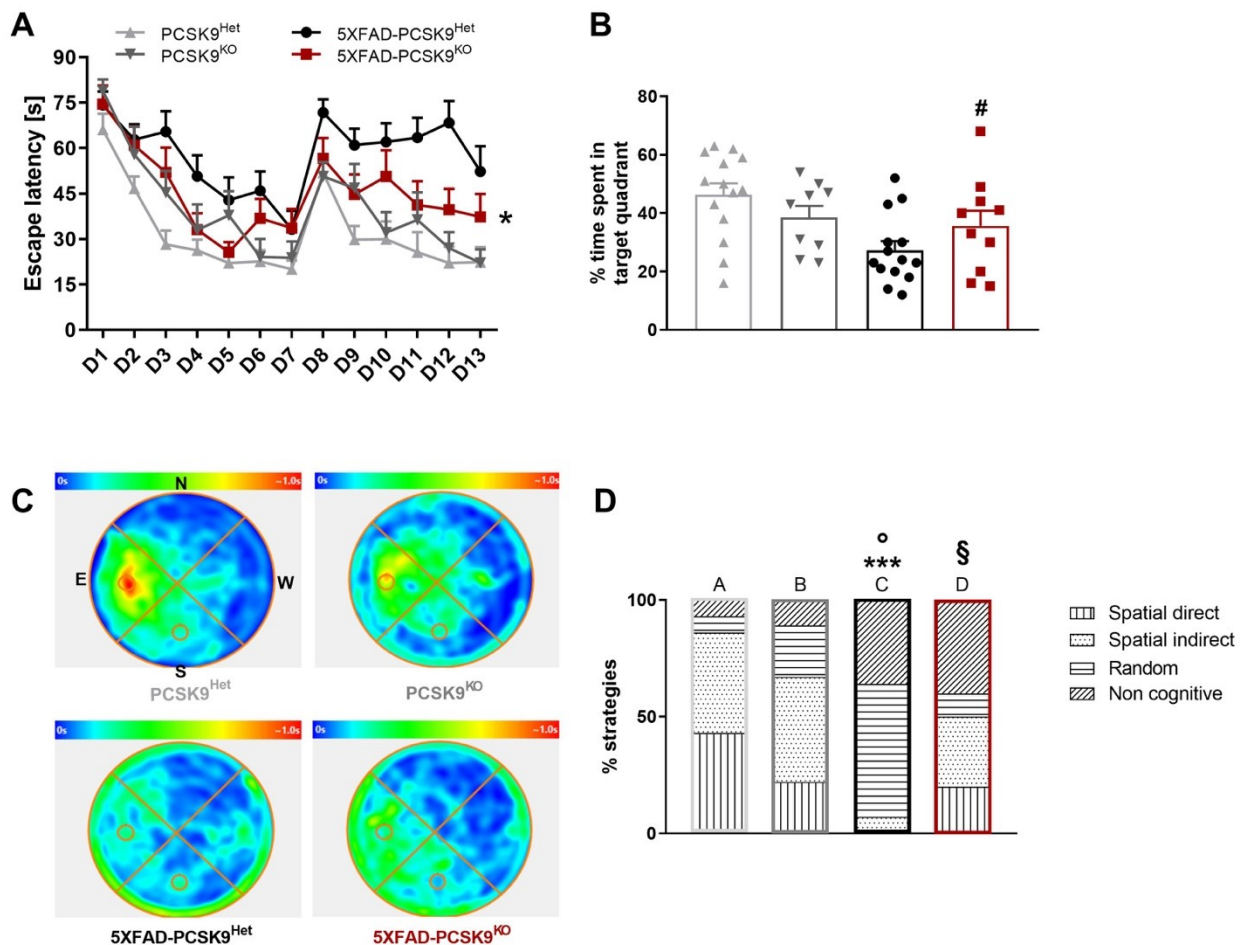


Figure 4: The loss of PCSK9 improves both learning in memory in AD mice. A Analysis of MWM learning curve

showing that the efficiency to solve the task is reduced in 5XFAD-PCSK9^{Het} mice and improved by PCSK9 loss. Except for 5XFAD-PCSK9^{Het} mice, the other three experimental groups acquired the task equivalently as illustrated by day block plots of escape latency. The time needed to find the platform (escape latency) was averaged for each day (4 trials/day) and analyzed by a two-way repeated measures ANOVA with training day and genotype as factors; * $p < 0.05$. **B** A decreased preference for searching for the platform in the trained target quadrant (east (E) quadrant) during the probe test in AD mice was only slightly improved by PCSK9 loss, indicating that in 5XFAD mice the acquisition of the memory for the platform location is impaired. The % of time spent in the target quadrant during 60 sec of probe test was analyzed by a two-way ANOVA with APP and PCSK9 expression as factors. # $0.10 > p > 0.05$ trend for a significant difference. **C** Occupancy plots for grouped data showing the position where experimental mice concentrated their searches in the probe test. The color scale represents the average search time during time. **D** The analysis of search strategy showed an increase in spatially targeted strategies in 5XFAD-PCSK9^{KO} mice compared to 5XFAD-PCSK9^{Het} mice. Data are analyzed by chi square test; * vs Wt-Het; ° vs Wt-KO; § 5XFAD-PCSK9^{KO}. °,§ $p < 0.05$; *** $p < 0.0001$. Data are shown as mean $\pm$ SEM. Experimental groups: Wt-PCSK9^{Het}, $n = 14$; Wt-PCSK9^{KO}, $n=10$; 5XFAD-PCSK9^{Het}, $n=15$; 5XFAD-PCSK9^{KO}, $n=12$.

3.3. Immunohistochemical analysis

3.3.1. Amyloid plaques

It is known that A β deposition in 5XFAD mice is a precocious event and a key pathology for AD development [36]. In order to investigate whether improved behavioral scores were associated to a reduction in AD-like neuropathology, brain slices from 10 mo Ctrl, 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO} mice were processed through IHC staining for plaques visualization. Different antibodies for A β and APP (anti-6E10 Ab) or A β 42 neurotoxic oligomers (anti-11A1 Ab), as well as ThS standard staining for β -sheet structure and thus marker for fibrillar and compact A β , were used. After staining, the number of protein aggregates as well as the mean % area occupied by A β or ThS stain were compared. Figure 5 provides representative images of hippocampal (DG, **Fig.5 A-B**) and cortical (Nctx, **Fig.5 E-F**) ThS⁺ amyloid plaques and their relative quantification (**Fig.5 C,D,G,H**). Our analysis demonstrated the selective presence of ThS⁺ β -sheet-rich protein aggregates in both 5XFAD-PCSK9^{KO} and 5XFAD-PCSK9^{Het} (**Fig.5 A-E**) but never in Ctrl (not shown). A significantly reduced number of ThS⁺ amyloid plaques by about 40% was observed both in DG ($p=0.004$) and Nctx ($p=0.005$) of 5XFAD-PCSK9^{KO} mice with respect to 5XFAD-PCSK9^{Het} mice (**Fig.5 C,G** respectively). In the same brain areas, the reduction in ThS⁺ plaque number was paralleled by a similar reduction in ThS⁺ plaque area (DG, $p=0.009$; Nctx, $p=0.014$; **Fig.5 D,H**). All statistical data are summarized in **Suppl. Table 7**.

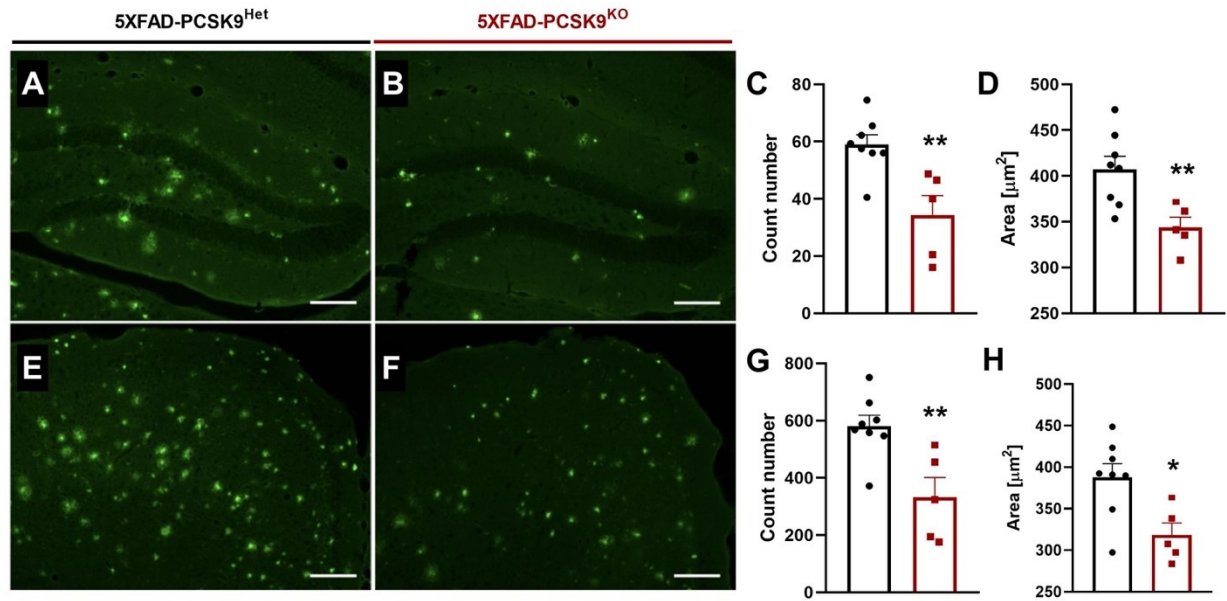


Figure 5: Plaque deposition is reduced in 5XFAD mice in absence of PCSK9. A-B, E-F Representative images showing ThS⁺ plaque in DG (A-B) and Nctx (E-F) of 10 mo 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO} mice. Scale bar=200 μm . C-D, G-H Quantitative analysis of plaque count (C,G) and area (D,H) in DG and Nctx of 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO} mice. Data are shown as mean $\pm$ SEM. Statistical analysis according to one-way ANOVA; * $p < 0.05$, ** $p < 0.01$. Experimental groups: 5XFAD-PCSK9^{Het}, $n=9$; 5XFAD-PCSK9^{KO}, $n=5$.

We further characterized amyloid plaques by means of IHC analyses with mouse anti-human A β /APP 6E10 (Fig.6 A, E, C, G) and mouse anti-human A β 11A1 (Fig.6 B, F, D,H). Since we observed that amyloidosis process was more pronounced in the Nctx than in the (Hip) of 10 mo AD mice (Fig. 5-6), we first evaluated if the ratio between hippocampal and cortical plaques loading was influenced by genotypes. Our results did not evidence any significant change induced by genotype in the ratio between A β -stained plaques, that was about 10% for both ThS⁺ and 11A1⁺ and 15% for 6E10⁺ plaques, suggesting that the spatial/regional distribution of A β plaques was maintained in the absence of PCSK9 (ThS: $p=0.850$; 6E10: $p=0.422$; 11A1: $p=0.218$) (Fig.6 M). Then, we noticed that in 5XFAD-PCSK9^{Het} mice the number and area of amyloid plaques were similar between ThS⁺ and 11A1⁺ plaques, and smaller than 6E10⁺ ones. In 5XFAD-PCSK9^{KO} mice, despite a global and significant decrease of amyloidosis was observed, the ratio between different stained plaques was maintained (chi-squared test, GD: count: $p=0.766$; area: $p=0.06$; Ctx: count: $p=0.4927$; area: $p=0.2020$) (Fig.6 I-L and Suppl.Table 7 for statistical report).

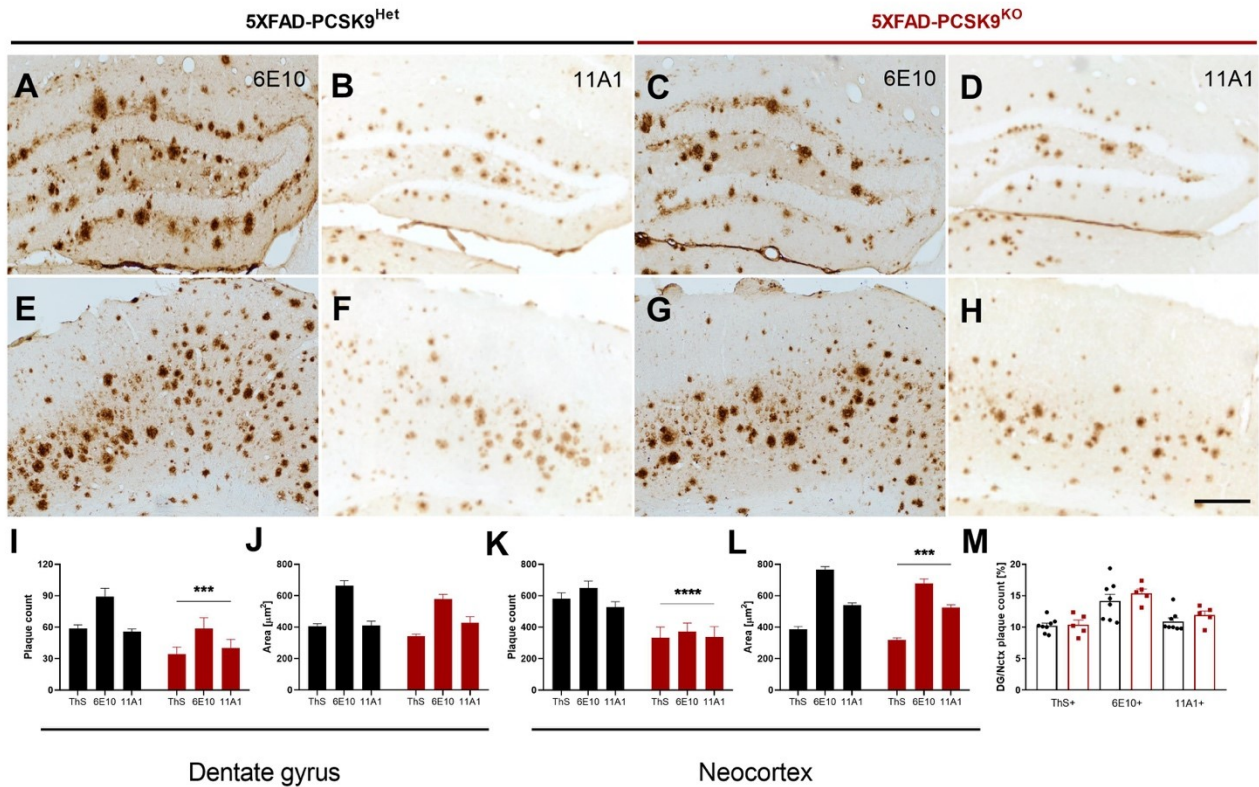


Figure 6: Spatial distribution and quantitative characterization of differently stained plaques within cortical and hippocampal regions. A-H Representative images showing 6E10⁺ and 11A1⁺ plaque in DG (A-B) and Nctx (E-F) of 10 mo 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO} mice. Scale bar=200 μ m. I-L ThS⁺, 6E10⁺ and 11A1⁺ amyloid plaque distribution in DG and Nctx of 5XFAD-PCSK9^{Het} (black bars) and 5XFAD-PCSK9^{KO} (red bars) mice. Globally, a decreased amyloidosis was detected in the 5XFAD-PCSK9^{KO} mice and the ratio between stain-specific was maintained within the experimental groups. M % of hippocampal/neocortical A β -positive plaques in 5XFAD-PCSK9^{Het} (black bars) and 5XFAD-PCSK9^{KO} (red bars) mice. Data are shown as mean $\pm$ SEM. Statistical analysis according to chi square test, one- and two-way ANOVA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Experimental group: 5XFAD-PCSK9^{Het}, n=9; 5XFAD-PCSK9^{KO}, n=5.

We then performed an analysis of the size distribution of amyloid plaques. Amyloid plaques in the hippocampal DG and Nctx of AD mice were stratified according to their size into four ranges. Our analysis demonstrated that the plaque size distribution was altered in 5XFAD-PCSK9^{KO} (Fig.7 A-F). Then, in order to identify which ranges of size are influenced by PCSK9 expression, we conducted a one-way ANOVA test followed by Bonferroni correction. In details, as concerns ThS analysis, we found a significant increase in the % plaque with a size $\leq 200 \mu\text{m}^2$ in DG (p=0.013) and $\leq 200 \mu\text{m}^2$ (p=0.006) in the Nctx, accompanied by a decrease in the range size $>1000 \mu\text{m}^2$ (p=0.0433) in the DG and in all ranges $>200 \mu\text{m}^2$ ($>200 \mu\text{m}^2$ - $= 500 \mu\text{m}^2$, p=0.089; $>500 \mu\text{m}^2$ - $= 1000 \mu\text{m}^2$, p=0.013; $>1000 \mu\text{m}^2$, p=0.04) in the Nctx of 5XFAD mice lacking PCSK9 compared to 5XFAD-PCSK9^{Het}. In addition, we found a trend towards a significant decrease in the % of 6E10⁺ plaques with medium and large size in the DG ($>500 \mu\text{m}^2$ - $= 1000 \mu\text{m}^2$, p<0.067; $>1000 \mu\text{m}^2$, p=0.079) and a significant

decrease in the % of large 6E10+ plaques in the Nctx ($>1000 \mu\text{m}^2$; $p=0.046$). No change was observed in the ranking analysis 11A1 % plaque size in DG, whereas a significant decrease in small and large size amyloid plaques was observed in the Nctx ($\leq 200 \mu\text{m}^2$, $p=0.038$; $>1000 \mu\text{m}^2$, $p<0.001$) (**Fig.7**). To investigate if reduced plaque area is a result of increased amyloid compaction or a consequence of a delayed amyloidosis, we further examined A β compaction expressed as the diffuseness index ((Area 6E10 – Area ThS) / Area 6E10) (not shown). The analysis did not demonstrate any influence of PCSK9 loss in the plaque diffuseness both in Nctx and Hip (DG: $p=0.2844$; Nctx: $p=0.3434$). Altogether, the data demonstrated that the absence of PCSK9 induced a global reduction in amyloidosis without although influencing regional distribution and maturation.

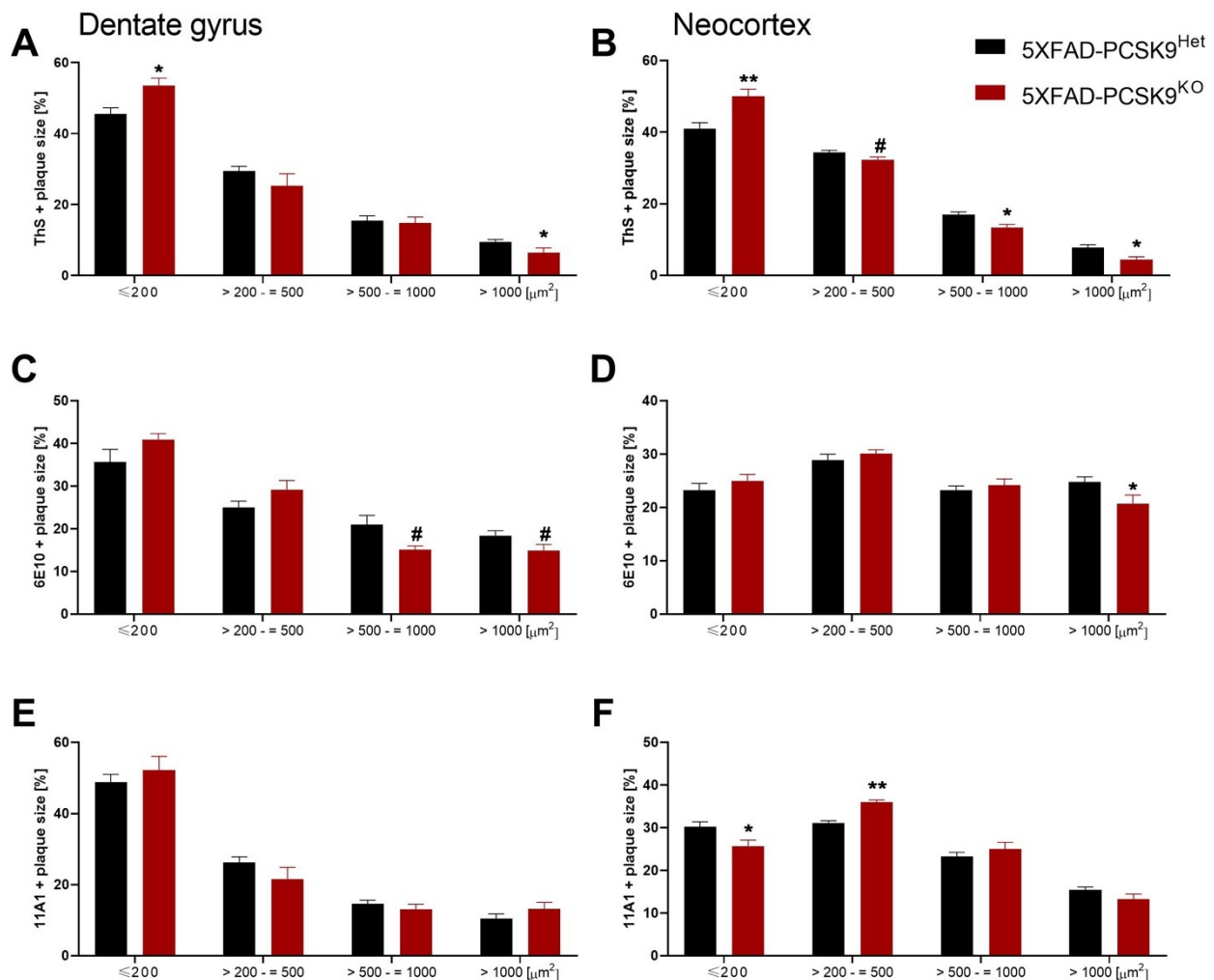


Figure 7: Size distribution of ThS⁺, 6E10⁺ and 11A1⁺ amyloid plaque in the hippocampal dentate gyrus (A,C,E) and neocortex (B,D,F) of 5XFAD-PCSK9^{Het} (black bars) and 5XFAD-PCSK9^{KO} (red bars) mice. Data are shown as mean $\pm$ SEM. Statistical analysis according to two-way ANOVA analysis followed by one-way ANOVA with Bonferroni

correction; * $p < 0.05$, ** $p < 0.01$; # $0.10 > p > 0.05$ trend for a significant difference. Experimental group: 5XFAD-PCSK9^{Het}, n=9; 5XFAD-PCSK9^{KO}, n=5.

3.3.2. BACE1 immunoreactivity

The BACE1 is highly expressed in the CNS and has been implicated in AD and data from literature demonstrated a significant increase of BACE1 protein expression in 10 mo 5XFAD mice [47]. In our experimental groups, the densitometric analysis of BACE1 ir did not show any statistical differences in the ir area in the stratum lucidum (SL) among groups. In addition, the analysis of BACE1 ir thickness in SL, as a morphometric parameter, did not reveal any difference among experimental groups (**Fig.8 A-F**). Furthermore, as also reported by others [48], we found a BACE1 ir elevation in presynaptic dystrophic neurites that surround amyloid plaques. In fact, as shown in higher magnification images, we found an increased BACE ir (DAB⁺ spots, brown) close to Congo Red⁺ amyloid plaques (red) in AD mice (**Fig.8 C,D**). Given the reduced amyloidosis in 5XFAD-PCSK9^{KO} mice, we asked whether BACE1-plaque associated dystrophic neurites were also reduced in these mice. BACE1-labelled brain slices were co-labelled with Congo Red stain and the number of double positive plaques was manually counted. The quantitative analysis showed that the loss of PCSK9 induces a significant reduction in BACE1-plaque associated dystrophic neurites in Nctx ($p < 0.001$) (not shown); however, the % of Congo Red⁺ / BACE⁺ plaques in 5XFAD-PCSK9^{KO} mice was comparable to that of 5XFAD-PCSK9^{Het} mice ($p = 0.160$) (**Fig.8 G** and **Suppl.Table 7** for detailed statistical report).

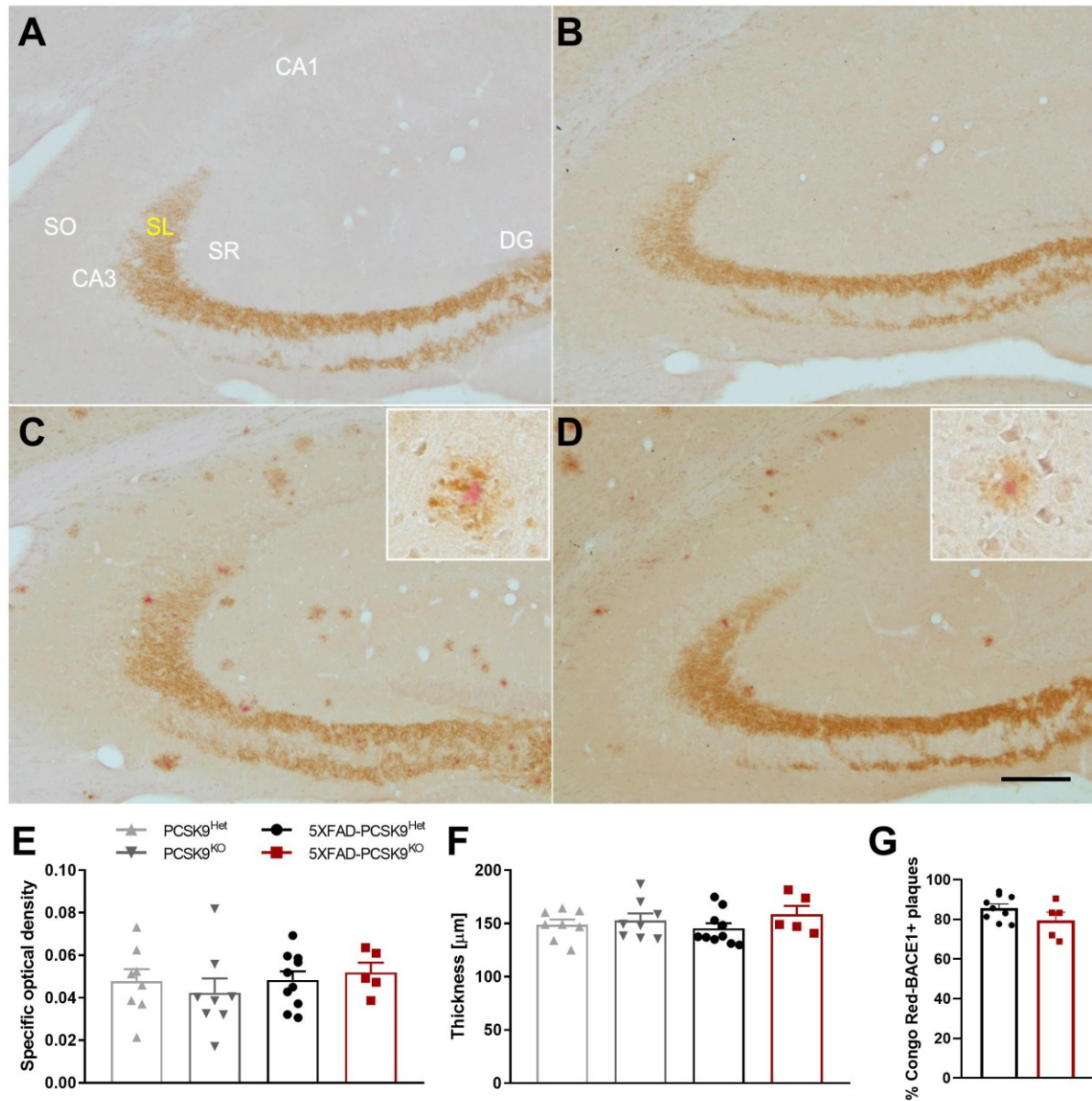


Figure 8: Representative photomicrographs of CA3 field of hippocampus of 10 mo Wt-PCSK9^{Het} (light grey bars), Wt-PCSK9^{KO} (dark grey bars), 5XFAD-PCSK9^{Het} (black bars) and 5XFAD-PCSK9^{KO} (red bars) mice showing immunoreactivity for BACE1 and Congo Red stained amyloid plaques. Scale bar=200 μm. E-F Densitometric analysis of BACE1 ir in hippocampal stratum Lucidum (SL) of CA3 field by analyzing specific optical area (E) and BACE1 ir thickness (F). G % of co-stained CongoRed-BACE1 plaques in CA3 field of hippocampus of 5XFAD-PCSK9^{Het} (black bars) and 5XFAD-PCSK9^{KO} (red bars). Data are shown as mean ± SEM and compared by one or two-way ANOVA test. Experimental groups: Wt-PCSK9^{Het}, n=8; Wt-PCSK9^{KO}, n=8; 5XFAD-PCSK9^{Het}, n=10; 5XFAD-PCSK9^{KO}, n=5. Abbreviations: DG = dentate gyrus; CA3 = cornu Ammonis 3; CA1 = cornu Ammonis 1; SR = Stratum Radiatum, SL = Stratum Lucidum, SO = Stratum Oriens.

3.3.3. Glial fibrillary acidic protein staining

In physiologic conditions, astrocytes contribute to neuronal homeostasis in the CNS, providing cholesterol and other neurotrophic factors. In response to various stimuli, astrocytes undergo morphological, molecular and functional changes known as astrogliosis. In this condition, these cells contribute to neuronal damage through various mechanisms, including enhanced pro-inflammatory cytokines production. We thus analyzed the impact of PCSK9 loss on the immunoreactivity for glial fibrillary acidic protein (GFAP), a marker for astrocyte reactivity. Brain slices from our mice models were immunolabelled with anti-GFAP Ab and the ir was quantified by means of image analysis. Representative images from several brain regions, such as, CC-Cg, DG hilus, CA1 and CA3 fields of hippocampus are shown in **Fig.9**. The semiquantitative analysis demonstrated that, compared to Ctrl, all analyzed areas are characterized by a significant increase in GFAP ir in 10 mo 5XFAD mice, whereas the absence of PCSK9 had no effect with the exception of CC-Cg and CA1 field of hippocampus (**Fig.9 I** and **Suppl.Table 7** for detailed statistical report).

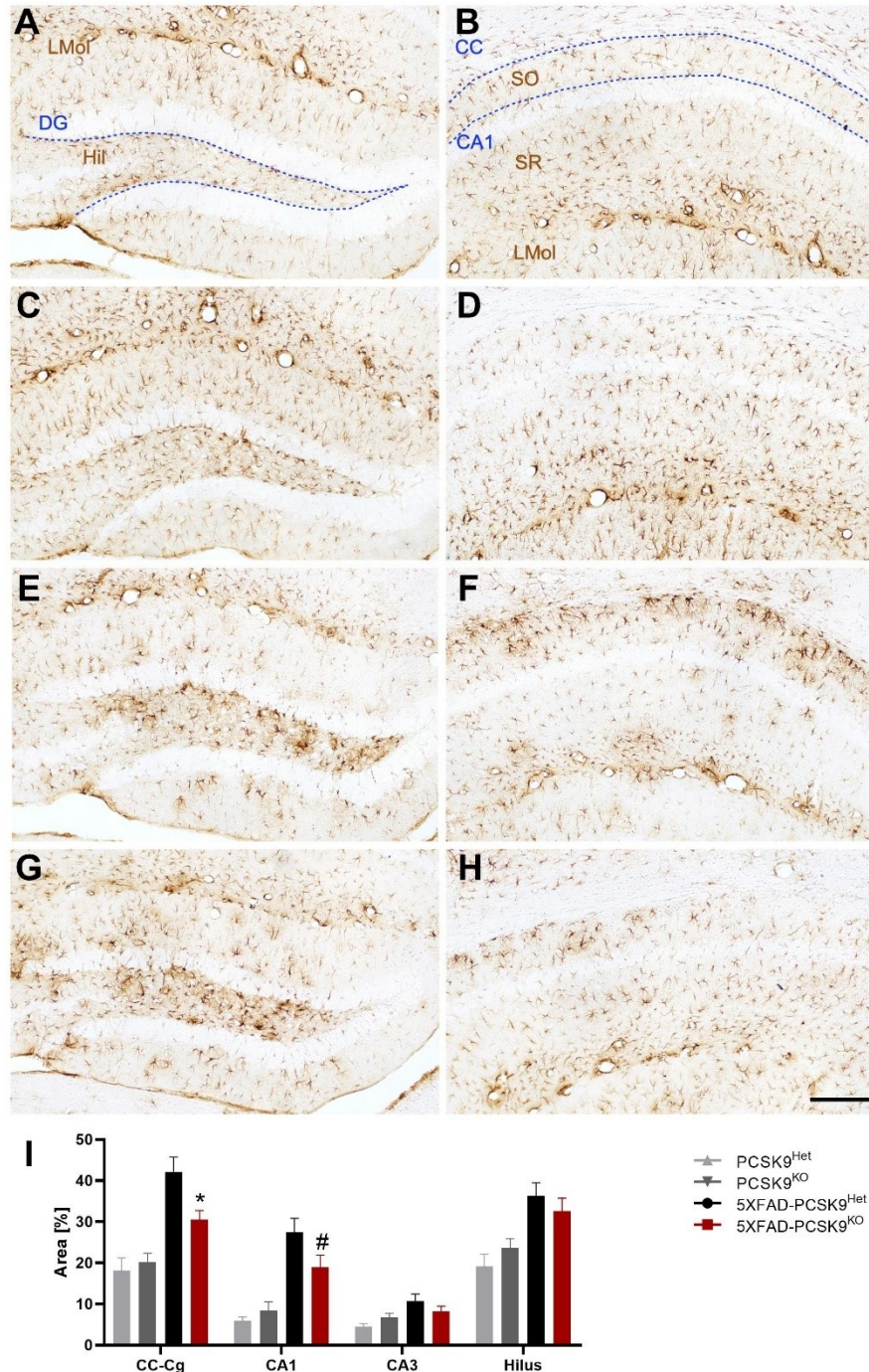


Figure 9: Astrocyte reactivity in AD mice is not influenced by PCSK9 loss. A-H. Representative photomicrographs of dentate gyrus (A, C, E, G) and CA1 (B, D, F, H) fields of hippocampus of 10 mo Wt-PCSK9^{Het} (light grey bars), Wt-PCSK9^{KO} (dark grey bars), 5XFAD-PCSK9^{Het} (black bars) and 5XFAD-PCSK9^{KO} (red bars) mice showing immunoreactivity for GFAP. Scale bar=200 μ m. I Quantitative analysis of GFAP ir in CC-Cg, Hilus, CA1, CA3 fields of hippocampus of 10 mo mice. Data are shown as mean $\pm$ SEM and compared by two-way ANOVA analysis of variance; * $p < 0.05$; # $0.10 > p > 0.05$ trend for a significant difference. Experimental groups: Wt-PCSK9^{Het}, n=5-6; Wt-PCSK9^{KO}, n=5-7; 5XFAD-PCSK9^{Het}, n=9; 5XFAD-PCSK9^{KO}, n=5). Abbreviations: DG = dentate gyrus; LMol = stratum lacunosum-moleculare; Hil = hilus; CC = corpus callosum; CA1 = cornu Ammonis 1; SR = Stratum Radiatum, SL = Stratum Lucidum, SO = Stratum Oriens.

3.3.4. Ionized calcium-binding adapter molecule 1 staining

Microglial cells, the CNS immune cells, contribute to A β clearance through its phagocytosis as well as of apoptotic cellular substances. To assess whether the reduced plaque deposition observed in 5XFAD-PCSK9^{KO} is associated with a decrease in neuroinflammation, we studied the reactivity of microglial cells, the CNS macrophages, visualized by IBA1 ir. In **Fig.10** are shown representative images of analyzed brain regions, such as the hippocampal hilus (**A,C,E,G**) and the somatosensory cortex (**B, D, F, H**) of experimental mice. The quantitative analysis demonstrated that the increased IBA1 ir in AD mice is counteracted by PCSK9 loss in both hilar region (**Fig.10 I**) and cortical areas (**Fig.10 J**). Furthermore, the reduced microgliosis is also accompanied by a significant decrease in the number of microglial cells (not shown). Statistical analysis is detailed in **Suppl. Table 7**.

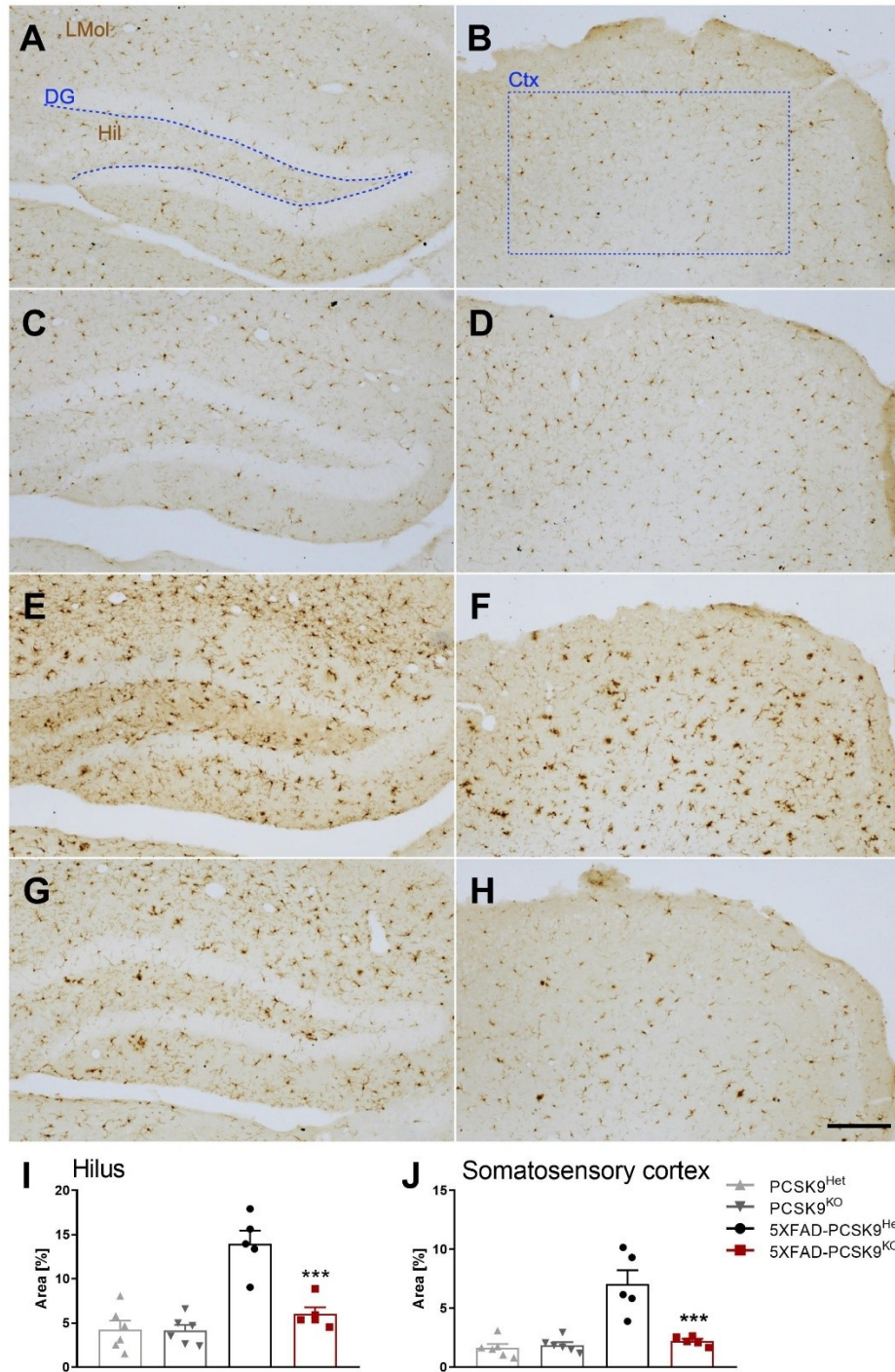


Figure 10: The loss of PCSK9 significantly reduced hippocampal and cortical microgliosis in AD mice. A-H Representative images of brain slices showing IBA1-positive microglial cells in the hilus of dentate gyrus (A,C,E,G) and somatosensory cortex (B,D,F,H) of Ctrl and AD mice. Scale bar = 200 μ m. E-J Quantitative analysis of microgliosis in somatosensory cortex and hilar region of hippocampus. The % area occupied by microglial cells, a parameter related to the reactivity of these cells, shows a significant reduction in 5XFAD-PCSK9^{KO} in both areas. Data are shown as mean $\pm$ SEM. Statistical analysis according to two-way ANOVA; ***p < 0.001 ****p < 0.0001. Experimental groups: Wt-PCSK9^{Het}, n=6; Wt-PCSK9^{KO}, n=6; 5XFAD-PCSK9^{Het}, n=5; 5XFAD-PCSK9^{KO}, n=5). Abbreviations: DG = dentate gyrus; LMol = stratum lacunosum-moleculare; Hil = hilus; Ctx = cerebral cortex.

3.4. Brain cholesterol and oxysterols quantification

We finally assessed whether the absence of PCSK9 in 5XFAD mice may lead to an alteration in brain total cholesterol (TC) and oxysterols content of our experimental models. We first observed a TC reduction of about 52% in 5XFAD mice as compared to Wt-B6SJL controls (**Suppl. Table 1**), demonstrating a significant alteration in cholesterol homeostasis in AD mice. Then, we compared TC levels in our experimental mice (**Table 2**). We found that TC levels were similar between the mice in the Ctrl group, irrespective of the presence or absence of PCSK9. In 5XFAD mice, a significant lower brain TC content was detected as compared to the Ctrl mice. This reduction was slightly but significantly recovered by PCSK9 loss (49%, **Suppl. Table 7**). In parallel, we have also quantified serum cholesterol levels in our mouse models. Serum TC was similar in 5XFAD mice and Wt-B6SJL mice (**Suppl. Table 3**). Additionally, PCSK9 loss led to a reduction of similar entity in both Ctrl and AD - 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO} - mice (**Suppl. Table 4**),

Brain	µg/mg cerebral tissue
Wt-PCSK9 ^{Het}	1.86 ± 0.17
Wt-PCSK9 ^{KO}	1.35 ± 0.24
5XFAD-PCSK9 ^{Het}	0.95 ± 0.13 **
5XFAD-PCSK9 ^{KO}	1.18 ± 0.17

Table 2: Brain cholesterol levels of 10 mo Wt-PCSK9^{Het}, Wt-PCSK9^{KO}, 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO}. Brain cholesterol levels are expressed as [µg cholesterol/mg of cerebral tissue]. Data are shown as mean ± SEM. Wt-PCSK9^{Het} n=14; Wt-PCSK9^{KO} n=10; 5XFAD-PCSK9^{Het} n=13; 5XFAD-PCSK9^{KO} n=12. Statistical analysis according to two-way ANOVA followed by one-way ANOVA with Bonferroni correction. A value of p<0.05 was considered statistically significant. ** p < 0.01 vs PCSK9^{Het}.

The most relevant oxidative bioactive metabolites of cholesterol, 24-, 25- and 27-OHC, were then quantified in the cerebral tissue and plasma of the above-described animal models. In details, we found that only 24- and 27-OHC were detectable in the brain. 5XFAD mice displayed 4-fold higher brain 27-OHC levels as compared to Wt-B6SJL controls (p<0.05; **Suppl. Table 2,7**). 24- and 27-OHC levels were comparable among the four considered experimental groups (**Table 3**). A parallel analysis of serum did not evidence significant differences in 24-, 25- and 27-OHC (**Suppl. Table 5**).

Brain	24-OHC [ng/ml]	25-OHC [ng/ml]	27-OHC [ng/ml]
Wt-PCSK9 ^{Het}	2.52 ± 0.20	n.d.	2.07 ± 0.24

Wt-PCSK9^{KO}	3.08 ± 0.24	n.d.	2.94 ± 0.75
5XFAD- PCSK9^{Het}	4.09 ± 0.70	n.d.	1.11 ± 0.29
5XFAD- PCSK9^{KO}	2.99 ± 54	n.d.	1.79 ± 0.38

Table 3 Brain hydroxysterols levels of 10 mo Wt-PCSK9^{Het}, Wt-PCSK9^{KO}, 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO}. Brain hydroxysterols are expressed as [ng hydroxysterols/mg cerebral tissue]. Data are shown as mean ± SEM. Wt-PCSK9^{Het} n=14; Wt-PCSK9^{KO} n=10; 5XFAD-PCSK9^{Het} n=13; 5XFAD-PCSK9^{KO} n=12. Statistical analysis according to two-way ANOVA. A value of p<0.05 was considered statistically significant. n.d: not detectable.

4. Discussion

Among AD pathogenic factors, altered brain cholesterol homeostasis in CNS is a well-recognized contributor, causing oxidative stress, neuroinflammation, synaptic and neuronal dysfunction and leading to cognitive decline [49]. Recent data showed that PCSK9, a key regulator of LDLR leading to its lysosomal degradation and increasing plasma LDL-cholesterol levels, is dynamically regulated and highly expressed in different neuropsychiatric disease states. However, its contribution to AD is still controversial and only little evidence is available on the beneficial effect of PCSK9 inhibition on A β pathology and cognitive functions. The limit of the available data is that they were obtained in animal models only partially mimicking AD phenotype (i.e., high fat-diet rats) [50, 51], or through the use of monoclonal antibodies that only target peripheral PCSK9, given their inability to cross the BBB [26]. The present study highlighted for the first time a role of PCSK9 in modulating neuroinflammatory response, through the expression levels of several key inflammatory markers in human U373 cell line after the exposure to A β fibrils (**Fig.1-3**); in addition, we reported several protective effects *in vivo* due to the full ablation of PCSK9 in adult 5XFAD mice, a severe mouse model of AD, by measuring behavioral phenotype and neuropathological outcomes as well as microglial density, distribution and morphology. In detail, we clearly demonstrated that disrupted hippocampal-dependent cognitive functions and worsened performance of spatial memory task in 10 mo 5XFAD mice (**Suppl.Fig.1**) were significantly improved in the absence of PCSK9 (**Fig.4**). In fact, while in the initial cue-guided training period (D1-D7) all mice demonstrated a similar performance, in the second training period (D8-D13) only 5XFAD mice lacking PCSK9 were able to learn and locate the position of the hidden platform through spatially directed strategies similarly to Ctrl. These data are in accordance with those recently obtained by others in the high fat cholesterol diet (HFCD)-model of AD-like conditions in rats [50] and in 6 mo 5XFAD mice [26] after a systemic

PCSK9 inhibition through monoclonal anti-PCSK9 antibodies injection; furthermore, here we clearly add new insights into the role of PCSK9 on hippocampal-dependent cognitive decline. In fact, the inability to form and retain new memories is among the most precocious and common features of human AD and such deficits are dependent on entorhinal cortex, perforant pathway, and hippocampus. We demonstrated that PCSK9 loss improves hippocampal-dependent memory impairment in 10 mo 5XFAD, and specifically ameliorates spatial working abilities (learning curve) as well as retention of spatial reference memory (probe test) without any influence of classical or Pavlovian conditioning but represent an amygdala-dependent behavior [26]. In addition, since recent data demonstrated an intact BBB permeability in 15 mo 5XFAD mice [52], we could speculate that measured changes are a direct consequence of PCSK9 loss in the CNS rather than an influence of peripheral PCSK9 inhibition [26]. Taken together, these data demonstrated that the inhibition of PCSK9 is able to preserve hippocampal-dependent learning abilities and memory in AD animal models. Mechanistically, we also found for the first time that PCSK9 deletion globally protects from SYN reduction that normally occur in 10 mo 5XFAD mice [53], thus preserving synaptic integrity (**Suppl.Fig.2**). In agreement with the data on cognitive improvement, also A β burden was influenced by PCSK9. In fact, PCSK9 loss induced a marked reduction in both count and area of ThS⁺ plaques in corticohippocampal regions of 5XFAD (**Fig.5**) with no effects either in spatial distribution or in quantitative parameters of 6E10⁺ and 11A1⁺ plaques (**Fig.6-7**). Supported by recent studies [50, 54], these results suggest PCSK9 role in promoting plaque formation since its inhibition attenuates A β aggregation affecting neither global BACE1 expression nor its accumulation in peri-plaque dystrophies (**Fig.8**). In fact, even if the role of PCSK9 in modulating BACE1 activity is still controversial, suggesting both increase or decrease in different experimental conditions [55], here we clearly demonstrated that % of Congo Red⁺ / BACE⁺ plaques was comparable between AD mice, thus leaving us to speculate that dendritic neurites formation in AD is not influenced by PCSK9 (**Fig.8**). Emerging evidence has also implicated PCSK9 in neuroinflammation, namely through a role in coordinating the induction of NF- κ B pathway and the activation of astrocytes and microglia [50, 54]. Our *in vitro* data demonstrated that recombinant PCSK9 exacerbated the A β fibrils-mediated induction of proinflammatory cytokines and inflammasome-related gene, such as *Pyrin*, *NLRP1*, *NLRC4* and *NLRP3*, in U373 astrocytome cell line (**Fig.1-3**). In the same way, the loss of PCSK9 in 5XFAD mice *in vivo* decreased microglial reactivity, representing a hallmark of inflammation in the brain, in corticohippocampal regions to the levels of Ctrl, in terms of global IBA1 ir and number of microglial cells. This result tallies well with recent studies showing a reduced brain damage and decreased microglial reactivity in ischemic rats treated with PCSK9 inhibitors [54], as well as a reduced activation of human peripheral blood monocytes induced by PCSK9 inhibition [56].

However, the marked increase in the cortical expression of homeostatic genes, such as *P2y12r*, *CD11b* and *Cx3cr1*, as well as phagocytic/disease-associated genes, such as *Ifitm3* and *Cst7*, of microglia observed in 10 mo 5XFAD mice was not modulated by PCSK9 loss (**Suppl.Fig.3-4**). These data, even if are not correlated with the analysis of relative protein expressions by IHC assay, let us to hypothesize that reduced amyloid plaque deposition is not a consequence of an accelerated A β phagocytosis in absence of PCSK9. In this view, increased expression of CD36 has been associated to an increased A β 1–42 uptake in the brain and lowered A β plaque burden in the hippocampus of AD mice [57-59]. However, our analysis revealed only a minor decrease in CD36 expression dependent on AD pathology but not PCSK9 loss. Lack of effect on CD36 was also confirmed by our *in vitro* experiments on astrocytes. Since gene expression analysis has been conducted in whole dissected cerebral cortex, the most A β –affected brain region in 5XFAD mice, we cannot exclude that PCSK9 loss may induce single cell type- and cell side- specific alterations that are underestimated with our technical approach. If on the one hand PCSK9 loss modulates microglial reactivity, on the other hand its absence did not cause a marked global reduction in GFAP ir, considered to represent an index of astrogliosis, with the exceptions of cingulate cortex and CA1 field of hippocampus (**Fig.9**). Since GFAP ir does not label all astrocytic populations especially in areas such as cerebral cortex, our data do not exclude that PCSK9 loss may have a more pervasive protective effect on other astrocytic populations. Additionally, it is known that GFAP+ astrocytes contain subpopulations with different functional roles, such as A1 neurotoxic astrocytes, the major astrocytic subtype that mediates astrocytic toxicity in AD [60]. Further studies with A1-specific markers may show alterations in astrocytic subpopulations that have been overlooked in present analysis. Finally, reduced brain cholesterol levels in 10 mo 5XFAD mice (**Suppl.Table 1**) was slightly counteracted by PCSK9 loss (**Table 2**). In addition, our results indicated an absence of changes in oxysterols (**Table 3**), thus suggesting that the ameliorated cognitive performances, reduced amyloidosis and, consequently, neuroinflammation caused by PCSK9 deletion could only partially be explained by a restoration of lipid homeostasis. The lack of prominent differences in brain cholesterol across the PCSK9 genotypes may be interpreted in light of a possible interfering effect of PCSK9 specifically on the brain cholesterol flux from astrocytes to neurons, that we previously hypothesized, but not necessarily accompanied by overall changes in total brain content. At the same way, also the expression of lipid-related genes was unaffected by PCSK9 loss with minor exceptions. In fact, both *ApoE* and *Lpl* were increased in AD mice without any interference of PCSK9 whether an increased *Lrp5* level in 5XFAD mice was slightly decreased, showing a trend towards a significant effect, parallel to PCSK9 loss. For the reasons mentioned above, we cannot exclude that cell-specific changes occur in 5XFAD in absence of PCSK9.

5. Conclusion

Present data strongly evidenced a protective role of PCSK9 genetic deletion against A β pathology, synaptic integrity, neuroinflammation and cognitive decline in a severe mouse model of AD, thus putting the premises to identify PCSK9 as pharmacological target for development of novel therapeutic strategies for AD, for which no treatment is yet available.

Authors Contributions:

Modena: Eleonora Daini, Eleonora Vandini: mouse breeding, *ex vivo* investigation; Martina Bodria, Antonietta Vilella: behavioural screening, *in vivo* and *ex vivo* investigation, data curation; Antonietta Vilella, Daniela Giuliani: *in vivo* study design, data collection and curation, formal analysis, supervision; Antonietta Vilella: original draft preparation; Michele Zoli, Daniela Giuliani: review & editing.

Parma: Franco Bernini, conceptualization, review & editing, funding; Francesca Zimetti, *ex vivo* investigations, supervision, formal analysis, original draft preparation; Bianca Papotti: *ex vivo* analyses, data curation, formal analysis, original draft preparation; Francesco Potì: experimental design; review & editing; Ilaria Zanotti: experimental design, review & editing. Lisa Elviri and Giulia Remaggi: analytical method for *ex vivo* analyses set up, *ex vivo* analysis, review & editing

Padova: Nicola Ferri, conceptualization, *in vitro* investigation, supervision, formal analysis, original draft preparation; Maria Giovanna Lupo: *in vitro* investigation, formal analysis, review & editing; Giovanni Panighel: *in vitro* investigation, formal analysis, review & editing.

All authors have read and agreed to the published version of the manuscript.

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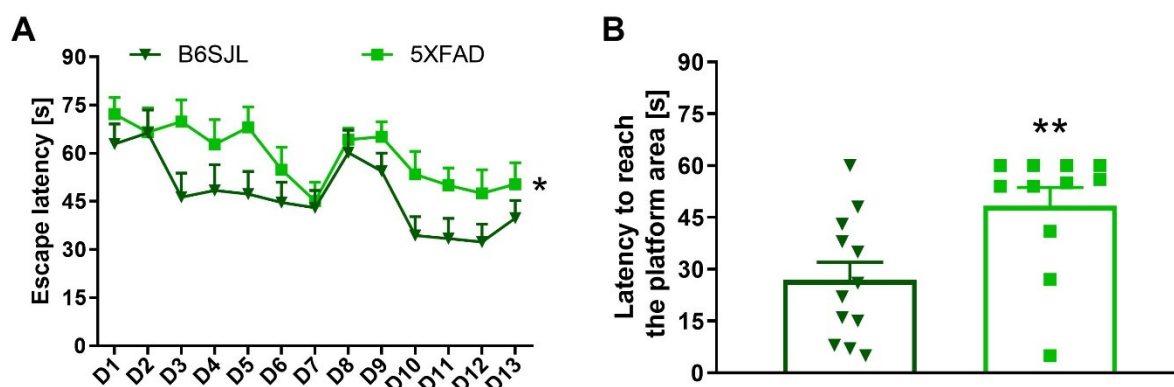
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Supplementary Materials

S1.1: Behavioral screening



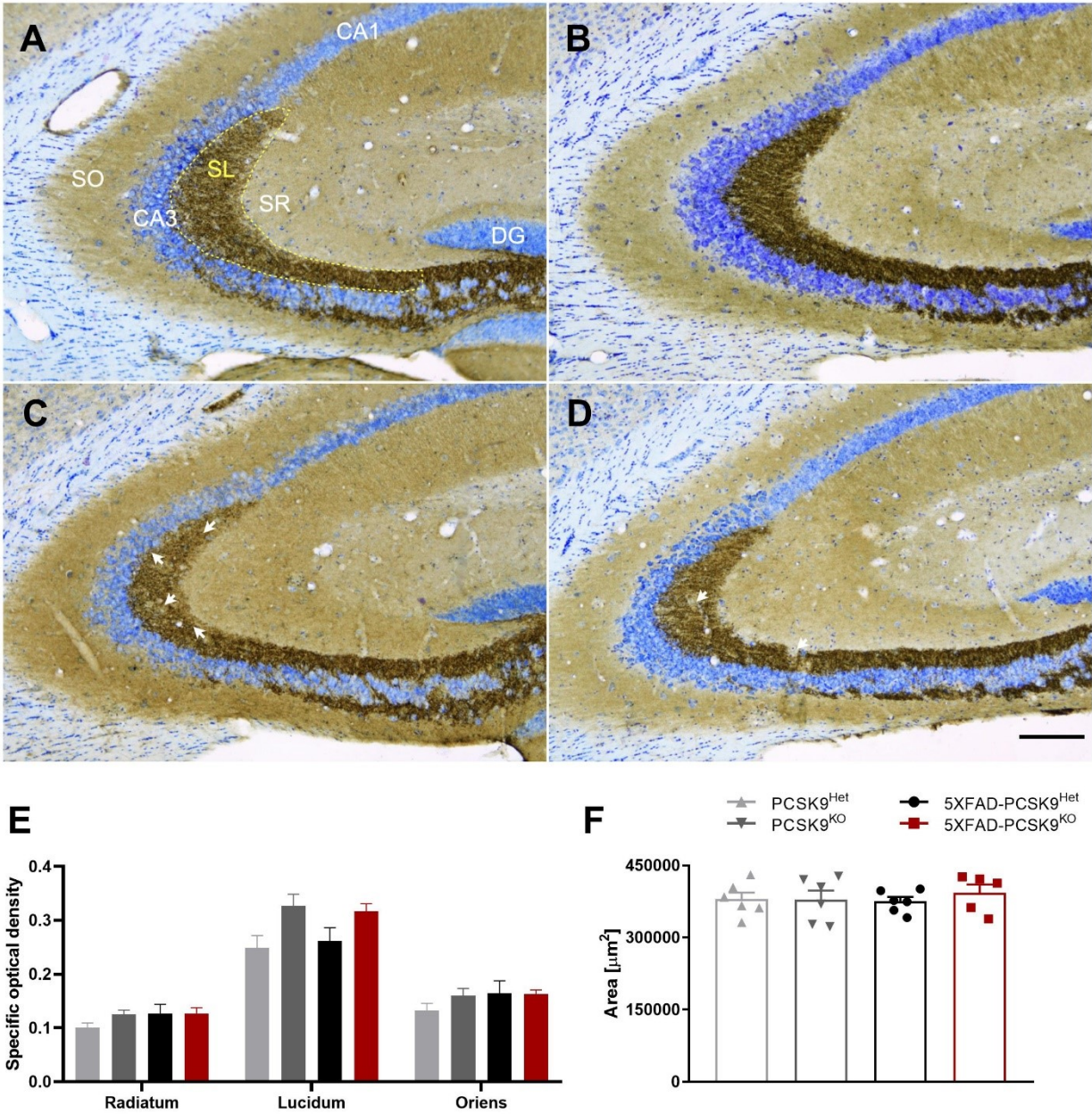
Suppl. Figure 1: Learning and memory are impaired in 10 mo 5XFAD mice. **Panel A** Analysis of MWM learning curve showing that the efficiency to solve the task is reduced in Wt B6SJL and 5XFAD mice. The time needed to find the platform (escape latency) was averaged for each day (4 trials/day) and analyzed by a two-way repeated measures ANOVA with training day and genotype as factors, * $p < 0.05$. **Panel B** A decreased time to reach the platform in the trained target quadrant (east (E) quadrant) during the probe test in AD mice was recorded, indicating that in 5XFAD mice the acquisition of the memory for the platform location is impaired. Statistical analysis according to one-way ANOVA, ** $p < 0.01$. Data are shown as mean $\pm$ SEM. Experimental groups: B6SJL, $n = 12$; 5XFAD, $n = 11$.

S1.2: Immunohistochemical analysis

Synaptophysin immunoreactivity

Loss of synapses at a fine structural level and reduction in synaptic markers have been well documented in early and late stages of AD as well as in AD mouse models and have been shown to correlate with the extent of cognitive deficits. To assess whether the improved cognitive abilities and reduction of amyloid plaques in 5XFAD-PCSK9^{KO} mice are accompanied by changes at synaptic level, we studied SYN ir as an index of synaptic integrity in the CA3 field of hippocampus (**Suppl. Figure 2**). In our experimental groups, SYN ir was primarily observed in neuropil with dense

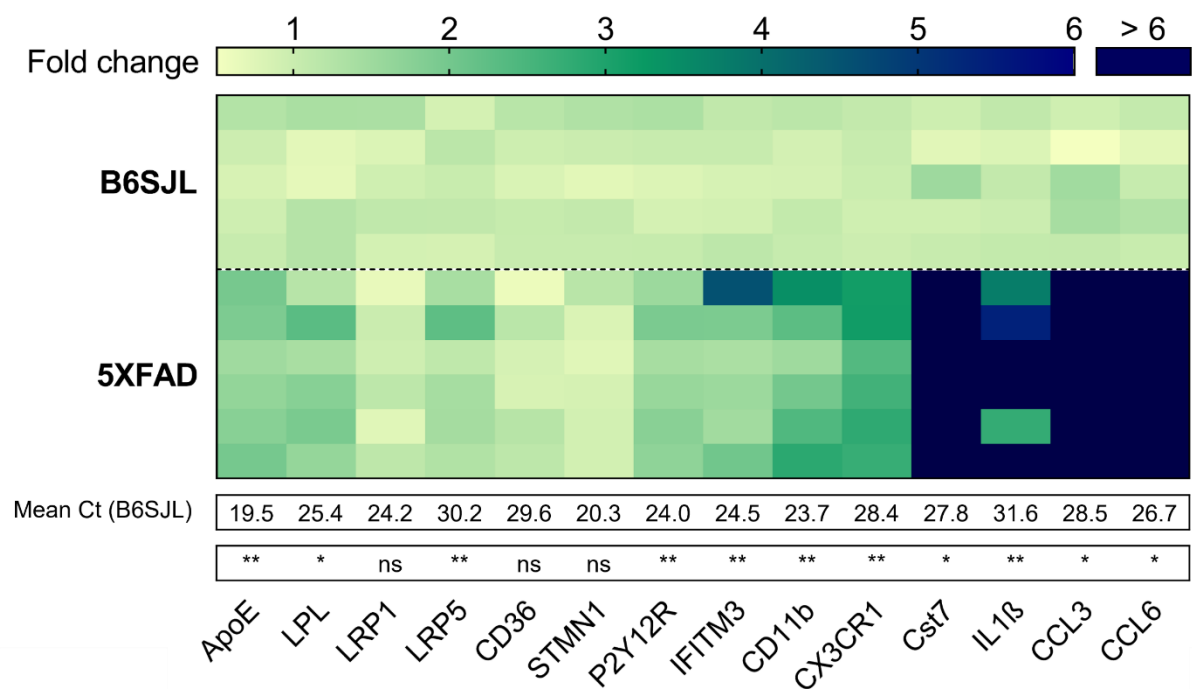
labeling in dendrites. Densitometric analysis showed that PCSK9^{KO} mice displayed significantly increase in the intensity of SYN ir in stratum lucidum (SL), but not in the stratum oriens (SO) or in the stratum radiatum (SR) whereas the genotype 5XFAD had no significant effect (Suppl. Figure 2 E). No significant changes in any quantitative morphometric parameter analyzed in SL, such as area (Suppl. Figure 2 F), SYN ir length and thickness, area/thickness ratio, area/CA3 length ratio, SYN ir SL/SL-CS3 thickness, were detected despite the presence of A β deposits (Suppl. Table 7 for detailed statistical report). Overall, our data suggest that the absence of PCSK9 improves SYN integrity, normally reduced in 10 mo 5XFAD mice (Kim et al., 2019). However, we cannot exclude that also changes in SYN puncta, as index of synapse loss, occur around amyloid plaques.



Suppl. Figure 2: Representative images of CA3 field of hippocampus of 10 mo Wt-PCSK9^{Het} (A) Wt-PCSK9^{KO} (B), 5XFAD-PCSK9^{Het} (C) and 5XFAD-PCSK9^{KO} (D) mice showing immunoreactivity for SYN. Scale bar=200 μ m Panel E-G: Densitometric analysis of SYN ir in hippocampal stratum Radiatum, stratum lucidum (dashed yellow line) and stratum oriens of CA3 field by analyzing specific optical area (E,) and total area (F). Amyloid deposits were clearly recognizable as round/oval structures negative for SYN ir in disturbed neuropil (white arrows in C,D). Data are shown as mean $\pm$ SEM and compared by two-way ANOVA test. Abbreviations: DG = dentate gyrus; CA3 = cornu Ammonis 3; CA1 = cornu Ammonis 1; SR = Stratum Radiatum, SL = Stratum Lucidum, SO = Stratum Oriens.

S3.1: *Ex-vivo* analysis of neuroinflammation and lipid metabolism

We preliminary tested the expression of specific gene targets in Wt B6SJL and 5XFAD mice (**Suppl. Figure3**). We found that most of lipid-related proteins (ApoE, LPL, LRP1, LRP5, CD36),

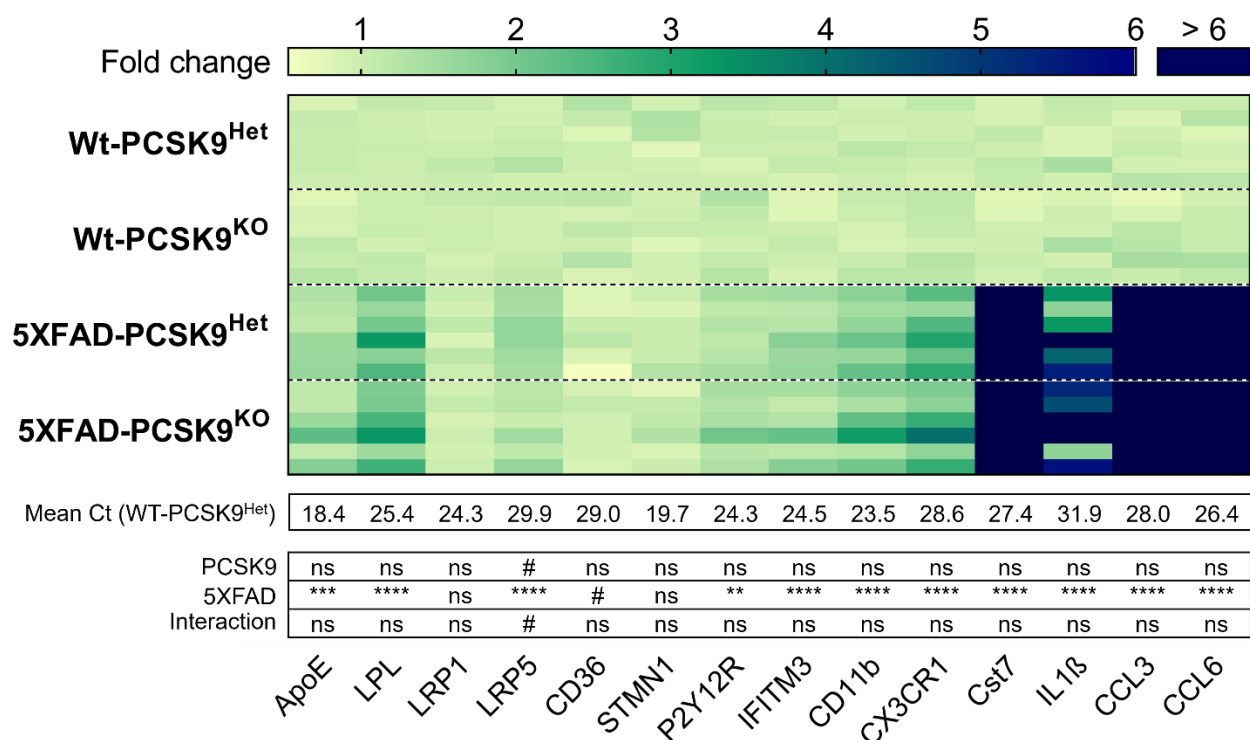


microglial factors (STMN1, P2Y12R, IFITM3, CD11b, CX3CR1, Cst7) and inflammatory mediators (IL1- β , CCL3, CCL6) were upregulated in 5XFAD mice with only minor exceptions (LRP1, CD36, STMN1).

Suppl. Figure 3: Effect of AD in the expression of lipid/cholesterol metabolism-, microglial- and cytokines- related genes. Heatmap of differential gene expression in Wt B6SJL and 5XFAD mice. Data are presented as fold change normalized over Wt B6SJL group mean and calibrated over GAPDH/RER1/CypA housekeeping genes. Fold changes are visualized through a chromatic scale ranging from yellow (<1) to blue (6) and dark blue (>6). The mean Ct of the group used as calibrator and the results of one-way ANOVA are indicated beneath the heatmap. Data are shown as mean $\pm$ SEM and analysed by two-way ANOVA; * $p < 0.05$; ** $p < 0.01$. Experimental groups: B6SJL, $n = 4-5$; 5XFAD, $n=4-6$.

Then we analysed the expression of above mentioned genes in the Nctx of Ctrlx and AD mice to test if the absence of PCSK9 modulates A β -mediated neuroinflammation and lipid metabolism genes expression (**Suppl. Figure4**). As concerns lipid-related proteins, the analysis demonstrated a significant increase in ApoE, LPL and slight, but not significant, decrease in CD36 expression in AD mice with no effects of PCSK9 loss; in addition, a trend towards a significant reduction in LRP5

expression was induced by PCSK9 loss in AD mice. However, among screened genes, LRP1 expression was not affected in AD mice (for statistical report see **Suppl.Table 7**). Analysis of microglial factors revealed no changes in the expression of P2Y12R, considered as a marker of homeostatic microglia. Conversely, CD11b and CX3CR1 expression was upregulated in AD mice regardless of PCSK9 loss as IFITM3 and Cst7, identifying phagocytic/disease-associated microglia. Additionally, analysis of STMN1, expressed both by neurons and proliferating microglia, showed a trend towards a significant effect dependent only on AD (for statistical report see **Suppl.Table 7**). Finally, all pro-inflammatory cytokines analyzed, IL1 β , CCL3 and CCL6 showed a significant increase in AD mice with no effects dependent on PCSK9 loss (**Suppl.Table 4**).



Suppl.Figure 4: Effect of PCSK9 loss in the expression of lipid/cholesterol metabolism-, microglial- and cytokines- related genes. Heatmap of differential gene expression in Wt-PCSK9^{Het}, Wt-PCSK9^{KO}, 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO} mice. Data are shown as fold change normalized over Wt-PCSK9^{Het} group mean and calibrated over GAPDH/RER1/CypA housekeeping genes. Fold changes are visualized through a chromatic scale ranging from yellow (<1) to blue (6) and dark blue (>6). The mean Ct of the group used as calibrator and the results of two-way ANOVA, with PCSK9 and 5XFAD factors and their interaction, are indicated beneath the heatmap; ** p < 0.01, ***p<0.001, ****p<0.0001, # 0.10 > p > 0.05 trend for a significant difference. n=6 for each experimental group.

S3.2: Brain cholesterol and oxysterols quantification

Brain	Cholesterol
Wt-B6SJL	1.83 ± 0.27
5XFAD	0.89 ± 0.05 ***

Supplementary Table 1. Brain cholesterol levels of Wt-B6SJL and 5XFAD mice. Brain cholesterol levels are expressed as [μg cholesterol/mg of cerebral tissue]. No differences were observed in brain weight among the different mouse groups (data not shown). Data are shown as mean $\pm$ SEM and analysed according to unpaired t-test. Wt-B6SJL n= 9; 5XFAD n=11. A value of $p<0.05$ was considered statistically significant. *** $p<0.001$ vs Wt-B6SJL.

Brain	24-OHC	25-OHC	27-OHC
Wt-B6SJL	3.27 $\pm$ 0.79	n.d.	0.59 $\pm$ 0.12
5XFAD	2.82 $\pm$ 0.23	n.d.	2.91 $\pm$ 0.74 *

Supplementary Table 2. Brain hydroxysterols levels of Wt-B6SJL and 5XFAD mice. Brain hydroxysterols are expressed as [ng hydroxysterols/mg cerebral tissue]. No differences were observed in brain weight among the different mouse groups (data not shown). Data are shown as mean $\pm$ SEM and analysed according to unpaired t-test. Experimental groups: Wt-B6SJL n= 3; 5XFAD n=4. A value of $p<0.05$ was considered statistically significant. n.d: not detected. * $p<0.05$ vs PCSK9^{Wt}.

S3.3: Serum cholesterol and oxysterols quantification

Serum	Cholesterol [$\mu\text{g/ml}$]
Wt-B6SJL	276.70 $\pm$ 33.05
5XFAD	249.70 $\pm$ 30.30

Supplementary Table 3. Serum cholesterol levels of Wt-B6SJL and 5XFAD mice. Serum cholesterol levels are expressed as [μg cholesterol/ml]. Data are shown as mean $\pm$ SEM and analysed according to unpaired t-test. Wt-B6SJL n= 9; 5XFAD n=11. A value of $p<0.05$ was considered statistically significant. *** $p<0.001$ vs Wt-B6SJL.

Serum	Cholesterol [$\mu\text{g/ml}$]
Wt-PCSK9^{Het}	161.40 $\pm$ 16.72
Wt-PCSK9^{KO}	69.33 $\pm$ 9.65 ***
5XFAD-PCSK9^{Het}	109.30 $\pm$ 17.17
5XFAD-PCSK9^{KO}	67.55 $\pm$ 9.65

Supplementary Table 4: Serum cholesterol levels of 10 mo Wt-PCSK9^{Het}, Wt-PCSK9^{KO}, 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO}. Serum cholesterol levels are expressed as [μg cholesterol/ml]. Data are shown as mean $\pm$ SEM. Wt-PCSK9^{Het} n=14; Wt-PCSK9^{KO} n=10; 5XFAD-PCSK9^{Het} n=13; 5XFAD-PCSK9^{KO} n=12. Statistical analysis according to two-way ANOVA. A value of $p<0.05$ was considered statistically significant. *** $p < 0.001$ vs Wt-PCSK9^{Het}.

Serum	24-OHC [ng/ml]	25-OHC [ng/ml]	27-OHC [ng/ml]
Wt-B6SJL	39.22	15.29	33.21
5XFAD	16.47 $\pm$ 3.72	23.69 $\pm$ 8.77	49.80 $\pm$ 5.20

Supplementary Table 5. Serum hydroxysterols levels of Wt-B6SJL and 5XFAD mice. Serum hydroxysterols are expressed as [ng hydroxysterols/ml serum]. No differences were observed in brain weight among the different mouse groups (data not shown). Data are shown as mean $\pm$ SEM. Experimental groups: Wt-B6SJL n= 1; 5XFAD n=3. Unpaired t-test was not applicable.

Serum	24-OHC [ng/ml]	25-OHC [ng/ml]	27-OHC [ng/ml]
Wt-PCSK9^{Het}	34.61 $\pm$ 12.50	12.85 $\pm$ 2.05	28.66 $\pm$ 2.78
Wt-PCSK9^{KO}	18.67 $\pm$ 2.83	13.65 $\pm$ 2.61	36.76 $\pm$ 10.26
5XFAD-PCSK9^{Het}	17.29 $\pm$ 4.53	14.23 $\pm$ 2.49	24.87 $\pm$ 9.31
5XFAD-PCSK9^{KO}	21.71 $\pm$ 7.07	33.56 $\pm$ 12.10	42.01 $\pm$ 14.87

Supplementary Table 6. Serum hydroxysterols levels of 10 mo Wt-PCSK9^{Het}, Wt-PCSK9^{KO}, 5XFAD-PCSK9^{Het} and 5XFAD-PCSK9^{KO}. Serum hydroxysterols are expressed as [ng hydroxysterols/ml serum]. Data are shown as mean ± SEM. Wt-PCSK9^{Het} n=4; Wt-PCSK9^{KO} n=7; 5XFAD-PCSK9^{Het} n=7; 5XFAD-PCSK9^{KO} n=7. Statistical analysis according to two-way ANOVA. A value of p<0.05 was considered statistically significant.

S4.1: Detailed statistical report

Supplementary Table 7: detailed statistical report.

Fig.1	Cytokine mRNA	IL6	***	F(3,8)=24.05, p=0.0002	one-way ANOVA
		IL-1 β	*	F(3,8)=7.34, p=0.011	
		TNF- α	**	F(3,8)=10.52, p=0.003	
Fig.2	MCP-1	MCP-1	**	F(3,4)=27.4, p=0.004	one-way ANOVA
Fig.3	Inflammasome mRNA	Pyrin	*	F(3,8)=5.53, p=0.023	one-way ANOVA
		NLRP1	ns	F(3,8)=2.75, p=0.112	
		NLRP3	***	F(3,8)=17.61, p=0.0007	
		NLRC4	**	F(3,8)=14.15, p=0.0015	
Fig.4	MWM	daily escape latency (A)	*	Genotype F(3,42)=9.538, p<0.001; days of training F(12,42)=23.697, p<0.001, Genotype*days of training F(36,42)=1.584, p=0.019	two-way repeated measure ANOVA
		% time spent in the target quadrant (B)	#	PCSK9 KO F(1,46)=0.0050, p=0.9438; 5XFAD F(1,46)=7.105, p=0.0108, PCSK9*5XFAD F(1,46)=3.794, p=0.0580	one-way ANOVA
		% strategies (D)		Wt-Het vs Wt-KO, p=0.6307; Wt-Het vs Tg-Het, p=0.0005; Wt-KO vs Tg-Het, p=0.0241; Wt-KO vs Tg-KO, p=0.5261; Tg-Het vs Tg-KO, p=0.0437	chi square test
Fig.5	ThS	DG count number (C)	**	F(1,12)=13.07, p=0.004	one-way ANOVA
		Nctx count number (G)	**	F(1,12)=11.994, p=0.005	
		DG Area (D)	**	F(1,12)=9.93, p=0.009	
		Ctx Area (H)	*	F(1,12)=8.45, p=0.014	
Fig.6	ThS 6E10 11A1	plaque count (I)	ns	PCSK9 KO F(1,35)=18.25, p<0.0001; plaque type F(2,35)=10.36, p=0.0003, PCSK9*plaque type F(2,35)=0.636, p=0.535	two-way ANOVA
		area (J)	ns	PCSK9 KO F(1,35)=3.342, p=0.076; plaque type F(2,35)=41.00, p<0.0001, PCSK9*plaque type F(2,35)=1.810, p=0.179	
		plaque count (K)	ns	PCSK9 KO F(1,34)=35.57, p<0.0001; plaque type F(2,34)=1.367, p=0.268, PCSK9*plaque type F(2,34)=0.428, p=0.655	
		area (L)	ns	PCSK9 KO F(1,34)=13.94, p=0.0007; plaque type F(2,34)=189.9, p<0.0001, PCSK9*plaque type F(2,34)=2.120, p=0.136	
		% Hip/Nctx plaque count (M)	ns	PCSK9 KO F(1,11)=1.200, p=0.297; plaque type F(1,22)=21.508, p<0.001, PCSK9*plaque type F(2,22)=0.330, p=0.722	
Fig.7	ThS	DG (A)	*	PCSK9 KO F(1,11)=0.04369, p=0.382; plaque size F(3,33)=167.62, p<0.0001, PCSK9*plaque	two-way ANOVA

					size F(3,33)=3.895, p=0.017	
					PCSK9 KO F(1,11)=1.747, p=0.2131; plaque size F(3,33)=341.89, p<0.0001, PCSK9*plaque size F(3,33)=9.919, p<0.0001	
					PCSK9 KO F(1,11)=0.03679, p=0.8514; plaque size F(3,33)=35.369, p<0.0001, PCSK9*plaque size F(3,33)=2.722, p=0.060	
					PCSK9 KO F(1,11)=1.692, p=0.2199; plaque size F(3,33)=9.874, p<0.0001, PCSK9*plaque size F(3,33)=1.913, p=0.147	
					PCSK9 KO F(1,12)=3.739, p=0.0771; plaque size F(3,36)=107.6, p<0.0001, PCSK9*plaque size F(3,36)=1.236, p=0.311	
					PCSK9 KO F(1,12)=3.203, p=0.0987; plaque size F(3,36)=88.79, p<0.0001, PCSK9*plaque size F(3,36)=5.803, p=0.0024	
Fig.8	BACE1	SL	Optical density (E)	ns	PCSK9 KO F(1,30)=0.029, p=0.866; 5XFAD F(1,30)=0.801, p=0.379, PCSK9 KO*5XFAD F(1,30)=0.632, p=0.434	two-way ANOVA
			BACE1 ir thickness (F)	ns	PCSK9 KO F(1,30)=0.028, p=0.867; 5XFAD F(1,30)=2.060, p=0.163, PCSK9 KO*5XFAD F(1,30)=0.574, p=0.455	
		Nctx	Count (not shown)	**	F(1,13)=27.011, p<0.001	one-way ANOVA
			% of Congo Red+ / BACE+ plaques (G)	ns	F(1,13)=2.247, p=0.160	
Fig.9	GFAP	CC/Cg		*	PCSK9 KO F(1,26)=2.209, p=0.151; 5XFAD F(1,26)=28.707, p<0.001, PCSK9 KO*5XFAD F(1,26)=4.606, p=0.043	
		CA1		#	PCSK9 KO F(1,23)=0.963, p=0.338; 5XFAD F(1,23)=26.72, p<0.001, PCSK9 KO*5XFAD F(1,23)=3.201, p=0.089	
		CA3	% area	ns	PCSK9 KO F(1,23)=0.016, p=0.899; 5XFAD F(1,23)=6.001, p=0.024, PCSK9 KO*5XFAD F(1,23)=2.266, p=0.148	
		Hilus		ns	PCSK9 KO F(1,23)=0.014, p=0.907; 5XFAD F(1,23)=15.856, p<0.001, PCSK9 KO*5XFAD F(1,23)=1.558, p=0.226	
						two-way ANOVA
Fig.10	Iba1	Hilus (I)		***	PCSK9 KO F(1,21)=16.942, p<0.001; 5XFAD F(1,21)=34.367, p<0.001, PCSK9*5XFAD F(1,21)=15.780, p<0.001	
		sCtx (J)	% area	***	PCSK9 KO F(1,21)=15.959, p<0.001; 5XFAD F(1,21)=25.110, p<0.001, PCSK9*5XFAD F(1,21)=19.302, p<0.001	
		Hilus (not shown)		***	PCSK9 KO F(1,21)=15.943, p<0.001; 5XFAD F(1,21)=25.206, p<0.001, PCSK9*5XFAD F(1,21)=26.832, p<0.001	
		sCtx (not shown)	Count (not shown)	**	PCSK9 KO F(1,21)=12.753, p=0.002; 5XFAD F(1,21)=12.369, p=0.002, PCSK9*5XFAD F(1,21)=11.922, p=0.003	
Table 2	TC	Brain		*	PCSK9 KO F(1,45)=0.72, p=0.399; 5XFAD F(1,45)=10.13, p=0.002, PCSK9*5XFAD F(1,45)=4.85, p=0.032	two-way ANOVA
Table 3	Hydroxysterols	Brain	24-OHC	ns	PCSK9 KO F(1,44)=0.05, p=0.83; 5XFAD F(1,44)=0.53, p=0.472, PCSK9*5XFAD	two-way

				F(1,44)=1.59, p=0.214	ANOVA
		27-OHC	ns	PCSK9 KO F(1,44)=0.02, p=0.881; 5XFAD F(1,44)=0.50, p=0.482, PCSK9*5XFAD F(1,44)=0.13, p=0.721	
Suppl.Fig. 1	MWM	daily escape latency	*	Genotype F(1,21)=4.587, p=0.0441; days of training F(12,252)=6.665, p<0.001; Genotype*days of training F(12,252)=0.9138, p=0.534	two-way repeated measure ANOVA
		latency to reach the platform area	**	F(1,22)=8.393, p=0.009	one way ANOVA
Suppl.Fig. 2	SYN	SR	ns	F(3,22)=1.246, p=0.321	one-way ANOVA
		SL	*	F(3,22)=3.303, p=0.043	
		SO	ns	F(3,22)=0.981, p=0.423	
		Area	ns	PCSK9 KO F(1,19)=0.318, p=0.5837; 5XFAD F(1,19)=0.080, p=0.7804, PCSK9*5XFAD F(1,19)=0.3654, p=0.5527	two-way ANOVA
		Lenght	ns	PCSK9 KO F(1,19)=0.064, p=0.8037; 5XFAD F(1,19)=0.472, p=0.5003, PCSK9*5XFAD F(1,19)=0.004681, p=0.9462	
		CA3 SYN ir thickness	ns	PCSK9 KO F(1,19)=0.064, p=0.8037; 5XFAD F(1,19)=0.472, p=0.5003, PCSK9*5XFAD F(1,19)=0.004681, p=0.9462	
		area/thickness ratio	ns	PCSK9 KO F(1,19)=0.066, p=0.4259; 5XFAD F(1,19)=0.2293, p=0.637, PCSK9*5XFAD F(1,19)=0.9657, p=0.3381	
		area/CA3 length ratio	ns	PCSK9 KO F(1,19)=0.0027, p=0.9591; 5XFAD F(1,19)=0.1577, p=0.6957, PCSK9*5XFAD F(1,19)=0.8586, p=0.3658	
SYN ir SL/SYN+CA3 thickness	ns	PCSK9 KO F(1,19)=2.193, p=0.1550; 5XFAD F(1,19)=0.1443, p=0.7082, PCSK9*5XFAD F(1,19)=0.1103, p=0.7434			
Suppl.Fig. 3	lipid/cholesterol metabolism-, microglial- and cytokines-related mRNAs	ApoE	****	F (1,10)=50.494; p<0.0001	one-way ANOVA
		LPL	*	F(1,10)=8.796, p=0.016	
		LRP1	ns	F(1,10)=0.398, p=0.544	
		LRP5	*	F(1,10)=6.552, p=0.0310	
		CD36	ns	F(1,10)=0.073, p=0.793	
		STMN1	ns	F(1,7)=0.619, p=0.461	
		CTX P2Y12R	***	F(1,10)=28.560, p=0.0005	
		IFTM3	#	F(1,10)=4.268, p=0.069	
		CD11b	**	F (1,10)=20.939; p=0.001	
		CX3CR1	****	F(1,10)=163.171; p<0.0001	
		Cst7	**	F(1,7)=26.961; p=0.002	
		IL1b	**	F(1,10)=19.061, p=0.002	
		CCL3	****	F (1,7)=94.198; p<0.0001	

Suppl.Fig. 4	lipid/cholesterol metabolism-, microglial- and cytokines-related mRNAs	CTX	CCL6	**	F (1,7)=25.233; p=0.002	two-way ANOVA
			ApoE	ns	PCSK9 KO F(1,20)=0.1314, p=0.7208; 5XFAD F(1,20)=15.44, p=0.0008, PCSK9*5XFAD F(1,20)=0.2725, p=0.6074	
			LPL	ns	PCSK9 KO F(1,20)=0.1618, p=0.6918; 5XFAD F(1,20)=46.79, p<0.0001, PCSK9*5XFAD F(1,20)=0.08013, p=0.7800	
			LRP1	ns	PCSK9 KO F(1,20)=0.9097, p=0.3516; 5XFAD F(1,20)=0.0042, p=0.9489, PCSK9*5XFAD F(1,20)=0.1496, p=0.7030	
			LRP5	#	PCSK9 KO F(1,20)=3.499, p=0.0761; 5XFAD F(1,20)=37.22, p<0.0001, PCSK9*5XFAD F(1,20)=3.478, p=0.0769	
			CD36	ns	PCSK9 KO F(1,20)=0.797, p=0.3826; 5XFAD F(1,20)=3.782, p=0.0660, PCSK9*5XFAD F(1,20)=0.1273, p=0.7249	
			STMN1	ns	PCSK9 KO F(1,20)=0.4456, p=0.5121; 5XFAD F(1,20)=0.4138, p=0.5274, PCSK9*5XFAD F(1,20)=0.3537, p=0.5587	
			P2Y12R	ns	PCSK9 KO F(1,20)=3.689, p=0.0691; 5XFAD F(1,20)=14.47, p=0.0011, PCSK9*5XFAD F(1,20)=0.1869, p=0.6702	
			IFTM3	ns	PCSK9 KO F(1,20)=0.1677, p=0.6865; 5XFAD F(1,20)=25.79, p=0.0001, PCSK9*5XFAD F(1,20)=0.2726, p=0.6074	
			CD11b	ns	PCSK9 KO F(1,20)=0.3005, p=0.5897; 5XFAD F(1,20)=29.43, p<0.0001, PCSK9*5XFAD F(1,20)=0.2737, p=0.6066	
			CX3CR1	ns	PCSK9 KO F(1,20)=0.2408, p=0.6290; 5XFAD F(1,20)=41.67, p<0.0001, PCSK9*5XFAD F(1,20)=0.0048, p=0.9454	
			Cst7	ns	PCSK9 KO F(1,20)=0.0351, p=0.8533; 5XFAD F(1,20)=33.84, p<0.001, PCSK9*5XFAD F(1,20)=0.0459, p=0.8324	
			IL1b	ns	PCSK9 KO F(1,20)=1.408, p=0.2494; 5XFAD F(1,20)=40.18, p<0.0001, PCSK9*5XFAD F(1,20)=1.34, p=0.2606	
			CCL3	ns	PCSK9 KO F(1,20)=0.0108, p=0.9183; 5XFAD F(1,20)=39.84, p<0.0001, PCSK9*5XFAD F(1,20)=0.008, p=0.9284	
			CCL6	ns	PCSK9 KO F(1,20)=0.1542, p=0.6987; 5XFAD F(1,20)=54.68, p<0.0001, PCSK9*5XFAD F(1,20)=0.1225, p=0.7300	
Suppl.Table 1	TC	Brain		***	t=4.18 df=14, p=0.0009	Unpaired t-test
Suppl.Table 2	Hydroxysterols	Brain	24-OHC	ns	t=0.64 df=5, p=0.553	Unpaired t-test
			27-OHC	*	t=3.09 df=4, p=0.036	
Suppl. Table 3	TC	Serum		ns	Wt-B6SJL vs 5XFAD t=0.58 df=16, p=0.567	Unpaired t-test
Suppl. Table 4	TC	Serum		ns	PCSK9 KO F(1,45)=21.47, p<0.0001; 5XFAD F(1,45)=3.48, p=0.686, PCSK9*5XFAD F(1,45)=3.03, p=0.088	two-way ANOVA
Suppl. Table 5	Hydroxysterols	Serum	24-OHC	ns	PCSK9 KO F(1,21)=0.84, p=0.369; 5XFAD F(1,21)=1.29, p=0.268, PCSK9*5XFAD	two-way

				F(1,21)=2.63, p=0.120	ANOVA
			25-OHC	ns PCSK9 KO F(1,21)=0.22, p=0.156; 5XFAD F(1,21)=2.42, p=0.135, PCSK9*5XFAD F(1,21)=1.84, p=0.190	
			27-OHC	ns PCSK9 KO F(1,21)=0.13, p=0.274; 5XFAD F(1,21)=0.01, p=0.945, PCSK9*5XFAD F(1,21)=0.16, p=0.691	
			24-OHC	not applicable	Unpaired t-test
Suppl. Table 6	Hydroxysterols	Serum	25-OHC	not applicable	Unpaired t-test
			27-OHC	not applicable	Unpaired t-test

CHAPTER VI: Three-Dimensional (3D) Printed Silver Nanoparticles/Alginate/Nanocrystalline Cellulose Hydrogels: Study of the Antimicrobial and Cytotoxicity Efficacy

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Abstract

Here, a formulation of silver nanoparticles (AgNPs) and two natural polymers such as alginate (ALG) and nanocrystalline cellulose (CNC) was developed for the 3D printing of scaffolds with large surface area, improved mechanical resistance and sustained capabilities to promote antimicrobial and cytotoxic effects. Mechanical resistance, water content, morphological characterization and silver distribution of the scaffolds were provided. As for applications, a comparable antimicrobial potency against *S. aureus* and *P. aeruginosa* was demonstrated by in vitro tests as function of the AgNP concentration in the scaffold (Minimal Inhibitory Concentration value: 10 µg/mL). By reusing the 3D system the antimicrobial efficacy was demonstrated over at least three applications. The cytotoxicity effects caused by administration of AgNPs to hepatocellular carcinoma (HepG2) cell culture through ALG and ALG/CNC scaffold were discussed as a function of time and dose. Finally, the liquid chromatography-mass spectrometry (LC-MS) technique was used for targeted analysis of pro-apoptotic initiation and executioner caspases, anti-apoptotic and proliferative proteins and the hepatocyte growth factor, and provided insights about molecular mechanisms involved in cell death induction.

Keywords: 3D printing; alginate/nanocrystalline cellulose/silver nanoparticles hydrogels; antimicrobial activity; HepG2 cytotoxicity

1. Introduction

The development of innovative biomaterials as advanced therapeutic solutions is continuously under investigation. To reach this aim, nowadays, natural, biocompatible, biodegradable materials combined with nano-systems are largely developed. Among applications, innovative biomaterial-based devices suitable for the treatment of infected wounds without inducing antimicrobial resistance or with anti-tumoral properties are particularly attractive. Bacterial wound infection in humans often represents the major cause of severe pathologies development and chronicization, rather than the main obstacle to repair of the soft tissues concerned [1–5]. Furthermore, several clinical observations showed that chronic inflammatory diseases are statistically correlated with cancer development as a consequence in different tissues and organs [6]. The typical initial management of such aggressive infections is enrolled by persistent and broad-spectrum antibiotic administration, that can sometime transform into a clinically relevant and potentially dangerous condition, due to the predisposition of the tissues to host resistant species (e.g., methicillin resistant *Staphylococcus aureus* (Gram+; aerobic)—MRSA). MRSA and resistant *Pseudomonas* (Gram–; anaerobic) spp., as well as several among bacteria inhabiting chronic ulcers, are particularly prone to produce biofilms, a sort of protective polymeric matrix that mainly acts as a physical barrier to the permeation and action of antimicrobial agents [7], making many traditional therapies ineffective. Advanced medications can be considered a consistent synergic aid for the prevention and/or management of the situations described. In general, polymeric hydrogel scaffolds are retained as worthwhile for their biocompatibility, biodegradability and capacity to keep the wound bed moist to prevent tissue dehydration while absorbing excessive exudates [8]. However, naturally occurring saccharide polymers can result in being too easily and quickly biodegradable in living microenvironments rather than indeed presenting poor mechanical properties in terms of consistency and elasticity [9,10]. Calcium alginate (ALG) is worldwide considered a highly absorbent, biocompatible and biodegradable hydrogel suitable for dressing purposes [11], it derives from seaweed, is generally regarded as safe and its known strength relies on the capacity to keep the wound environment moist, favoring healing as well as the formation of granulation tissue and being easily removable with painless dressing changes [12,13]. Crystalline nanocellulose (CNC) is a promising biomaterial derived from different cellulose sources by means of various processing methodologies [14]. The cellulose nanoparticles are characterized by elevated surface area and crystallinity, they have a very large elasticity modulus, and they have high strength, low toxicity and high biocompatibility. For these reasons CNC is a good candidate to be considered in many fields, including biomedical applications and especially in the use of 3D printing for tissue-engineered scaffolds and wound healing [15–17]. CNC has been employed in hydrogel preparation for strengthening mechanical

properties such as elasticity and resistance to breakage [18]. Antimicrobial substances, especially silver, were already included in calcium alginate pharmaceutical forms [19]. L. Inbathamizh et al. described the effectiveness of plant-derived silver nanoparticles (AgNP) in terms of antimicrobial activity and anticancer activity on the hepatocellular carcinoma cell line (HepG2) using concentrations around 1 mg/mL [20]. Furthermore, recently Zhang et al. reviewed the literature on the topic, focusing on the clarification of anticancer mechanisms of action of AgNP, individuated in activities such as induction of stress to the cell endoplasmic reticulum and production enhancement of reactive oxygen species, that brings a cascade of events that cause damages to organelles and DNA provoking cell death [21], highlighting the wide spectra of potential applications of AgNPs. A particular focus was dedicated to their potential employment as next-generation anticancer therapeutic agent, investigated by both academic and industrial research in order to circumvent adverse effects caused by chemo- and radiation-therapies [22], rather than fighting antibiotic resistance through a specific antimicrobial activity [23]. Incorporation of AgNPs in calcium alginate-based hydrogels reinforced with cellulose nanocrystals, to properly improve the mechanical resistance, might be advantageous in order to combine the pharmaceutical properties of the metal nanoparticles with the physical features provided by the hydrogel intended as a biocompatible drug-delivery platform. Moreover, their preparation by means of ad hoc developed processes can be tailored in order to tune their shape, size, structure, moisture, mechanical characteristics and thus overall efficiency. The most versatile technique, suitable to manage hydrogel materials, considered almost semi-solid for their high viscosity, was identified in a previously reported 3D printing method by Elviri et al. [24]. The technique permits the easy setting of loaded drug amounts and arbitrarily choosing the size/dimension parameters with high reproducibility [24], in order to obtain hydrogels characterized by very high surface area/volume ratio and to limit drug dosages [25]. Here, ALG and CNC were chosen as the main excipients for the production of robust and highly reproducible 3D printed medication prototypes embedding AgNPs; their characterization was performed from a physical/mechanical point of view. In vitro antimicrobial activity against *S. aureus* and *P. aeruginosa* and cytotoxicity toward HepG2 cells were assayed. HepG2 cells present epithelial morphology and grow in vitro adherent to the culture substrate. This cell line also shows the peculiarity of being highly differentiated both from a functional and morphological point of view, resulting in being very useful in investigations concerning toxicity, cellular trafficking at the molecular level, and drug targeting [26]. Finally, investigations on cytotoxicity effects caused by the administration of AgNPs to HepG2 cells through scaffold inoculation were proposed by the liquid chromatography-mass spectrometry technique, deepening what are the molecular mechanisms involved in cell death induction evaluating the expression of protein markers [27].

2. Materials and Methods

2.1. Materials

Sodium alginate (Ph.Eur. grade; molecular weight by gel filtration chromatography (GFC) 180–300 kDa; slowly soluble in water) was from Carlo Erba (Milan, Italy). Tribasic sodium citrate dihydrate (Lot. 1986C100), urea and calcium chloride were from Carlo Erba. Trypsin, bovine serum albumin (BSA), dimethylsulphoxide, thiazolyl blue tetrazolium bromide (MTT), Tris-HCl, ethylene diamine tetraacetic acid (EDTA), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, trifluoroacetic acid (TFA), acetonitrile were from Sigma-Aldrich® (Darmstadt, Germany). Dulbecco's Modified Eagle's Medium (DMEM) was from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Fetal Serum Bovine (FBS) and Dulbecco's phosphate buffered saline (PBS) were from Euroclone (Milan, Italy). AgNP suspension (nominal concentration: 1000 ppm) was prepared by AgNO₃ reduction in water with NH₄, stabilized with poly-vinyl-alcohol (PVA), [28]. Dynamic Light Scattering particle size characterization of the AgNP suspension (1000 ppm) indicated an average hydrodynamic radius of about 60–80 nm. The sol shows a moderate polydispersity (polydispersity index 0.4). Protein assay dye reagent concentrate was from Bio-Rad. CNC suspension was obtained by acid hydrolysis of cotton linter following the procedure described by Basile et al. [29].

2.2. Methods

2.2.1. Ink Preparation for 3D Printing

Ink formulations for 3D printing were developed following the same preparation principles and critical parameters used for scaffold production in previous research works (e.g., viscosity, polymer concentrations, homogeneity) [29–31]. In particular, ALG (5% w/v) alone or ALG (5% w/v) and CNC (3% w/v) powders were accurately weighted and homogeneously dispersed in an ultrapure water or in a aqueous suspension of AgNP (different concentrations). The blends were then kept under magnetic stirring at room temperature (22–24 °C) over night to let sodium alginate completely dissolve, physically embedding CNC and AgNP. Once ready to use, the formulations were stored at 4 °C in the dark for up to one week.

2.2.2. Three-Dimensional (3D) Printing and Scaffold Production

A 3D printer built in-house was specifically designed for manipulation of aqueous viscous materials intended for hydrogel scaffold production. The surface plate is cooled at –14 °C, while the viscous material (ranging 8–40 k cP) [32], instantaneously solidifies during construction through layer by layer deposition. At the end of the printing procedure, the solid (frozen polymeric blend) structure

was fixed (30 min in 3M CaCl₂ solution) in its hydrogel form through the formation of the characteristic “egg box structure” [33]. Scaffolds were then washed with ultrapure water for the removal of calcium chloride excesses and stored at 4 °C.

2.2.3. Scaffold Characterization

Water content was determined by gravimetric analysis: 5 scaffolds of each type were gently tamponed on filter paper and weighted, then placed in oven at 40 °C (till constant weight) and weighted again. The % water content was calculated using the formula:

$$100 - (100 \times Dw)/Ww$$

where “Dw” stands for “dry weight” and “Ww” for “wet weight”. The mechanical parameters evaluated were elasticity (Young’s modulus) and elongation % at break. The tests were performed by a tractional dynamometer (AG M1, Acquat, Milan, Italy); scaffold nominal sizes were: 20 layers × 5 cm × 1.5 cm; thicknesses were determined by a digital thickener (Mitutoyo, Japan) taking measurements from 6 distinct points along the scaffolds. Distance between clips was prefixed at ±25 mm, traction speed 25 mm/min, 5 DaN top head. Force applied by the tensile tester (N) and net movement (mm) was continuously recorded and digitalized by PowerLab 400 board and Scope 3.5 software. Elongation at break (% strain) and Young’s modulus were calculated from the relevant stress–strain curves, taking into consideration the linear portion, corresponding to the elastic behavior of the specimens. In particular, Young’s modulus was calculated using the formula:

$$E = \sigma/\varepsilon$$

where σ corresponds to stress (applied force/cross section area) and ε to strain (net elastic elongation). Elongation percentage was calculated as the ratio $(100 \times \varepsilon)/\text{specimen length}$. All analyses were conducted in triplicate for each type of sample.

2.2.4. Elemental (Ag) Distribution within Scaffolds and Overall Morphology

The distribution of silver nanoparticles in the scaffold was evaluated by a scanning electron microscope (Jeol JSM 6400)(Jeol Spa, Milan, Italy) equipped with an Oxford Instruments Link Analytical Si (Li) energy-dispersive system detector (SEM-EDS). All the samples were carbon coated before the analysis. The data was processed by INCA built-in software. Scaffolds were characterized as well in terms of macro/micro-porosity; 5-layer (1.6 cm × 1.6 cm) specimen were de-hydrated by washing in increasing percentages of ethanol, 10 min each, till absolute ethanol. Then, Critical Point Drying (Balserz Union, FL, USA) was employed (70 atm, 37 °C) to eliminate ethanol and obtain dried samples with an unaltered structure. Scaffolds were finally accurately cut/broken in order to obtain pieces exposing the cross sections, rather than surface area. The last step consisted in sample

gold sputtering, producing a thin gold coating for optimal electrical conduction. Finally, after proper fixing on ad hoc stubs, scaffolds were observed through a Philips 501 SEM at magnifications ranging from 80× to 320×. Photographs were processed by means of ImageJ software (NIH, Bethesda, MD, USA), measuring the filaments patterned by the printer and collecting Feret's diameters of 150 randomly taken micropores both of the surface and the cross section of the scaffolds.

2.2.5. Antimicrobial Activity

Antimicrobial activity evaluation against two strains of bacteria (Gram⁺ and Gram[−]) was assessed. Multi-drug resistant strains of *Staphylococcus aureus* (ATCC 25923) (Manassas, VA, USA) and *Pseudomonas aeruginosa* (ATCC 27853) (Manassas, VA, USA) were considered for such test, since they are responsible of frequent infections in chronic wounds [34]. Diffusion disk method (or Kirby-Bauer technique) was adopted and ad hoc—modified in order to assay 3D printed hydrogel scaffolds [35]. 15 layered scaffolds ($15 \times 15 \text{ mm}^2$) were produced using the developed formulation, ALG and ALG/CNC AgNP-free scaffolds, as control, and cut with circular 6 mm diameter stamps. After that, samples were sterilized in 70% v/v ethanol [29], rinsed and stored in sterile water at 4 °C until use. AgNPs were tested at different concentrations, ranging from 100 ppm to 1 ppm, in order to define the MIC. Bacteria were seeded in pure culture and inoculated in a Mueller Hinton Broth at 37 °C under aerobic conditions for 1–2 h (0.5 McFarland). Bacterial suspension was seeded through the use of sterile tampons on a Mueller Hinton Agar terrain (carefully covering the entire Petri dish). Scaffolds were then applied by the use of sterile forceps. Each test was conducted in duplicate. A negative and positive control were established: a negative one with the scaffold only, to ensure it was not contaminated and a positive one consisting in a Petri dish seeded with bacteria but free of scaffolds. Finally, all the plates were incubated at 37 °C for 18–24 h. Results were evaluated based on the presence/absence of the inhibition ring, followed by its diameter determination. Bacterial sensibility to antimicrobial agent is directly proportional to this latter parameter [36].

2.2.6. Cytotoxic Activity towards Hepatocellular Carcinoma (HepG2)

Cells HepG2 Seeding on Scaffolds Seven layered, 20 mm-diameter scaffolds made with ALG, ALG/CNC or ALG/CNC-AgNP were produced and sterilized as reported above. AgNPs were used at different concentrations, ranging from 100 to 250 ppm. The scaffolds were placed in 12-well plates and washed with ultrapure sterile water to eliminate the ethanol. HepG2 were then seeded onto scaffolds or on the nude plate (control cells) at the concentration of 1.3×10^6 cells/well; cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS). In particular, the seeding procedure implied the deposition of 150 µL of cell suspension (containing the

1.3×10^6 cells), to let cells to interact with the scaffold, and after one hour further 850 μL of cell-free medium were added to reach 1 mL of total volume. Plates were kept in an incubator at 37 °C and 5% of CO_2 . The experiment was replicated over 7 days, setting as sampling time points day 2 and 7. To evaluate the cytotoxicity of the AgNP only, HepG2 cells were seeded on the 12-well plates and exposed to a solution of 100 or 200 ppm AgNP. The nanoparticles were, sterilized by filtration through a 0.45 μm filter before addition to the cells. This experiment was conducted over 48 h, taking into consideration 24 h and 48 h as sampling time points. Viability MTT Assay AgNP cytotoxicity on HepG2 cells was indirectly evaluated through MTT viability assay. At each time point, 200 μL of MTT (1mg/mL)-enriched DMEM at 5% FCS were added to each well and incubated for two hours at 37 °C under a continuous flow of 5% CO_2 in the dark. The scaffolds were then transferred into clear plates. The formazan crystals were dissolved in 500 μL /well DMSO under shaking for 10 min. An aliquot of 150 μL was thus taken and absorbance was finally measured at a wavelength of 570 nm using a Spark™ 10M multimode microplate reader. The absorbance value of only DMSO and scaffold alone was subtracted. Viability percentages were obtained keeping ALG absorbance values (positive control) minus the absorbance of the negative controls as the 100% of viability.

2.2.7. Cells Collection for Proteomic Analysis

Cells grew on a nude plate (control–) were washed once in phosphate buffered saline (PBS 1X), then, trypsin solution was added (500 μL /well) and plates incubated for 5 min; finally, the reaction was stopped by addition of fresh medium (DMDM + FCS) and cells were collected in 1.5 mL vials after centrifugation. Cultured scaffolds were transferred in a new sterile plate, then, 3 mL/well of sodium citrate (55 mM) and EDTA (50 mM) at pH 7.4 were added under orbital shaking for 15 min to dissolve the calcium alginate-based scaffolds to remove cells entrapped in it. Once terminated the content of each well was collected and centrifuged to separate cells from the supernatant (stored at –80 °C); the pellet was then resuspended in 3 mL of PBS to wash cells, centrifuged, the supernatant was eliminated and cells were stored at –80 °C. Cell culture medium was removed from each culture well designated to proteomic analysis and stored in 1.5 mL Eppendorf at –80 °C until use.

2.2.8. Protein Extraction and Sample Preparation

Total proteins were extracted from cell pellets using 8M urea lysis buffer in Tris-HCl pH 8. Lysates were sonicated for 10 min at 4 °C and incubated for 45 min on ice. Extracts were centrifuged at 4 °C for 10 min at 15,000 rpm and then assayed for protein dosage according to Bradford's method. Buffer exchange and protein concentration was performed using 50 mM ammonium bicarbonate in Amicon Ultra 0.5 mL centrifugal filters (Sigma-Aldrich) (Darmstadt, Germany) with a 3-kDa MWCO. Then

proteins were reduced and alkylated with DTT (5 mM for 30 min at 37 °C), followed by IAA (14 mM for 30 min in the dark), and addition of DTT (5 mM for 15 min at room temperature). Finally, trypsin in 1:50 (enzyme:protein) ratio was used overnight at 37 °C to digest proteins.

2.2.9. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

The resulted tryptic peptides were reconstituted in 50 µL of 0.1% (v/v) formic acid aqueous solution and 50% (v/v) acetonitrile and analyzed by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) selected reaction monitoring (SRM) approach to identify and quantify proliferative and apoptosis marker proteins. Samples were analyzed as biological (n = 3) and technical replicates (n = 3) using an Agilent HP 1260 liquid chromatography (Agilent Technology, Santa Clara, CA, USA) equipped with a 200-vial capacity sample tray and coupled to a QTRAP 400 triple quadrupole mass spectrometer (ABSCIEX), interfaced with an electrospray source (ESI). An Aeris C18 (150 × 2.1 mm, 5 µm) column (Phenomenex, Torrance, Ca, USA) was used for peptide separation. The injection volume was 10 µL. An elution system based on a solvent gradient [solution A: 0.1% aqueous formic acid (v/v)/solution B: 0.08% formic acid in acetonitrile (v/v)] was delivered at 0.2 mL/min. A flow rate of 200 µL/min and a 120 min linear gradient was applied for peptide separation. Source parameters were set as follows: ESI voltage 5.5 kV, declustering potential 70 eV, ion source temperature of 350 °C, collision energy (CE) 40 V. The sheath gas (nitrogen, 99.999% purity) and the auxiliary gas (nitrogen, 99.998% purity) were delivered at flow-rates of 45 and 5 arbitrary units, respectively. As for quantitative analysis, experiments were performed under positive ion-SRM conditions using nitrogen as collision gas (medium nitrogen pressure) and a 20 ms-dual time for each transition monitored. The SRM transitions monitored were reported on Table S1 (Supplementary Material). The analytes were relatively quantified among samples by normalization with total protein content.

2.2.10. Software for Bioinformatic Analysis and Mass Spectrometry (MS)

Data Processing A panel of 6 proliferative and apoptotic proteins (ki-67, HGF, BCLX, Casp8, Casp9 and Casp3) were considered for the assay. FASTA protein sequences was obtained from Uniprot Human proteome database and SRM transitions for each protein were simulated by Skyline (v. 20.0, SCIEX) (Redwood City, CA, USA), setting trypsin as digestion mode with no missed cleavage and carbamidomethylation of cysteins as structural modification (Table S1). Uniqueness of candidate peptide sequences were assessed by BLASTp tool (basic local alignment search tool; www.ncbi.nlm.nih.gov link NCBI BLAST) search (algorithm: blastp; ATRIX PA 30; GAP

COASTS: existence 10, extension 1; DATABASE: non redundant protein sequences) from NCBI (National Center for Biotechnology Information) (Bethesda, MA, USA).

2.2.11. Statistical Analysis

Data of MTT assay were analyzed by ANOVA one way using the Prism statistical analysis package version 6 (GraphPad Software). Data are given as mean $\pm$ standard deviation (SD). A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Three-Dimensional (3D) Scaffold Preparation and Characterization

Based on the definition of the optimal parameters for the development of polymer ink [24], ALG and CNC were mixed in different ratio in order to obtain an homogeneous solution with viscosity suitable for the printing of scaffolds. When CNC was mixed in the 3 or 4% w/v range with a 5% w/v ALG solution, scaffolds with controlled and reproducible 3D structural quality were obtained. Higher percentage of CNC (>5%, w/v) resulted in too viscous solutions and lowest percentage of CNC (0.05) being $86.24 \pm 0.45\%$, $87.12 \pm 0.64\%$ ($n = 5$), respectively. Elasticity of hydrated scaffolds was determined by traction tests and subsequently elaborated stress-strain curves (Figure 1). Nanomaterials 2020, 10, x FOR PEER REVIEW 7 of 16 3.1. Three-Dimensional (3D) Scaffold Preparation and Characterization Based on the definition of the optimal parameters for the development of polymer ink [24], ALG and CNC were mixed in different ratio in order to obtain an homogeneous solution with viscosity suitable for the printing of scaffolds. When CNC was mixed in the 3 or 4% w/v range with a 5% w/v ALG solution, scaffolds with controlled and reproducible 3D structural quality were obtained. Higher percentage of CNC (>5%, w/v) resulted in too viscous solutions and lowest percentage of CNC (0.05) being $86.24 \pm 0.45\%$ $87.12 \pm 0.64\%$ ($n = 5$), respectively. Elasticity of hydrated scaffolds was determined by traction tests and subsequently elaborated stress-strain curves (Figure 1).

ALG/CNC and ALG based scaffolds resulted being resistant to breakage till the application of 58 ± 0.44 and 5.4 ± 0.53 N, respectively. Elasticity resulted significantly higher ($p = 0.031$) in CNC containing scaffolds (average E resulted 0.32 ± 0.04 MPa) with respect to ALG-based scaffold (0.43 ± 0.03 MPa). No significant differences were observed by using CNC at the 3% or 4% (w/v). Optimal elasticity values for the purpose are not trivial to be defined, these results suggest that the 3D ALG/CNC based scaffolds could be applicable as covering advanced dressing. All the subsequent work was carried out by using the scaffolds prepared with 3% (w/v) CNC.

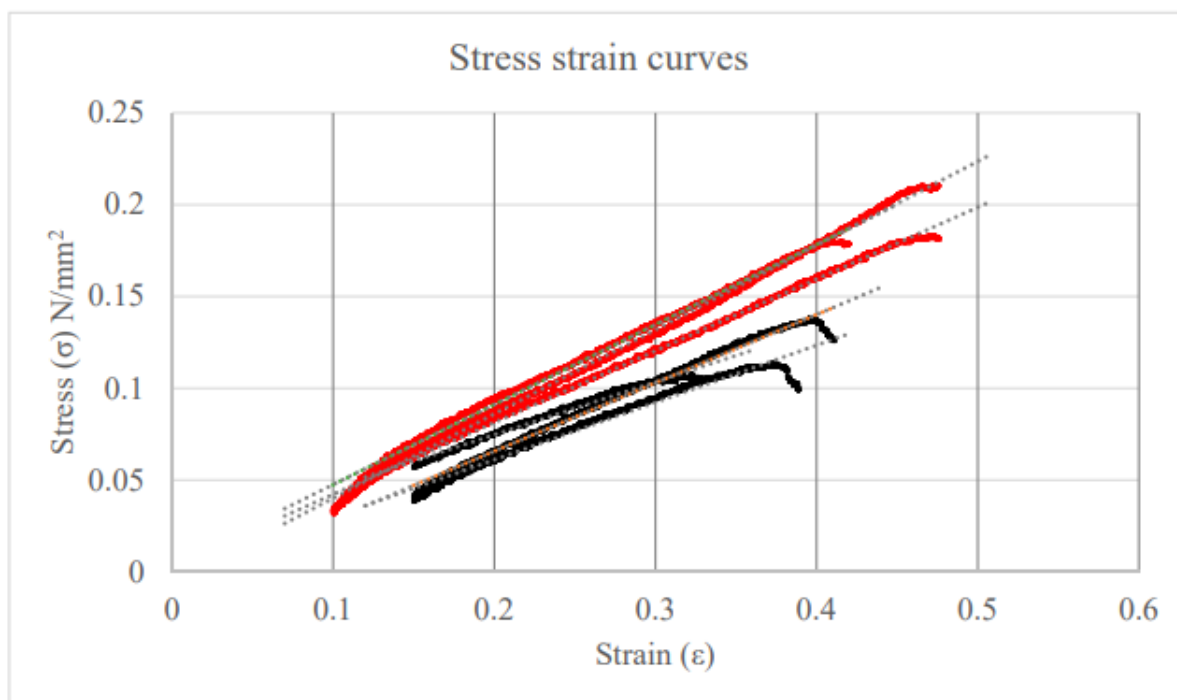


Figure 1. Relevant stress-strain curves: ALG controls ($Y = 0.32X - 0.0045$, $r^2 = 0.989$ $n = 3$) in RED; alginate/nanocrystalline cellulose (ALG/CNC, 5% and 3% w/v) + silver nanoparticles (AgNP, $Y = 0.43X - 0.0041$, $r^2 = 0.998$ $n = 3$) in black.

3.2. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS)

CAD design for the 3D printing of scaffolds with the nominal filament and macropore values is presented in Figure 2. As for morphology, SEM images of ALG and ALG/CNC scaffolds exhibited rough homogeneous surfaces with optimal macro pore size, ranging from 174 to 201 μm , demonstrating that the computational CAD parameters set during the design phase are predictable and respected over the whole manufacturing process (Figure 3A,C). The cross-sectional micrographs of ALG scaffolds revealed a regular interconnected and layered pore structure (Feret diameter ranging from 11 to 45 μm) in the interior region (Figure 3B). Interestingly, the case of ALG/CNC scaffold the regular formation of hollow tubular fibers (Feret diameter ranging from 11 to 126 μm) was observed (Figure 3D). These interesting results can be related only to the biomaterial blend and the gelation parameter process and can be further investigated as a function of the CNC ratio and the processing parameters to create ad hoc 3D systems suitable for applications in regenerative medicine or drug delivery. These porosity values indicate that the topography of the system is suitable for capillary absorption of exudate, wound transpiration and nutrients diffusion through the structure coming from surrounding healthy tissue. Finally, EDS Ag map clearly evidenced how AgNPs are homogeneously embedded and distributed in both biomaterials (Figure 3E,F).



Figure 2. Schematic representation of expected scaffold micro-dimensions

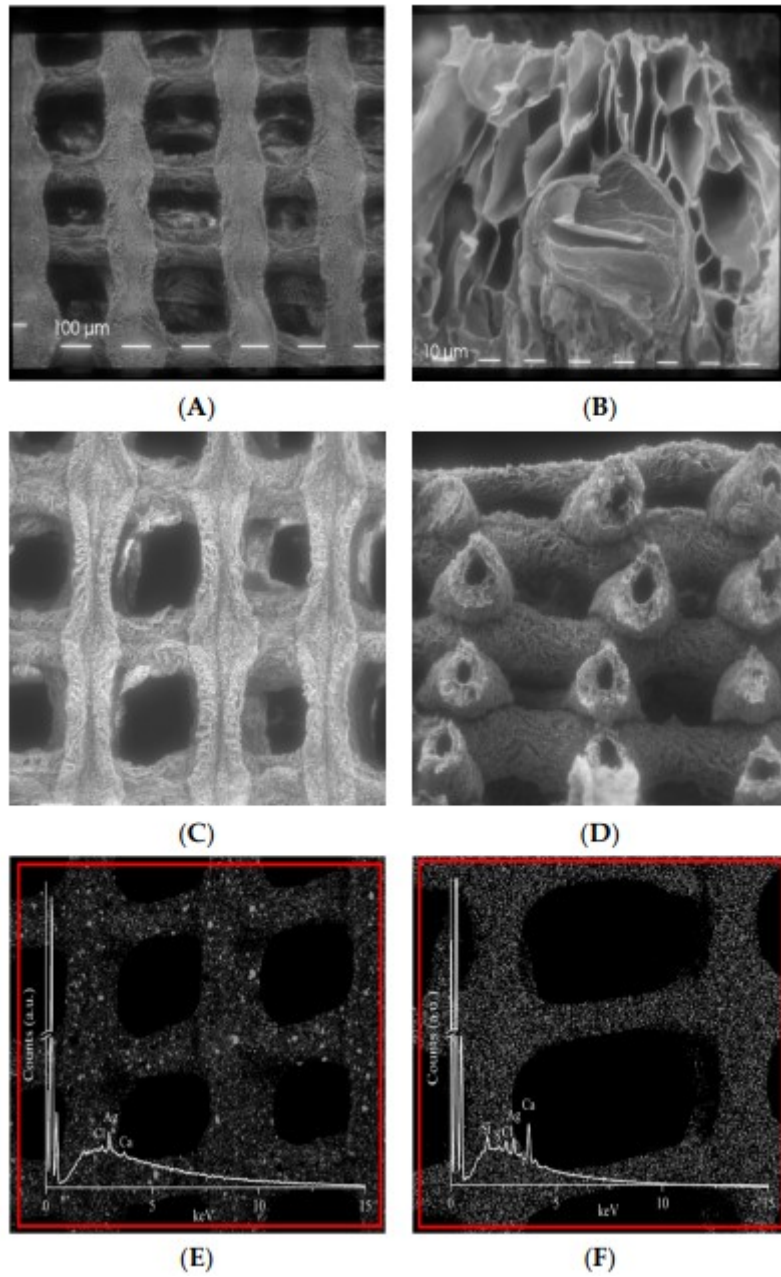


Figure 3. Scanning electron microscope images at different magnification of: (A) ALG 5% (w/v) scaffold (80×); (B) ALG 5% (w/v) scaffold (360×); (C) ALG/CNC (5% and 3% w/v) scaffold (80×); (D) ALG/CNC (5% and 3% w/v) scaffold cross Section (80×). Energy-dispersive X-ray spectroscopy

(EDS) map distribution and EDS spectrum of Ag in: (E) ALG 5% (w/v) scaffold (80×); (F) ALG/CNC (5% and 3% w/v) scaffold (80×).

3.3. Antimicrobial Activity

Assay In order to evaluate the antimicrobial efficacy of the developed devices, ALG, ALG/CNC (AgNP-free) and ALG/CNC + AgNP 3D scaffolds were compared in terms of inhibition of bacterial growth in bacterial inoculated Petri dishes. Different AgNP concentrations were tested to assess MIC, when embedded in the 3D scaffolds and relevant data are reported in Table 1. As expected, antimicrobial assay demonstrated how controls (tested scaffolds not containing AgNP) did not show any antimicrobial activity whereas AgNP-loaded scaffolds demonstrated the capability to inhibit growth of both the resistant bacteria species, inducing an inhibition ring of 6 mm. By decreasing concentrations of AgNP from 100 to 1 µg/mL, MIC was established being very close to 10 µg/mL. By applying scaffolds up to three times, analogous results were obtained. These findings suggest that the ALG/CNC hydrogel formulation can be effective at low dose of AgNPs, with a possible controlled release of the active agent driven by the biomaterial, thus reducing therapeutic side effects and preparation costs.

Table1. Determination of antimicrobial activity of 3D-printed developed scaffolds.

SCAFFOLD	<i>Staphylococcus aureus</i>		<i>Pseudomonas aeruginosa</i>	
	Ø Inhibition Diameter (mm)			
ALG	0	0	0	0
ALG/CNC	0	0	0	0
ALG/CNC + AgNP (100 µg/mL)	6	6	6	6
ALG/CNC + AgNP (10 µg/mL)	6	6	6	6
ALG/CNC + AgNP (5 µg/mL)	0	0	0	0
ALG/CNC + AgNP (1 µg/mL)	0	0	0	0

3.4. Cytotoxic Activity Towards HepG2 Cells

3.4.1. Cell Viability Evaluation

As a last application, HepG2, epithelial adherent hepato-carcinoma cells, were used as the cancer model. This cell line is particularly useful for investigating intracellular trafficking, estimating toxicity of xenobiotics or drugs and testing of novel anti-cancer active principles. Initially, aqueous nanoparticle suspension at 100 and 200 ppm were tested to explore which AgNP concentration could be appropriate for scaffold toxicity experiment. Cytotoxicity effect provided by AgNP at both concentration levels results in being clearly high and fast when the suspension is directly administered to cells. Cells vitality immediately drops close to zero over 24 h (Figure 4) and is maintained until 48

h (data not shown). These findings suggested the use of the lowest AgNP dose (100 ppm) to develop a 3D scaffold presenting cytotoxic activity vs. HepG2 cells meanwhile lowering potential side effects. However, ALG/CNC+ AgNP (100 ppm) scaffolds did not exhibit any cytotoxic effect and HepG2 cells' vitality was comparable to that obtained by using ALG and ALG-CNC control scaffolds within 7 days (Figure 5). After 14 days the vitality of HepG2 cells significantly decreased on scaffolds containing AgNPs (100 ppm). Interestingly, running this experiment it was noticed that ALG scaffold free of CNC tended to partially deform and/or dissolve while CNC-including scaffolds did not change even under optical microscopy evaluation (images not shown). This can be due to the formation of a tight network of hydrogen bonds between CNC particles and the alginate matrix, that can contribute to a major stability of the scaffold. The third experiment, AgNP concentration was increased to 250 ppm to better investigate the phenomenon that previously occurred. Analysis performed at day 2 revealed that treatments with AgNPs embedded in both ALG and ALG/CNC scaffolds induced a significant reduction of cell viability. In particular, the decrease was statistically significant ($p < 0.01$) in the case of ALG/CNC + AgNP (decrease $> 40\%$) (Figure 6). At day 7, both scaffolds still showed significant cytotoxicity raise compared to control ($p < 0.001$ and $p < 0.01$, respectively). Cytotoxicity of ALG + AgNP scaffolds showed an increasing pattern over 7 days, making us hypothesize that AgNP concentration in the well solution is increased by being released from alginate melting, and thus having a stronger effect. As already, noticed ALG + AgNP scaffolds lose their structure and dissolve in the presence of HepG2 cells. These observations suggest the efficacy of the developed scaffold to perform cytotoxic effects and the possibility to test different ALG/CNC ratio in order to develop an erodible system capable of cytotoxic effects towards HepG2 cells in a controlled way over time.

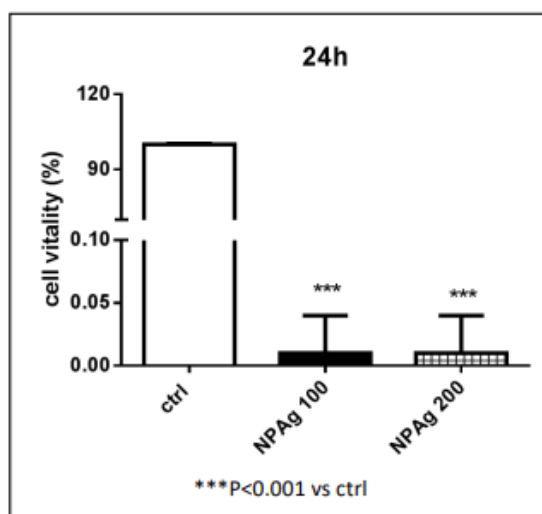


Figure 4. Cell viability % of hepatocellular carcinoma (HepG2) cells NOT TREATED (ctrl), TREATED with AgNP suspension at 100 (NPAg 100) and 200 (NPAg 200) ppm.

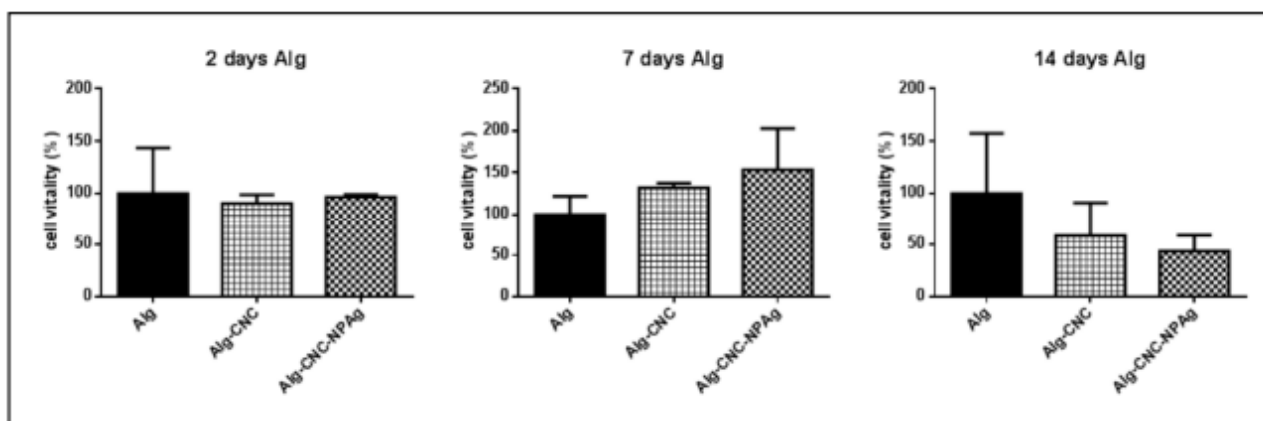


Figure 5. Cell viability % of HepG2 cells on ALG (5% w/v), ALG/CNC (5% and 3% w/v) and ALG/CNC (5% and 3% w/v) + AgNP (100 ppm) scaffolds.

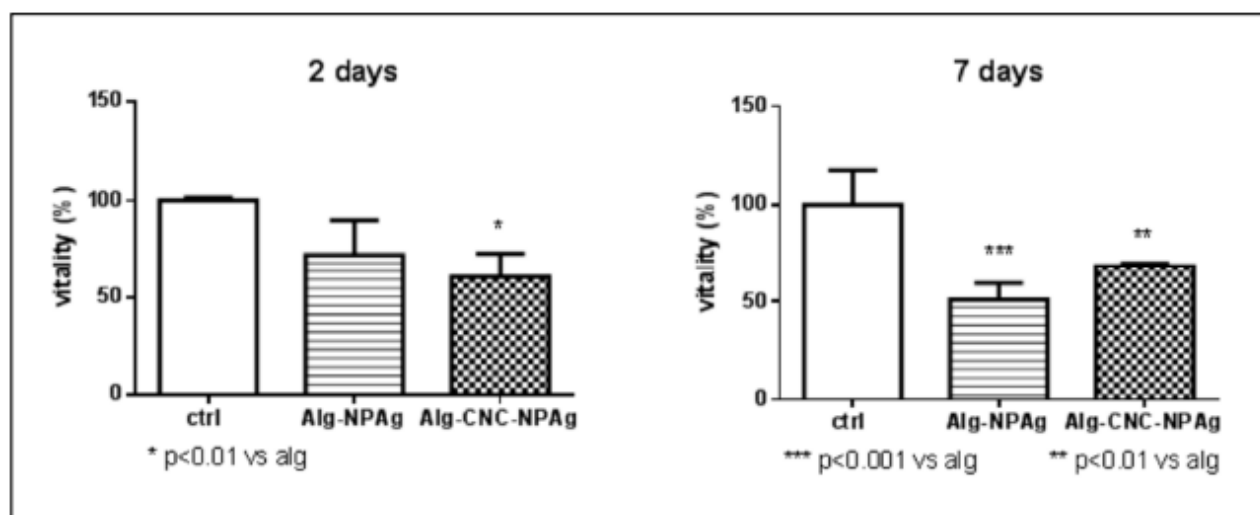


Figure 6. Cell viability % of HepG2 cells NOT TREATED (ctrl), TREATED with ALG (5% w/v)-AgNP (250 ppm) and ALG/CNC (5% and 3% w/v) + AgNP (250 ppm) scaffolds.

3.4.2. Evaluation of Apoptosis Induction through LC-MS/MS Protein Determination

Several studies available in the literature reported that AgNPs enhance programmed cell death mechanisms. In an endeavour to further investigate the complex process of AgNP cytotoxic/activity on HepG2 cells, specific proteins were determined by LC-MS/MS SRM targeted analysis. In particular, pro-apoptotic initiation caspases (8 and 9), executioner caspases (3), anti-apoptotic Bcl-xL class proteins, proliferative proteins like Ki-67 and the growth factor HGF (hepatocyte growth factor) were selected. The presence/absence of such proteins and their relative quantitation was carried out by analyzing cells after 2 days and 7 days of culture on ALG (control), ALG + AgNPs (250 ppm) and ALG/CNC + AgNPs (250 ppm). Protein quantitation by means of Bradford assay was employed to estimate protein quantity in each sample and to normalize data (Table S2). At day 2, Ki-

67 and HGF are significantly ($p > 0.05$) more expressed in ALG only with respect to both the treating conditions (AgNP-loaded scaffolds) (Figure 7).

These outcomes suggested that proliferative activity induced by these two proteins is significantly reduce on the AgNP-containing scaffolds. Confirmation of this hypothesis emerged through the analogue analysis run at day 7 (Figure 8); the expression of Ki-67 and HGF proteins dramatically drop in Ag-NP treated cells ($p > 0.05$) with respect to those cultured on ALG only. Taking into consideration anti-apoptotic BcL-xL protein, there is a noticeable decrease of its concentration in AgNP-enriched scaffolds at both 2 and 7 days (Figures 7 and 8). The other target proteins, such as pro-apoptotic one (caspases), increased over time in terms of expression for treated scaffolds, strengthening the thesis of AgNP-mediated apoptosis induction (Figures 7 and 8). These outcomes are partially superimposable with those obtained from viability assays and reveals that cells cultured on ALG/CNC + AgNP expressed the lowest viability scores which could be explained by the verified hypothesis of a direct cell apoptosis induction.

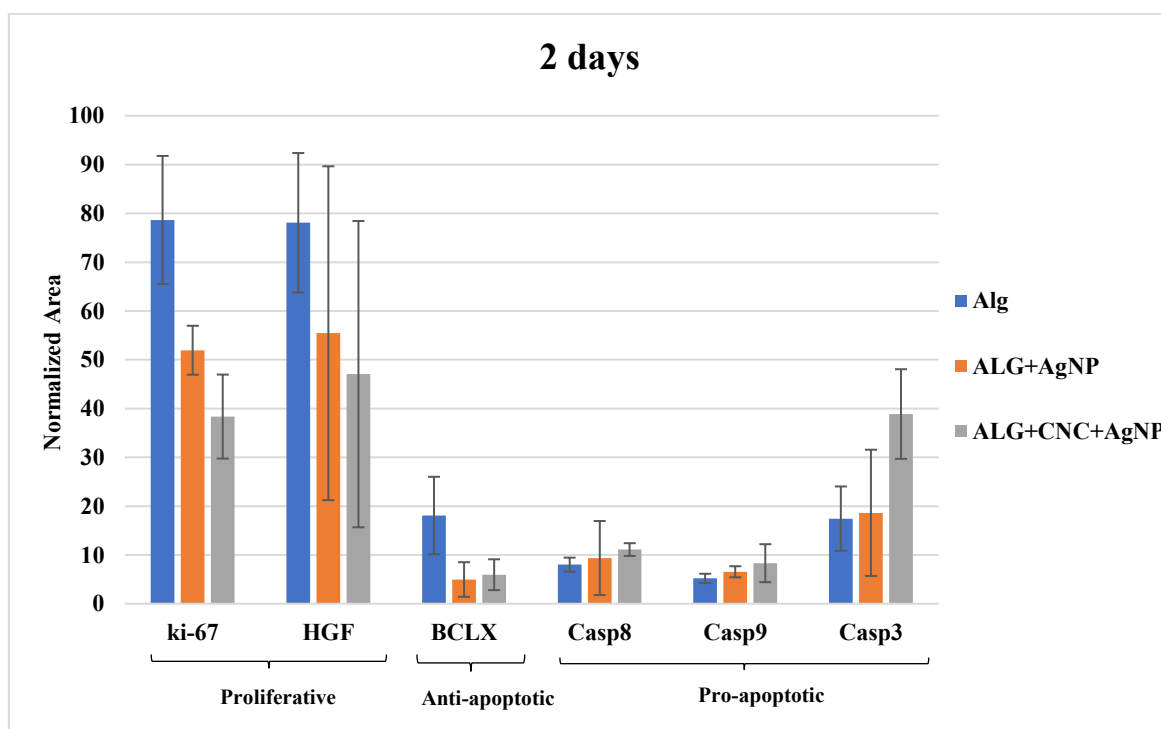


Figure 7. Liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) SRM targeted protein expression at day 2.

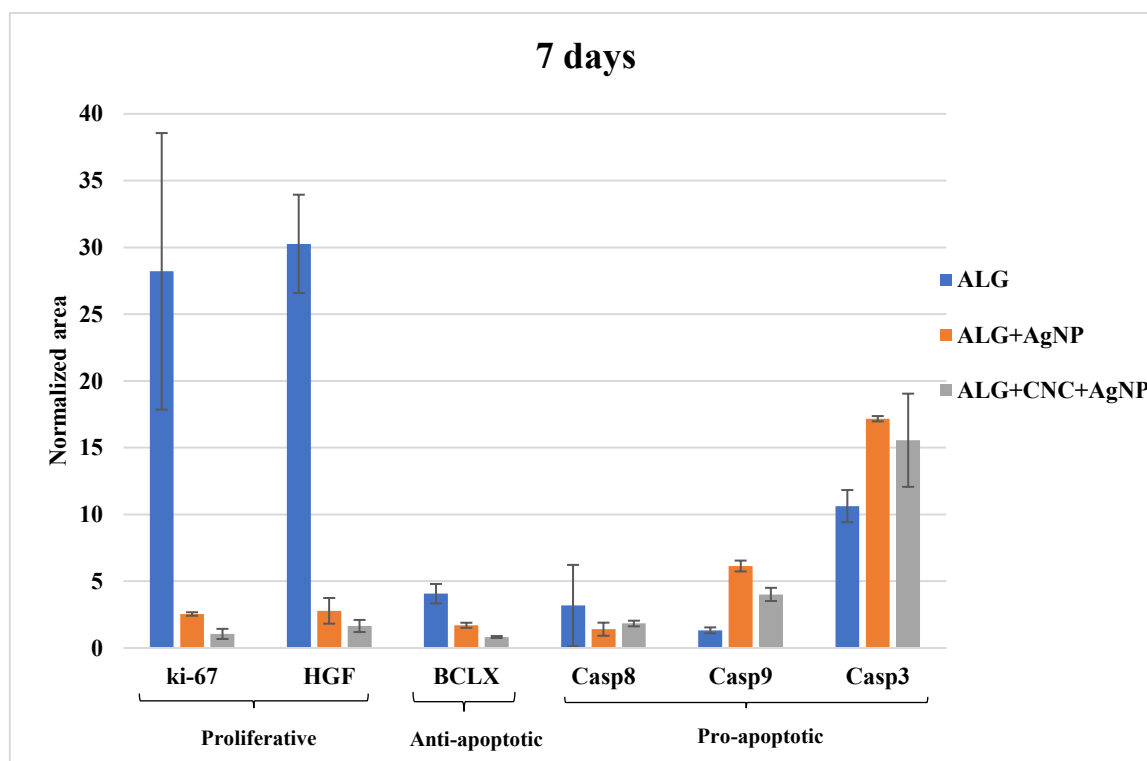


Figure 8. LC-ESI-MS/MS SRM targeted protein expression at day 7.

4. Conclusions

Silver nanoparticles supported in 3D printed ALG/CNC hydrogels were successfully prepared and characterized. Antimicrobial activity and cytotoxicity were evaluated for the first time. The results of an antimicrobial test against *S. Aureus* and *P. aeruginosa* confirmed a considerable antimicrobial activity against both Gram⁺ and Gram[−] bacteria with low nanoparticle dose (MIC 10 µg/mL) embedded in the composite biomaterial. In the presence of HEPG2 cells, the data pinpointed that AgNPs embedded in ALG only are rapidly dispersed in the culture medium because of low mechanical properties and solubility. The use of CNC in the ALG/AgNP mixture allowed us to overcome this issue and to obtain scaffolds with improved mechanical properties against HEPG2 cells, making AgNP cytotoxicity more effective over time. Finally, target MS analysis of marker proteins provided evidence about the impact of AgNPs on chosen cellular mechanisms. Hence, the use of this composite can be a promising approach to create new active materials for clinical applications.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2079-4991/10/5/844/s1>: Table S1: List of peptides and SRM transitions monitored, Table S2: Bradford assay protein quantitation (n = 2).

Author Contributions: L.E.: Conceptualization, project administration, methodology, data curation, draft revision. G.R.: Writing-original draft and investigation. C.B.: Investigation. R.B.: Review. I.Z.: Supervision and Investigation. F.B.: Review, Financial support. L.B.: Investigation. C.G.: Investigation and supervision. M.P.: Investigation. M.C.O.: Investigation. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials

Table S1. List of peptides and SRM transitions monitored.

Accession n.	Protein Name	Peptide Sequence	Precursor Mz	Prec. Char.	Product Mz	Fragment Ion
Q07817	BCL-X	EVIPMAAVK	479,28	2	616,35	y6
Q07817	BCL-X	EVIPMAAVK	479,28	2	229,12	b2
Q07817	BCL-X	EAGDEFELR	533,25	2	693,36	y5
Q07817	BCL-X	EAGDEFELR	533,25	2	288,20	y2
Q14790	CASP-8	MLEESNLSFLK	655,84	2	937,50	y8
Q14790	CASP-8	MLEESNLSFLK	655,84	2	808,46	y7
Q14790	CASP-9	MLEESNLSFLK	655,84	2	1066,54	y9
Q14790	CASP-8	MLEESNLSFLK	655,84	2	245,13	b2
Q14790	CASP-8	EQDSESQTLDK	640,29	2	691,36	y6
Q14790	CASP-8	EQDSESQTLDK	640,29	2	476,27	y4
P55211	CASP-9	LFFIQAC[+57]GGEQK	699,35	2	877,38	y8
P55211	CASP-9	LFFIQAC[+57]GGEQK	699,35	2	408,23	b3
P55211	CASP-9	ELFRPHMIEDIQR	421,72	4	303,18	y2
P55211	CASP-9	ELFRPHMIEDIQR	421,72	4	634,81	b10
P55211	CASP-9	FSSLHFMVEVK	441,90	3	246,18	y2
P55211	CASP-9	FSSLHFMVEVK	441,90	3	545,29	y9
P42574	CASP-3	SGTDVDAANLR	559,78	2	873,44	y8
P42574	CASP-3	SGTDVDAANLR	559,78	2	758,42	y7
P42574	CASP-3	EEIVELMR	509,77	2	647,35	y5
P42575	CASP-3	EEIVELMR	509,77	2	548,29	y4
P42578	CASP-3	GTSLDC[+57]GIETDSGVDDDMAC[+57]HK	808,99	3	1012,89	y18
P42579	CASP-3	GTSLDC[+57]GIETDSGVDDDMAC[+57]HK	808,99	3	876,33	y7
P46013	ki-67 b	ADVEEEFLALR	646,33	2	1006,52	y8
P46013	ki-67 b	ADVEEEFLALR	646,33	2	877,48	y7
P46013	ki-67 b	VEDAADSATKPENLSSK	587,96	3	767,37	y15
P46013	ki-67 b	VEDAADSATKPENLSSK	587,96	3	229,12	b2
P46013	ki-67 b	VEDAADSATKPENLSSK	587,96	3	201,09	b6
P46013	ki-67 b	VEDAADSATKPENLSSK	851,93	3	831,89	y16
P46013	ki-67 b	SQPDVPDTPSSKPSK	600,30	3	792,40	y15
P46013	ki-67 b	SQPDVPDTPSSKPSK	600,30	3	216,10	b2
P14210	HGF	GEEGGPWC[+57]FTSNPEVR	911,40	2	701,36	y6
P14210	HGF	GEEGGPWC[+57]FTSNPEVR	911,40	2	802,41	y7
P14210	HGF	HIFWEPDASK	615,30	2	251,15	b2
P14210	HGF	HIFWEPDASK	615,30	2	517,26	y5

Table S2. Bradford assay protein quantitation (n=2).

Treatment	2 Days	7 Days
	extracted proteins (µg)	
ALG	146.5	439.3
ALG	152.0	442.1
ALG+AgNPs (250ppm)	158.6	263.1
ALG+AgNPs (250ppm)	487.6	244.4
ALG/CNC+AgNPs (250ppm)	164.8	288.8
ALG/CNC+AgNPs (250ppm)	140.5	328.0

CHAPTER VII: The adaptation of lipid profile of human fibroblasts to alginate 2D films and 3D printed scaffolds

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IF 4.117

Personal Contribution

Giulia Remaggi: Writing-original draft and investigation

Abstract

Background: The investigation of the interactions between cells and active materials is pivotal in the emerging 3D printing-biomaterial application fields. Here, lipidomics has been used to explore the early impact of alginate (ALG) hydrogel architecture (2D films or 3D printed scaffolds) and the type of gelling agent (CaCl_2 or FeCl_3) on the lipid profile of human fibroblasts.

Methods: 2D and 3D ALG scaffolds were prepared and characterized in terms of water content, swelling, mechanical resistance and morphology before human fibroblast seeding (8 days). Using a liquid chromatography-triple quadrupole-tandem mass spectrometry approach, selected ceramides (CER), lysophosphatidylcholines (LPC), lysophosphatidic acids (LPA) and free fatty acids (FFA) were analyzed.

Results: The results showed a clear alteration in the CER expression profile depending of both the geometry and the gelling agent used to prepare the hydrogels. As for LPCs, the main parameter affecting their distribution is the scaffold architecture with a significant decrease in the relative expression levels of the species with higher chain length (C20 to C22) for 3D scaffolds compared to 2D films. In the case of FFAs and LPAs only slight differences were observed as a function of scaffold geometry or gelling agent.

Conclusions: Variations in the cell membrane lipid profile were observed for 3D cell cultures compared to 2D and these data are consistent with activation processes occurring through the mutual interactions between fibroblasts and ALG support. These unknown physiologically relevant changes add insights into the discussion about the relationship between biomaterial and the variations of cell biological functions.

Keywords: Ceramides, Fatty acids, Lysophosphatidic acids, Lysophosphatidylcholines, Human fibroblasts, 3D printing, Alginate, Liquid chromatography-mass spectrometry

1. Introduction

In the recent years the development of innovative active materials in combination with new production technologies (i.e. micro- and nanosystems, 3D printing, electrospinning, etc.) are of growing interest in several research and application fields (i.e. drug delivery, regenerative medicine, gene therapy, in vitro diagnostic tests, etc.) [1–3]. The driving idea is to overcome essential limitations of traditional approaches (i.e. introduction of more reliable diagnostic tests, improvement of drug delivery systems, bio-fabrication of tissues or organ-like systems etc.), and many efforts are made to evaluate the effectiveness of these systems through in vitro and in vivo studies. In such a context, the investigation of the interactions between cells and biomaterials is pivotal to obtain a more comprehensive mechanistic understanding of the cause-effect relationships of the whole system. The capability to offer thorough pictures of these complex living processes allow to drive new development strategies in the huge application landscape of biomaterials. However, many challenges still remain open, as it is well known that cell behavior depends upon several factors, including microenvironment dimensions, structure and chemico-physical composition. Biomaterials interact with the cells at various length scales, from that of individual cells (micrometers) to the that (nanometers) of single molecules (i.e. proteins, lipids). These interactions are based on both physical contact and chemical binding and depend over time as a function of the dimension of the system. Individual cells interact via integrin with a biomaterial for days or weeks, while individual proteins, or lipids or glycosaminoglycans interact through secondary bonds and hydrophobic interactions on time scales as short as seconds and minutes [4,5]. A complex network of non-covalent kinetically rapid interactions such as hydrogen bonds, van der Waals and hydrophobic interactions can affect the more driving and thermodynamically stable ionic interactions. The spatial architecture, surface area, interstitial pore distribution and dimensions of native extracellular matrix (ECM) strongly influence the cell migration, proliferation and differentiation [6]. Biomaterial surfaces can induce changes either in the cell membrane fluidity and permeability, which in turn regulate cellular and tissue functions, or in cell phenotype, including morphology, proliferation and biochemical properties [7,8]. For example, the surface and inner pore size of a scaffold influence the migration speed of cells: in collagen-GAG scaffolds the smaller is the average pore size, the lower is fibroblast migration speed [9–11]. Nonetheless, it has been shown that prostate cancer cells migrate faster than fibroblasts through the same scaffold [12]. It is clear that the knowledge of these effects at the molecular level can help to develop and tune optimal biomaterials and scaffolds as a function of the application. Alginate (ALG) is a widely investigated biomaterial used in drug delivery and in many biomedical applications thanks to its excellent properties, such as biocompatibility, low toxicity, low cost and ability to undergo spontaneous gelation under mild conditions [13–16]. ALG is a natural occurring

anionic polymer extracted from the brown algae cell wall. It is an unbranched binary copolymer consisting of the repetition of the monomer units D-mannuronic acid (M) and L-glucuronic acid (G), held together by β 1-4 bonds [17]. ALG, as cross-linked hydrogel presents structure, flexibility, porosity and diffusive transport characteristics similar to the ECM of human native tissues and is used in regenerative medicine as dressings to keep a wound moist, minimize bacterial infection and accelerate the healing process [18]. As for hydrogel formation, during ionotropic gelation a network of interactions involving different junction zones (i.e. eOH , COO^- and eO^- groups) of consecutive G-residues of the ALG chains and the cation occurs. Calcium chloride (CaCl_2) represents one of the most used crosslinking agent for the formation of the ALG ionotropic hydrogel. The divalent cations bind only to the glucuronic acid blocks of the ALG chains forming a cross-linking model called “egg-box”, resulting in a gel structure. Conversely, chelation with the trivalent Fe^{3+} ions allows for more spherical shapes [18]. In general, the speed of gelation is an important parameter to control the uniformity and shape of the gel. Slower gelation leads to the production of more uniform structures and greater mechanical integrity [19]. Furthermore, better cell adhesion and proliferation have been observed on ALG matrices when the latter is gelled with trivalent ions, such as Fe^{3+} [20]. Among applications ALG is widely exploited for the controlled release of drugs and proteins, for the transport of cells to a specific site [21–23] and to perform two dimensional (2D) and three dimensional (3D) cell studies to understand cell-matrix interactions. Although cells in a living system are exposed to complex 3D biological environments, biological phenomena is still extensively investigated by means of 2D substrates. 2D assays present major limitations to accurately describe the space constraints of cells in vivo and can induce different cell activities and/or loss of the original cell phenotype. 2D films are still widely used in several cell culture experiments, but 3D printing manufacturing process is rapidly gaining a prominent role as innovative technology in the medical or diagnostic fields to shape biopolymers in a variety of architectures to progressively replace two dimensional systems [24–29]. With the aim to improve the basic knowledge available in the cell-biomaterial interaction field, in this study the effects of different architectures and gelling media used for the preparation of ALG hydrogels were investigated for the first time on the targeted lipid profile of human fibroblasts. Controlled and reproducible 2D films (2D-ALG) and 3D printed ALG scaffolds (3D-ALG) were produced by gelation with CaCl_2 or iron chloride (FeCl_3), and a selected targeted lipid profile of human fibroblasts seeded on them was evaluated. Cell biochemistry can be studied at different complementary levels (i.e. the transcriptome, proteome, lipidome or metabolome) to gain information useful to frame their behavior [30,31]. Here we decide to focus our attention on lipids, as they play a crucial role in the physiology of cells, tissues and organs as demonstrated by a large number of genetic studies [32]. The deregulation of lipid metabolic pathways therefore leads to the onset of diseases,

including cardiovascular disorders, cancer and diabetes [33–35]. Lipidomics is nowadays a consolidated field capable of a comprehensive analysis of lipids in complex biological systems. Lipidomics aims to profile the lipid structures and quantity in a biological sample, to assess their metabolic functions and transformations that occur in different physiological and pathological conditions [36]. The birth of lipidomics has been possible thanks to technological advances in the field of analytical instrumentation such as mass spectrometry (MS) [37–39]. This set of techniques is the golden standard approach for the investigation of the lipids in cells by virtue of their ability to perform the simultaneous identification and quantification of thousands of analytes in the same biological sample. Here a liquid chromatography-electrospray-tandem mass spectrometer, with a triple quadrupole mass analyzer, was used for the identification and relative quantitative detection of lipids belonging to the following classes, selected as powerful mediators of cell functions: ceramides (CER), fatty acids (FFA), lysophosphatidic acids (LPA) and lysophosphatidylcholines (LPC). CER and FFA are lipid species that modulate membrane rigidity, creating microdomains, and altering membrane permeability, thus regulating cell membrane functions [40]. Moreover, CER enhance the bioavailability of drugs by acting as lipid delivery systems, they play a structural role in liposome formulations and enhance the cellular uptake of amphiphilic drugs, such as chemotherapies [41]. In a recent study FFA have been chemically linked with biological drug molecules to enhance oral absorption of therapeutic peptides and to provide a platform for oral peptide drug development [42]. LPA derivatives are bioactive phospholipids present in biological fluids that regulate many important fibroblast functions, including proliferation, migration and contraction. Alteration in normal LPA signaling may contribute to a range of diseases, including neurodevelopmental and neuropsychiatric disorders, pain, cardiovascular disease, bone disorders, fibrosis, cancer, infertility, and obesity [43]. Therefore, therapies targeting LPA biosynthesis and signaling may be feasible for the treatment of devastating human diseases [44]. LPC are present as minor phospholipids in the cell membrane and blood plasma, promote inflammatory effects [45] and play a role in the pathway of fibrotic injury in human cardiac fibroblasts [46].

2. Materials and methods

2.1. Reagents

Acetonitrile, methanol, hexane and isopropanol were supplied from Sigma-Aldrich (Taufkirchen, Germany). Water was purified (0.055 uS/ cm, TOC 1 ppb) with a Purelab pulse + Flex ultra-pure water system (Elga Veolia, Milan, Italy).

2.2. Scaffold preparation

ALG formulation (Ph.Eur. grade; molecular weight by gel filtration chromatography (GFC) 180–300 kDa; slowly soluble in water, Carlo Erba, Italy) was prepared by dissolving the sodium ALG 6% (w/v) in deionized water. The formulation was left under stirring overnight on a magnetic plate until a homogeneous mixture was obtained. The 2D-ALG films were obtained by pouring the 6% ALG formulation onto a glass plate and then stretching it using a 0.6 mm film-stretcher. The two casting solutions were left to dry in an oven at 40 °C for minutes and 2 and a half hours, respectively. At this point, the dried alginate films were immersed in the solutions of CaCl₂ 3% (w/v), (anhydrous CaCl₂, lot. 419,887/1, Fluka Chemie GmbH, Switzerland) or FeCl₃ 3% (w/v), (anhydrous FeCl₃ 98%, lot. H29Y005, Alfa Aesar, United States) to cross-link for one hour. Subsequently, films were detached from the glass plates and rinsed twice in deionized water for 15 min. The 3D-ALG scaffolds were made by using a 3D printer built in house (38). The machine is based on a three cartesian axes system that allows the movement on the horizontal plane of printing plate and the vertical translation of the nozzle. ALG solution was loaded into a 10 ml syringe and was extruded by a pump acting on a syringe mounting a 26G needle, deposited, layer by layer, on the printing plate at a velocity of 3 mm/s and instantaneously frozen (−14 °C) with a series of Peltier cells which allows the material, to freeze instantly and maintain the three-dimensional structure. At the end of the print, the steel plate with the scaffold is removed and immersed in the gelling solution (CaCl₂ 3% (w/v) or FeCl₃ 3% (w/v)) for one hour. At the end of the gelling process, the scaffold is rinsed in deionized water to remove all traces of the gelling agent.

2.3. Evaluation of water content

To determine the water content, 3D (5-layer square with a side of 1.4 cm) and 2D (2 mm-thick) ALG scaffolds gelled in CaCl₂ (ALG_Ca) or in FeCl₃ (ALG_Fe) were prepared. After being molded, the scaffolds were gelled for 1 h in the respective gelling agents and subsequently rinsed in deionized water. The scaffolds were lightly buffered on absorbent paper and weighed on an analytical balance to determine the wet weight (W_w). Subsequently, the scaffolds were dried in an oven at 40 °C overnight and weighed again to determine their dry weight (W_d). Five replicates were analyzed. The water content (W_c) was calculated as:

$$WC = (W_w - W_d) / W_w \times 100$$

2.4. Swelling test

The swelling behavior of the scaffolds was evaluated over time (1 min, 30 min, 1 h, 2 h, 6 h, 24 h) by measuring their weight. To perform the test, 5-layer square ALG scaffolds were created, with a side

of 1.4 cm. After printing, the scaffolds were gelled for 1 h in the respective gelling agents and subsequently rinsed in deionized water and dried in an oven at 40 °C overnight before weight measurement. Subsequently, each scaffold was placed in deionized water and the weights were taken at each time interval. Before each measurement, the scaffolds were swabbed on absorbent paper to eliminate surface drops. Five replicates were analyzed. The swelling ratio (Qs), defined as the average percentage of swelling, was assessed through the following equation:

$$Q_s = (W_s - W_d) / W_d$$

where Ws represents the weight of the scaffold at a certain time and Wd is its initial weight.

2.5. Mechanical properties

The mechanical resistance of ALG_Ca or ALG_Fe was measured by using 3D 20-layers scaffolds and 2 mm-thick ALG films having size of 5 cm × 1.5 cm. Thickness was determined (n. of replicates = 6) with a digital micrometer (Mitutoyo, Japan). Each scaffold was fixed on a tensile tester (AG M1 Acquati, Italy) loaded with a 5 daN cell. Force and time signals were digitalized by means of a PowerLab 400 board and recorded with the Scope v.3.5 software. Elongation at break (% strain) and Young's modulus were determined from the relevant stress-strain curves, taking into consideration the linear portion. Three replicates were analyzed.

2.6. SEM analysis

SEM analysis were carried out to study the morphology and pore distribution of 2D-ALG and 3D-ALG. 2D-ALG (n = 3) or 3D-ALG 10- layers square (1.5 × 1.5 cm; n = 3) gelled in CaCl₂ or in FeCl₃ were prepared. The samples were immersed in increasing concentrations of ethanol (10%, 20%, 30%, 40%, v/v in dH₂O) 10 min for each step. Subsequently, the samples were subjected to freeze-drying for 24 h in the Christ Alpha 2–4 LSC plus Freeze Dryer and gold sputtered through a metallizer (Balzers). Surface and section images of 3D samples were captured at different magnifications, ranging from 80× to 640×. Photographs of 2D film surfaces were acquired at 300× magnification. For the acquisition of the images of 2D-ALG a scanning electron microscope (Sigma HD, Carl Zeiss, Jena, Germany) at EHT 1.00 kV was used while a SEM 501 (Philips) was employed for 3D scaffold characterization. All images were analyzed by ImageJ software (NIH, Bethesda USA) for the measurement of macro- and micro-structures, mean pore size (Feret diameter) and distribution.

2.7. Cell culture

Primary human skin fibroblasts, coded as C86, were derived from a forearm biopsy of a healthy donor after signature of an informed consent. Cells were maintained in Dulbecco's Modified Eagle's

Medium (DMEM, Gibco) culture medium, supplemented with 1% (v/v) of L-glutamine (Gibco), 1% of Streptomycin and Penicillin (Gibco), 1% of Na-pyruvate (Gibco), 1% of NEAA (Non-Essential AminoAcids, Gibco) and 10% of FBS (Fetal Serum Bovine, Euroclone). After overnight disinfection in ethanol 70% (v/v in dH₂O), 2D-ALG and 3D-ALG were deposited in 12-well plates (Constar) and washed with sterile water. The following conditions have been tested: positive control (represented by the seeding of cells directly in the well); 2D-ALG_Ca; 3D-ALG_Ca; 2D-ALG_Fe; 3D-ALG_Fe; negative control (represented by films and scaffolds without cells). 500 k cells per well were seeded (150 µl) directly on 2D and 3D scaffolds to observe the impact of microenvironment on cell behavior and 850 µl of 10% FCS medium were added to reach the volume of 1 ml. Cells were incubated and maintained for 8 days at 37 °C, 5% CO₂ (Incubator Nabco, Chicago, IL, USA).

2.8. MTT assay

The MTT assay was carried out as follows: 500 µl of a solution consisting of MTT (1 mg/ml) and 5% FCS medium were added to each well. The cells were incubated for 2 h at 37 °C. After two hours, the MTT and medium solution was aspirated, and the films and scaffolds were transferred into new plates. 500 µl of DMSO (dimethylsulfoxide) were added to each well and the plates were stirred on a tilting plate for 15–20 min at room temperature. From each well 200 µl were taken and placed in a 96-well plate (Constar, Flat Transparent) for spectrophotometric reading. Each sample was analyzed in duplicated. 200 µl of DMSO was added to two wells of the plate to be used as a control. Spectrophotometry reading was carried out at a wavelength of 570 nm. The spectrophotometer used is a TECAN Spark 10 M spectrophotometer. The data relating to the analysis were processed by subtracting the absorbances obtained from the cell-free supports (negative controls).

2.9. Lipid extraction

The extraction of lipids from cellular matrices was performed by using a mixture of hexane and isopropanol (3:2; v/v). Cells grown on plastic and ALG films were detached using trypsin, centrifuged and resuspended in iced sterile water. To detach the cells grown on the 3D scaffolds an EDTA-Na citrate (EDTA 50 mM; Na citrate 55 mM) solution was used to dissolve the scaffolds. The contents of the wells were centrifuged and the pellet was resuspended in iced sterile water. The cell suspension obtained was transferred to cryotubes and cell lysis was obtained by 2 freezing cycles in liquid nitrogen and thawing at 37 °C for 10 min. Subsequently, the samples were transferred into glass tubes and 360 µl of hexane and 240 µl of isopropanol were added. After being vortexed for 1 min, the tubes were centrifuged (2000 rpm × 5 min). The organic phase was transferred to new glass tubes, and the

procedure was repeated twice. The supernatants obtained were evaporated under nitrogen stream and resuspended in 50 μ L of methanol before LC-MS analysis.

2.10. Liquid chromatography-mass spectrometry analysis

The analyses were carried out by using an HP1200 Agilent LC system (Agilent Technologies, USA) equipped with an electrospray QTRAP 4000 mass spectrometer (ABSCIEX, CA, USA). The chromatographic separation was carried out on C18 (50 $\times$ 2.1 mm, 5 μ m) column (Phenomenex, CA, USA). Mobile phase was (A) methanol and (B) acetonitrile. The mobile phase was filtered through a 0.45 μ m cellulose membrane before use. Flow-rate was 0.2 ml/min and the injected volume was 10 μ L. The system was controlled by the Analyst software v. 1.4. (ABSCIEX). Source parameters were: negative ion (NI) ESI voltage, -4.5 kV; declustering potential, -50 V; entrance potential, -10 V; source temperature, 350 $^{\circ}$ C and positive ion (PI) ESI voltage, 5.5 kV; declustering potential, 50 V; entrance potential, 10 V; source temperature, 350 $^{\circ}$ C. Quadrupoles were tuned to unit resolution. As for quantitative analysis, experiments were performed under PI- or NI-SRM conditions using nitrogen as collision gas (medium nitrogen pressure). The SRM transition considered in this study are reported in the Table S1 of the supplementary material. The analytes were relatively quantified among samples and normalized for MTT assay. The chromatograms were analyzed through the MultiQuant software (version 2.1).

2.11. Statistical analysis

Comparisons between groups were made by one-way ANOVA with Tukey's correction for multiple testing. The data from LC/MS analysis have been expressed as % of the individual lipid specie on the total amount of lipids on each biomaterial. Given the large number of observations that are highly correlated with each other, no statistical analysis was made for comparison of lipid species profiles within the four biomaterials.

3. Results and discussion

3.1. 2D-ALG and 3D-ALG preparation and characterization

In the first part of the work the preparation and characterization of the 2D-ALG and 3D-ALG (Fig. 1) were addressed in order to study the effects of the different gelling media on the final hydrogel properties. In general, ALG presents different chelating affinity for its cross-linking cations, as a function of their charge and dimensions, resulting in hydrogels having different properties such as swelling, elasticity and stability. The determination of the water content, a parameter that allows to evaluate the ability of the scaffolds to absorb biological fluids and keep the tissues hydrated, displayed

that both 2D- and 3D-ALG_Fe retain very similar percentage of water ($93 \pm 1\%$) compared to 2D- and 3D-ALG_Ca ($90.1 \pm 0.8\%$). The swelling tests showed that, in general, ALG_Ca immersed in deionized water tend to swell up to 6 h. ALG_Fe, on the other hand, showed this behavior only up to 2 h (Fig. 2). The ALG scaffolds were also submitted to stress-strain tests. The maximum stress (MPa) was calculated by dividing the force applied at the breaking point (N) by the cross-section area of the specimens (mm^2). Another test used was the tensile test that allowed to determine Young's modulus, (modulus of elasticity). The viscoelastic behavior of the scaffolds was therefore determined. 3D-ALG_Ca showed greater mechanical strength (5.7 ± 1.1 N) and therefore required greater strength to be broken, while 3D-ALG_Fe scaffolds were less resistant (4.9 ± 1.0 N). 3D-ALG_Ca scaffolds were characterized by greater elasticity (6.5 ± 0.4 MPa), than 3D-ALG_Fe, which were extremely rigid (0.72 ± 0.05 MPa). The gelling agent, therefore, caused a change in the elastic behavior of the ALG, making it particularly elastic and resistant when gelled with CaCl_2 and excessively rigid and less resistant when crosslinked with FeCl_3 . 2D-ALG were also characterized for their mechanical behavior. As for 3D-ALG, a macroscopic difference in terms of consistence was noticed between samples gelled by the two media. In particular, 2D-ALG_Ca appeared stiff but much less rigid compared to 2D-ALG_Fe. Measurements showed an average Young's modulus of 3.3 ± 1.5 MPa and 26 ± 8 MPa for 2D-ALG_Ca and 2D-ALG_Fe, respectively. 2D-ALG_Ca showed an elastic behavior only during the first phase of longitudinal traction, followed by deformation of the samples (plastic behavior) and consequent rupture. 2D-ALG_Fe showed instead a linear increase of stress over strain until breakage, that occurred very early due to the rigidity of the tested material. The higher elasticity and plasticity of 2D-ALG_Ca was demonstrated as well by the calculation of percent elongation, whose mean value resulted $20.3 \pm 9.9\%$. In comparison, 2D-ALG_Fe showed a significantly lower strain, with a maximum percent elongation of $3.0 \pm 1.5\%$. While 2D-ALG_Ca highlighted suitable features for potential adoption as material for regenerative medicine, 2D-ALG_Fe demonstrated overall weak mechanical properties, being very rigid, devoid of elasticity and capability to flex or strain without breakage. As for morphological characterization, SEM analysis was carried out to evaluate the microstructure of the ALG scaffolds. The scaffolds should have a high porosity and an interconnected pore structure suitable for penetration, as well as adhesion, proliferation and cell differentiation. Fig. 3 displays the pore size distribution, on surface of 3D-ALG_Ca scaffolds (Fig. 3a–b) and of 3D-ALG_Fe (Fig. 3d–e). In the first case the average pore size, expressed as Feret's diameter, was between 11 and 45 μm ; while in the second case the average pore size was higher (between 11 and 126 μm). This difference could be attributed to the gelling agent which leads to the formation of more or less large pores during the gelation process of the biomaterial. In both cases, the pore size was adequate for the penetration of fibroblasts which had a diameter ranging from 15 to

20 μm . The longitudinal cross-section of 3D-ALG_Ca exhibited high porosity with homogenous distribution (5–40 μm) (Fig. 3c). 3D-ALG_Fe showed regular tubular morphology mimicking a vessel-like structure (Fig. 3f), where the inner diameters of the hollow filaments range from 50 μm to 140 μm . This morphological behavior strongly depends on the gelling conditions and can be useful to design scaffolds with tunable shapes for further applications in regenerative medicine or drug delivery. The differences observed between the effects of the two gelling media can correlate with the differences between the mechanical properties described above. In fact the 3D-ALG_Ca showed greater mechanical strength, compared to the less resistant 3D-ALG_Fe. 2D-ALG present much less porous compared to 3D-ALG. Few pores having dimensions of 10 to 20 μm are randomly spread on the surfaces of overall compact structures. 2D-ALG_Fe (Fig. 3h) showed the presence of cracks in the polymeric structure, intermitted by rougher and more corrugated surfaces compared to 2D-ALG_Ca (Fig. 3g).

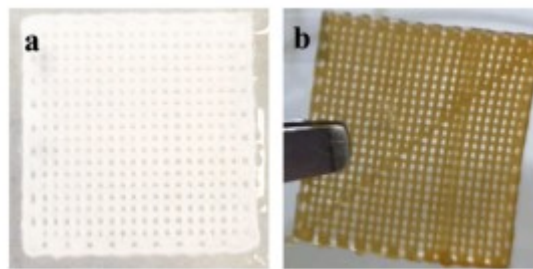


Fig. 1. 3D printed alginate scaffolds gelled by: (a) CaCl_2 and (b) FeCl_3 .

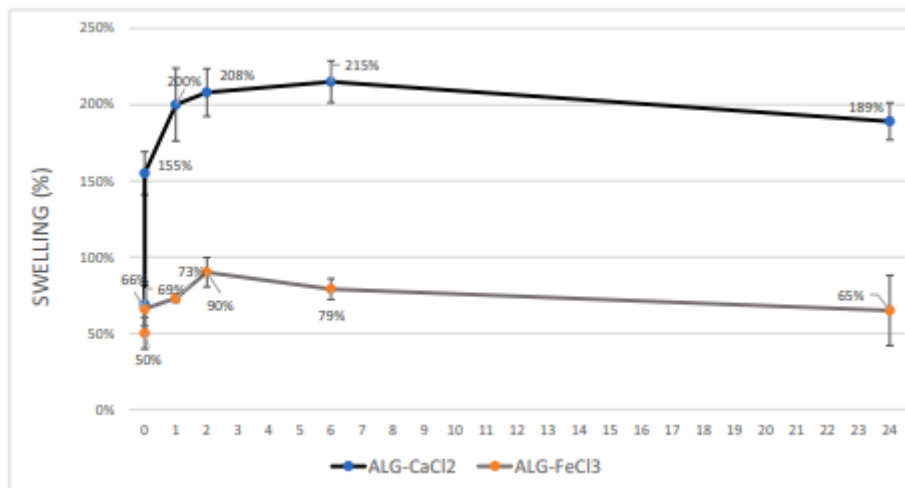


Fig. 2. Swelling test of 3D-ALG_Ca (dark grey) and 3D-ALG_Fe (light grey) scaffolds at different time points (n = 5).

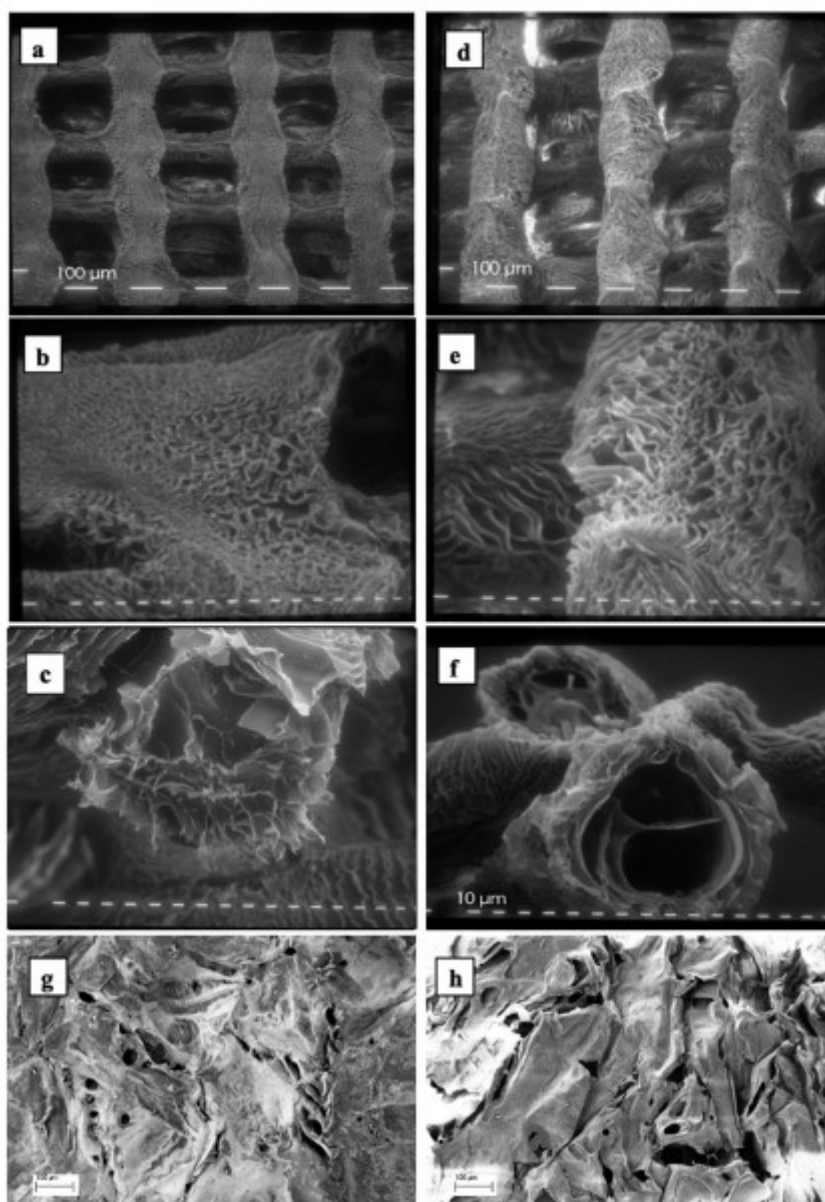


Fig. 3. SEM image of 3D- and 2D-ALG gelled with (a-c-g) CaCl_2 and (d-f-h) with FeCl_3 (4b). (a, b) 80 $\times$ magnification; (b, e) 240 $\times$ magnification; (c) 640 $\times$ magnification; (f) 320 $\times$ magnification; (g, h) 300 $\times$ magnification.

3.2. LC-MS/MS SRM analysis of the fibroblast lipid profile

The expression of CER, LPC, LPA and FFA was evaluated to understand how and if the geometry of 2D_ and 3D_ALG or the gelling agents are able to affect their relative expression levels in dermal human fibroblasts. Before LC-MS/MS analysis, the viability of the cells in contact with the 2D- and 3D-ALG was quantitatively assessed after 8 days by MTT colorimetric assay. Biocompatibility was demonstrated for 2D- and 3D-ALG_Ca (approx. 70% vs cells grown on Petri dish), and for 2D- and 3D-ALG_Fe (approx. 26% vs cells grown on Petri dish) (Fig. 4). In this latter case, a lower vitality percentage was obtained, but it should be noted that these supports were more difficult to handle

because of a reduced structural stability in the cell culture media. It was observed that the cell culture medium impacted on the scaffold structure. In particular, ALG_Fe tended to dissolve in the medium over the experimental time (images not shown), whereas ALG-Ca retained their structure. Such results could be in agreement with the different mechanical properties of the hydrogels obtained with calcium and iron or even with the affinity of the substances present in the culture medium toward the cations.

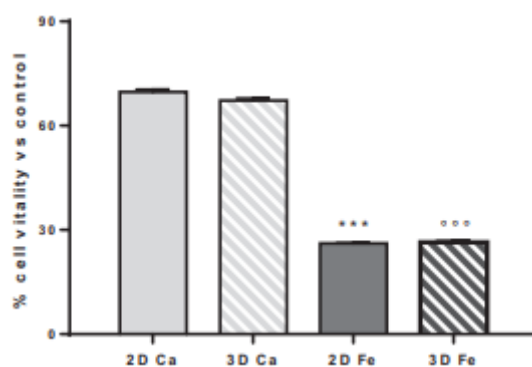


Fig. 4. Cell vitality evaluated by MTT assay. *** $p < 0.001$ vs 2D Ca; °°° $p < 0.001$ vs 3D Ca.

3.3. Ceramides

Nineteen CER d18:1 containing species were determined by targeted MS analysis. Among them, a remarkable variability was observed as a function of the scaffold preparation. The results obtained clearly show that the relative expression of these lipids was significantly affected by both the structure of the scaffolds and the gelling agents employed (Fig. 5). The most abundant species, represented by CER d18:1–16:0 and CER d18:1–22:0, showed a peculiar expression in response to the structure and the composition of the supports. The former reveals significantly higher amount on 2D-ALG_Ca than on 2D-ALG_Fe and on 3D-ALG_Ca, while the opposite was observed in the latter. In addition, CER d18:1–22:0 was more abundant when cells were grown on 2D-ALG_Fe compared to 3D-ALG_Fe. In the relatively less expressed species, a well-defined trend is evident: the short chain ceramides (CER d18:1–16:1, CER d18:1–20:0) present higher expression on the 2D-ALG_Ca compared to 3D-ALG_Ca, while the opposite occurs for long chain ceramides (CER d18:1–22:2, CER d18:1–22:5). The influence of the support composition is evident for many lipid species, that presented higher abundance on 2D-ALG_Ca (CER d18:1–16:0, CER d18:1–16:1, CER d18:1–20:0, CER d18:1–22:0, CER d18:1–22:2, CER d18:1–22:5) or 3D-ALG_Ca (CER d18:1–22:0, CER d18:1–22:2, CER d18:1–22:5) compared to the respective support made with FeCl₃. These findings suggest that, although the biomaterial and the geometry are the same, its surface morphology and the presence of Ca²⁺ or Fe³⁺ ions could significantly increase the production and the quali- and quantitative distribution of this class of compounds. In addition, LC-MS/MS chromatograms revealed that

ceramides exist as a balance mixture of two isomers (data not shown). In particular, two isomeric forms were observed for CER d18:1–16:0, CER d18:1–20:0, CER d18:1–22:0, CER d18:1–22:1, CER d18:1–22:2, with one isomer predominating over the second isomer, regardless of the architecture of the scaffold and the gelling agent.

3.4. LPC

The most representative LPC monitored were 16:0, 18:0 and 18:1 in all samples, whereas the other species were present in much lower amount (Fig. 6). LPC 16:0, LPC 18:0 and LPC 18:1 show a very similar relative expression level in all the tested conditions. On the other hand, many minor components of this class reveal significantly higher abundance when cells were grown on 2D-ALG than on 3D-ALG. This is the case for LPC 20:2, LPC 20:3, LPC 20:4, LPC 22:4, LPC 22:5 and LPC 22:6, when the gelling agent was FeCl₃, and for LPC 20:2, LPC 22:5 and LPC 22:6 when CaCl₂ was employed. Interestingly, the influence of the gelling agent was observed for LPC 18:2, LPC 20:4 and LPC 22:0, where higher abundance was observed in 3D-ALG_Ca than in 3D-ALG_Fe. All together these findings suggest that, although the main LPC composition is maintained, both the gelling agent and the scaffold structure may somehow affect the LPC distribution. As previously, LPC were detected as two isomeric forms, but no significant differences were observed in terms of relative intensities between the two gelling media or scaffold geometries.

3.5. LPA

LPA are usually present at very low concentrations in cell membranes and their detection is challenging. Using the LC-MS/MS method here proposed, the most abundant lipid detected was the 16:0 specie in all samples (Fig. 7). The data collected for LPA showed that the relative trend of these lipids was generally maintained with some slight differences especially related to the geometry of the scaffold. A significant increase of abundance in 2D-ALG_Fe vs 3D-ALG_Fe was observed for LPA 17:0 and LPA 20:1, while statistical difference was not reached for LPA 22:0. Consistently, a trend toward increased expression on 2D-ALG_Ca and 2D-ALG_Fe compared to respective 3D-ALG was observed for LPA 18:0. The abundance of this lipid specie was also influenced by the gelling agent, since an increase of expression in 2D- and 3D-ALG_Ca versus 2D- and 3D-ALG_Fe was observed, despite not statistically significant.

3.6. FFA

Among the detected FFA, the most abundant species were 20:0 and 22:0. No significant differences were observed in the general trend of FFA on the different supports (Fig. 8). Only an increase in the

expression level of two minor species (FA 20:4 and FA 22:1) was observed when cells were grown on 2D-ALG_Ca vs 3D-ALG_Ca. These data suggest that FFA are not susceptible of significant influence neither from the geometry nor from the gelling agent.

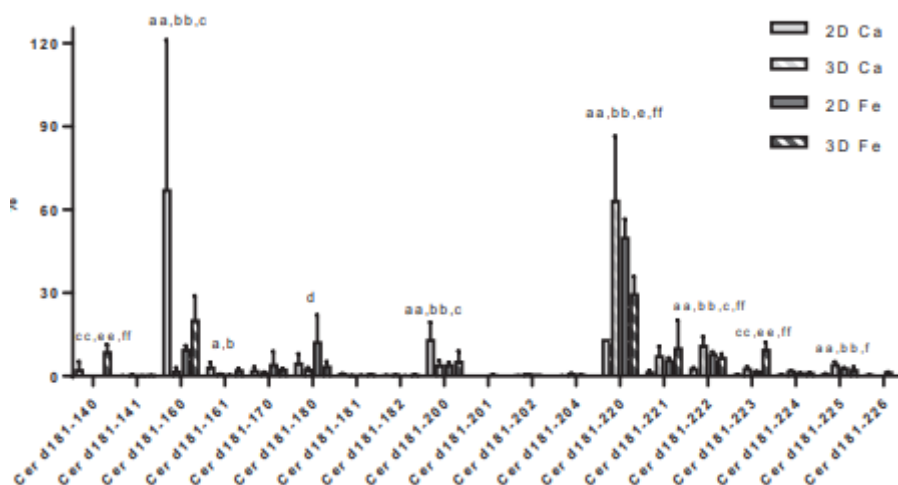


Fig. 5. Percentage abundance of ceramides in human fibroblast cells grown on 2D- and 3D-ALG_Ca or 2D- and 3D-ALG_Fe. a: $p < 0.05$ for 2D Ca vs 3D Ca, aa: $p < 0.01$ for 2D Ca vs 3D Ca; b: $p < 0.05$ for 2D Ca vs 2D Fe, bb: $p < 0.01$ for 2D Ca vs 2D Fe; c: $p < 0.05$ for 2D Ca vs 3D Fe, cc: $p < 0.01$ for 2D Ca vs 3D Fe; d: $p < 0.05$ for 2D Fe vs 3D Ca; e: $p < 0.05$ for 2D Fe vs 3D Fe, ee: $p < 0.01$ for 2D Fe vs 3D Fe; f: $p < 0.05$ for 3D Fe vs 3D Ca, ff: $p < 0.01$ for 3D Fe vs 3D Ca.

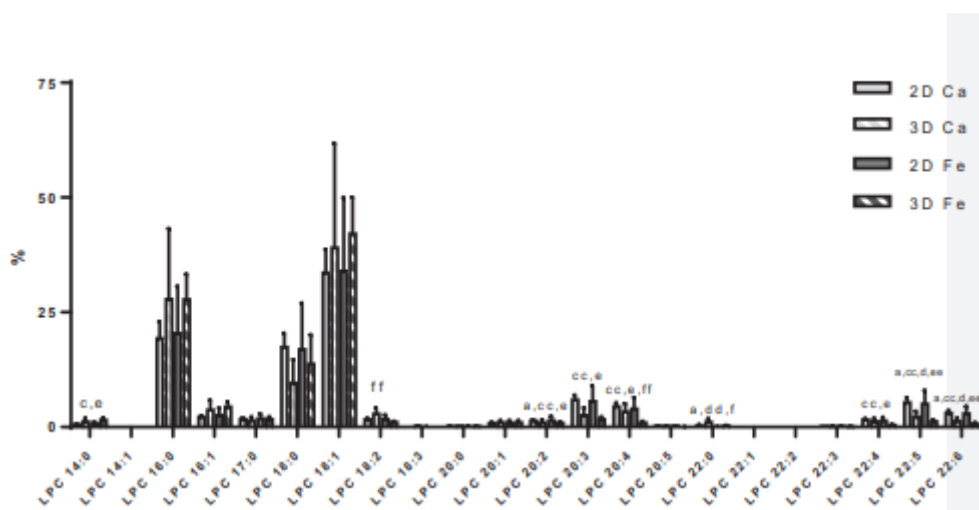


Fig. 6. Percentage abundance of LPCs in human fibroblast cells grown on 2D- and 3D-ALG_Ca or 2D- and 3D-ALG_Fe. a: $p < 0.05$ for 2D Ca vs 3D Ca; c: $p < 0.05$ for 2D Ca vs 3D Fe, cc: $p < 0.01$ for 2D Ca vs 3D Fe; d: $p < 0.05$ for 2D Fe vs 3D Ca; dd: $p < 0.01$ for 2D Fe vs 3D Ca; e: $p < 0.05$ for 2D Fe vs 3D Fe, ee: $p < 0.01$ for 2D Fe vs 3D Fe; f: $p < 0.05$ for 3D Fe vs 3D Ca, ff: $p < 0.01$ for 3D Fe vs 3D Ca.

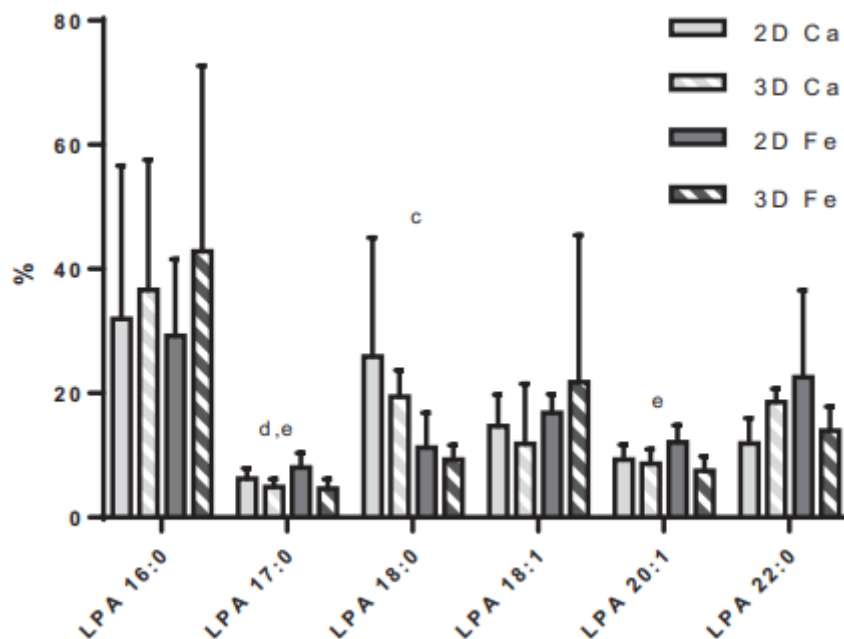


Fig. 7. Percentage abundance of LPAs in human fibroblast cells grown on 2D- and 3D-ALG_Ca or 2D- and 3D-ALG_Fe. c: $p < 0.05$ for 2D Ca vs 3D Fe; d: $p < 0.05$ for 2D Fe vs 3D Ca; e: $p < 0.05$ for 2D Fe vs 3D Fe.

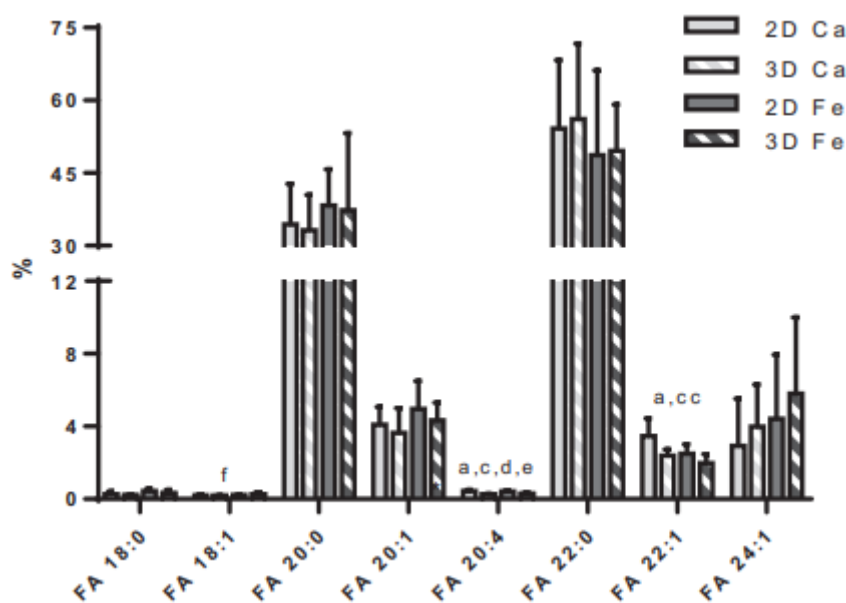


Fig. 8. Percentage abundance of FFAs in human fibroblast cells grown on 2D- and 3D- - and 3D-ALG_Fe. a: $p < 0.05$ for 2D Ca vs 3D Ca; c: $p < 0.05$ for 2D Ca vs 3D Fe, cc: $p < 0.01$ for 2D Ca vs 3D Fe; d: $p < 0.05$ for 2D Fe vs 3D Ca; e: $p < 0.05$ for 2D Fe vs 3D Fe.

4. Conclusions

3D cell cultures can add unknown physiologically relevant aspects compared to 2D. Here the behavior of fibroblasts as well as their lipid profile in contact with ALG systems was demonstrated to be influenced both by the architecture (2D or 3D) and the type of gelling agent. 2D- and 3D-ALG were prepared by using two gelling agents (CaCl_2 or FeCl_3) and characterized from a chemical-physical point of view through determination of the water content, swelling tests and mechanical resistance tests and from a morphological point of view through SEM analysis. The water content determination has shown that ALG_Fe had a slightly higher water content than ALG_Ca, i.e. 92% and 90% respectively. The mechanical resistance tests have shown that the ALG_Ca had a greater elasticity than ALG_Fe, which were extremely rigid. Thus, the former needed more strength to break. The swelling tests carried out showed that ALG_Ca swell and expand up to 6 h. On the other hand, ALG_Fe, showed this behavior only up to 2 h. Finally, the average pore size of scaffolds gelled with both solutions, was suitable for fibroblast (diameter ranging from 15 to 20 μm) adhesion and growth. A LC-MS/MS targeted approach was exploited to investigate the effects of cell-biomaterial interaction on the profile of selected lipids belonging to CER, LPC, LPA and FFA classes. The results clearly indicate that significant differences exist in the distribution of CER species in the fibroblasts and that these differences are determined by activation through biomaterial interactions. LPC distribution exhibited some differences among samples, whereas LPA and FFA resulted in more constant trends. Generally, cell-biomaterial engineered substrate interactions is strongly influenced by mechanical factors and cell membranes exhibit very different behaviors depending on the elasticity of the substrate microenvironment they are anchored. In the case of CER, as abundant bioactive signaling lipids present in the cell membrane, both the architecture and the gelling media affected the relative distribution of these species, playing a fundamental role in the organization of specific membrane regions. In the case of LPC, present as minor phospholipids in the cell membrane, our data suggests that the main parameter affecting their cell membrane lipid distribution is the scaffold architecture with a significant decrease in the relative expression levels of the species with higher chain length (C20 to C22) for 3D-ALG compared to 2D-ALG. As a final conclusion, different ALG scaffolds have the capabilities to affect the relative distribution profile of the main cell membrane lipids and this aspect could result in changes in the cell membrane properties and in a variation of the cell biological functions (e.g. signaling).

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Declaration of Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Materials

Table 1. SRM transitions monitored for the selected analyzed lipids.*

Analyte	Q1 (m/z)	Q3 (m/z)	Declustering potential (V)	Collision Energy (V)
Cer d18:1-14:0	510.5	264.2	75	35
Cer d18:1-14:1	508.5	264.2	75	35
Cer d18:1-16:0	538.5	264.2	75	35
Cer d18:1-16:1	536.5	264.2	75	35
Cer d18:1-17:0	552.5	264.2	75	35
Cer d18:1-18:0	566.6	264.2	75	35
Cer d18:1-18:1	564.5	264.2	75	35
Cer d18:1-18:2	562.5	264.2	75	35
Cer d18:1-20:0	594.5	264.2	75	35
Cer d18:1-20:1	592.5	264.2	75	35
Cer d18:1-20:2	590.6	264.2	75	35
Cer d18:1-20:4	586.6	264.2	75	35
Cer d18:1-22:0	622.6	264.2	75	35
Cer d18:1-22:1	620.5	264.2	75	35
Cer d18:1-22:2	618.5	264.2	75	35
Cer d18:1-22:3	616.5	264.2	75	35
Cer d18:1-22:4	614.6	264.2	75	35
Cer d18:1-22:5	612.5	264.2	75	35
Cer d18:1-22:6	610.5	264.2	75	35
LPC 14:0	468.2	184	70	30

LPC 14:1	466	184	70	30
LPC 16:0	496.2	184	70	30
LPC 16:1	494.2	184	70	30
LPC 17:0	510.4	184	70	30
LPC 18:0	524.3	184	70	30
LPC 18:1	522.3	184	70	30
LPC 18:2	520.3	184	70	30
LPC 18:3	518.2	184	70	30
LPC 20:0	552.3	184	70	30
LPC 20:1	550.3	184	70	30
LPC 20:2	548.3	184	70	30
LPC 20:3	546.3	184	70	30
LPC 20:4	544.2	184	70	30
LPC 20:5	542.3	184	70	30
LPC 22:0	580.4	184	70	30
LPC 22:1	578.4	184	70	30
LPC 22:2	576.3	184	70	30
LPC 22:3	574.4	184	70	30
LPC 22:4	572.3	184	70	30

LPA 16:0	409.2	153	-50	-25
LPA 17:0	423.3	153	-50	-25
LPA 18:0	437.3	153	-50	-25
LPA 18:1	435.3	153	-50	-25
LPA 20:1	463.3	153	-50	-25
LPA 22:0	493.3	153	-50	-25

FFA				
18:0	283	283	-70	-35
18:1	281	281	-70	-35
20:0	311	311	-85	-30

20:4	303	303	-85	-28
20:1	309	309	-85	-30
22:0	339	339	-90	-35
22:1	337	337	-90	-35
24:1	365	365	-90	-3

* EP ($\pm 10\text{V}$) and CXP ($\pm 11\text{V}$) voltage were kept constant for all analytes.

CHAPTER VIII: Alginate Self-Crosslinking Ink for 3D Extrusion-Based Cryoprinting and Application for Epirubicin-HCl Delivery on MCF-7 Cells

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Personal Contribution

G.R: Conceptualization, methodology, software, validation, investigation, writing-original draft preparation.

Abstract

3D-printed hydrogels are particularly advantageous as drug-delivery platforms but their loading with water-soluble active compounds remains a challenge requiring the development of innovative inks. Here, we propose a new 3D extrusion-based approach that, by exploiting the internal gelation of the alginate, avoids the post-printing crosslinking process and allows the loading of epirubicin-HCl (EPI). The critical combinations of alginate, calcium carbonate and d-glucono- δ -lactone (GDL) combined with the scaffold production parameters (extrusion time, temperature, and curing time) were evaluated and discussed. The internal gelation in tandem with 3D extrusion allowed the preparation of alginate hydrogels with a complex shape and good handling properties. The dispersion of epirubicin-HCl in the hydrogel matrix confirmed the potential of this self-crosslinking alginate-based ink for the preparation of 3D-printed drug-delivery platforms. Drug release from 3D-printed hydrogels was monitored, and the cytotoxic activity was tested against MCF-7 cells. Finally, the change in the expression pattern of anti-apoptotic, pro-apoptotic, and autophagy protein markers was monitored by liquid-chromatography tandem-mass-spectrometry after exposure of MCF-7 to the EPI-loaded hydrogels.

Keywords: 3D printing; internal gelation; alginate; biomaterial ink; epirubicin-HCl; MCF-7

1. Introduction

Three-dimensional (3D) printing is a rapidly growing technology that offers many opportunities for manufacturing biomaterials with tunable properties. The flexibility in the design of this technique allows to fabricate 3D structures with customizable features and intricate architecture and, most importantly, ease of personalization [1]. The extrusion-based approach is probably the most widely utilized method in 3D printing to create structures with a single material via an individual additive manufacturing process [2]. The computer-aided design (CAD) associated with this technology allows to achieve high automation/repeatability and precise control of microstructures, to prepare complex structures in a range of dimensions and material using the layer-by-layer deposition [2,3]. These features are particularly advantageous in the preparation of modified release systems as the conventional drug-delivery platforms often restrict their application in the pharmaceutical industry, due to the incapability of adapting to individual pharmacokinetic traits. Advanced drug-delivery devices with personalized drug dosing and/or complex drug-release profiles, personalized topical treatment devices, and novel dosage forms such as 3D-printed multilayer polypills are just some of the many possible applications of 3D printing in the drug-delivery field [4]. Hydrogels are one of the most feasible classes of ink materials for 3D printing, and this field has been rapidly advancing [5]. A polymer hydrogel is a 3D cross-linked network of flexible polymer chains that contains a large amount of water but retains the properties of solids [6], and they have attracted increasing attention for bioprinting due to the high drug-loading efficiency and excellent biocompatibility [7,8]. Many synthetic and naturally derived hydrogel-forming polymers were proposed as ink material for 3D printing. However, not all the hydrogel materials are suitable for this application, as the printability of a hydrogel is determined by the rheological properties (viscosity, shear-thinning, and yield stress) and cross-linking mechanisms (physical crosslinking and chemical cross-linking) [5]. The interest in natural polymers (often referred to as biopolymers due to their human, animal, plant, and bacteria origins) is due to their excellent biocompatibility and cell affinity and increasing efforts have been focusing on the use of polysaccharides (sugars linked by O-glycosidic bonds), glycosaminoglycans (polysaccharides with amine functionality), and polypeptides/proteins to obtain 3D-printable hydrogels [9]. Alginate (ALG) is a biodegradable, non-toxic, and non-immunogenic natural polymer extracted from marine brown algae biomass, and it is among the most used biopolymers for biomedical applications [10]. ALG is an anionic and hydrophilic block copolymer consisting of β -D-mannuronic acid (M) and α -L-guluronic acid (G) residues in different proportions, mainly depending on the source. Physical properties and the molecular weight of ALG strictly depend on the sequence of M and G units, as well as the cross-linking which occurs by the co-operative binding of divalent cations (such as Ca^{2+} , Ba^{2+} , Zn^{2+} , or Sr^{2+}) with the carboxylic groups of the G-block regions of the

copolymer. This interaction results in the formation of a complex interconnected bulk structure in the shape of an “egg-box” [11,12]. The essential role of the crosslinking process in the alginate scaffold design has been extensively reported in the literature [13], but when it comes to 3D printing, this role is even more important. The major drawback of single component alginate bioink in the form of low-concentration sols (<6 wt%) is the lack of the mechanical strength required to ensure the structural fidelity immediately after extrusion to avoid collapse and deformation. To tackle this problem, He and coworkers proposed to extrude an alginate sols directly into a CaCl_2 solution to maintain the structural integrity of whole 3D scaffolds during the printing process [14]. Although this method worked for the preparation of high strength 3D hydrogel constructs with built-in microchannels, its application in drug delivery can be limited by the fast drug release in the crosslinking solution. Moreover, the direct contact with the highly concentrated calcium salt solution needed for the crosslink lead to a rapid and inadequately controlled gelation resulting in hydrogels with poor structural homogeneity and limited mechanical properties [15,16]. Several other strategies were proposed to prepare 3D printed porous alginate scaffolds, but they all involve contact with calcium salt solutions to ensure the structural integrity of the whole 3D scaffold [17]. Internal gelation is a cross-linking technique that involves the gradual generation of cross-linking ions (Ca^{2+}) into the polymeric mixture through a CaCO_3 –GDL (d-glucono- δ -lactone) system. This methodology ensures a slow and more modular gelling process allowing the production of homogeneous hydrogels with well-defined porosity, mechanical properties and the possibility to include water-soluble actives in the formulation [16,18]. It is clear that a bio-ink based on this technique could offer several advantages for 3D printing, but to date, few efforts have been made in this direction. Recently, Hazur and coworkers used internal ge-lation as a pre-crosslinking technique, to form an homogeneous alginate bioink able to maintain a shape fidelity after printing [19]. The authors suggested the feasibility of this method by observing the high cell viability of printed cells embedded in the formulated bioink, however, to fully solidify the 3D-printed structures, a post-crosslinking step was still performed, slowly covering the alginate hydrogels with CaCl_2 . The general aim of this work was to explore the internal gelation technique to prepare an alginate ink capable of completely self-crosslinking after printing. Avoiding the post-printing crosslinking step would make this method more suitable for the preparation of drug delivery platforms for small hydrophilic molecules. To present a proof of principle, we decided to load our 3D-printed platforms with epirubicin-HCl (EPI), a water-soluble 4’epiisomer of doxorubicin with a well-established application in breast cancer treatment, one of the most prevalent diseases among women worldwide [20,21]. EPI induces DNA damage by inhibiting the topoisomerase II activity, inducing apoptosis in cancer cells [22]. EPI local delivery has been extensively studied mainly through the development of nanostructured delivery

sys-tems [23,24], but few efforts were made to prepare polymeric platforms for local delivery. One of these attempts proposed EPI-loaded gelatin hydrogel carriers for intravesical usage exhibiting a promising anti-cancer effect on a urothelium carcinoma [25]. This study can be divided into two main parts: the development of an alginate ink based on the combination of ALG/CaCO₃/GDL to obtain a formulation compatible in terms of viscosity and gelation time with the extrusion-based 3D. Then hydrogels functionalized with EPI were printed, characterized and subsequently tested in vitro on MCF-7 cells, a commonly used human breast cancer target cell line [26–28]. Finally, using a state of the art liquid chromatography-mass spectrometry technique, the protein expression of pro and anti-apoptotic biomarkers was evaluated by target analysis to further validate the potentiality of this approach.

2. Results and Discussion

2.1. Optimization of the Self-Crosslinking Formulation for 3D Printing

The goal of this work was the development of an ALG-based self-crosslinking bioink to be used in a 3D-printing extrusion-based system. Thus, fine control on the gelation speed is fundamental to obtain homogeneous formulations with such initial viscosities (8–40 kCP) to be printed by the custom low-temperature extrusion 3D-printing system developed for this work [8,29–31]. Increasing the viscosity of the initial solution (working at high alginate concentration or preparing blends of polymers) has been a strategy often used to obtain ALG bioinks suitable for 3D printing. However, the high viscosity of the initial solution results in a longer gelation time due to fewer alginate chains mobility and slower diffusion of the crosslinking ions into the bulk material [16]. The internal gelation method, consisting of a combination of a slowly hydrolyzing lactone (GDL) with water-insoluble carbonate salt as a donor of the crosslinking ions, allows instead to generate these ions directly from the inside the material solution, resulting in a hydrogel with suitable viscosity and capable of retaining shape after 3D-printing. As a first step, we tried to establish the best combinations of GDL concentration and scaffold production parameters that would allow us to obtain self-crosslinked 3D hydrogels. The concentration of GDL in the bioink formulation is the critical parameter to modify gelation properties of ALG [16], as too-fast gelation can lead to difficulties in printing the material, while too-slow gelation results in low-extrusion printing precision and large variation in the device shape after printing. Our strategy to avoid the use of a too-high concentration of GDL (that would have started the gelation in the syringe used for the extrusion of the alginate) was to use a low temperature to maintain the 3D structure after printing, making the extrusion take place on a Peltier cell at $-14\text{ }^{\circ}\text{C}$. However, it should be noted that the ink preparation and the printing process occur at room temperature, whereas only the post-printing cross-linking reaction was performed at low temperature

(2 °C). As the temperature significantly affects the kinetic of the ALG GDL-mediated self-crosslinking reaction, and thus the ink viscosity over time, the optimization of the whole scaffold production process required the study of the effect of the molar ratio between CaCO₃ and GDL on the ALG gelation properties. As a starting point, we took into consideration some previous studies that have shown that is possible to prepare self-crosslinking alginate hydrogels scaffolds with defined dimensions for tissue-engineering applications with a molar ratio between CaCO₃ and GDL of 0.5 [15] and 2, in the case of alginate–hyaluronan blends [16]. As a preliminary test, a solution of ALG (6% w/v) and CaCO₃ (80 mM) was mixed, in a 1:1 v/v ratio, with GDL 80 mM and loaded into a syringe to be printed. A four-layer scaffold was successfully printed on the plate of Peltier cell (−14 °C) and left to crosslink first for 10 min at −20 °C to avoid 3D structure collapse and then at 2 °C. The scaffold was monitored over time, and only after 5 days it easily detached from the plate and was able to retain its 3D structure at room temperature. However, once immersed in water, it dissolved immediately, clearly indicating that a complete dehydration process of the formulation occurred instead of a cross-linking reaction. Starting from this evidence, we decided to fix the initial concentration of ALG and CaCO₃ on 6% (w/v) and 160 mM, respectively. Once the experimental conditions for the formation of the bioink were fixed, the influence of GDL on the printing time was evaluated. For this purpose, GDL solutions at increasing concentrations (from 120 mM to 180 mM) were prepared and mixed with the ALG/CaCO₃. A series of technological parameters such as the ink working time, the number of printable layers, the scaffold curing time at 2 °C, the scaffold detachment from the plate, and the retention of the 3D structure in water were evaluated. Table 1 summarizes the main findings for the different combinations of ALG/CaCO₃/GDL tested. The formulation with the highest GDL concentration (180 mM) was immediately discarded, as the too-fast gelation (ink working time: 8 min) allowed to print hydrogels with only two overlapping layers. The formulation crosslinked with 160 mM GDL presented a longer printing time of approx. 11 min, enough to obtain a three-layer scaffold able to be easily detached from the plate (Figure 1a) and to maintain the 3D structure if left for 2 to 4 days at 2 °C. Similarly, the formulation with 140 mM GDL showed a good printability with an extended printing time with the possibility of preparing hydrogels with four overlapped layers and a similar self-gelling capacity (Figure 1b). The poor crosslinking ability confirmed by a difficult detachment from the plate and the fast dissolution of the hydrogel 3D structure when placed in water forced us to discard the 120 mM GDL formulation (Figure 1c). The data reported on Table 1 evidenced a linear relationship ($Y = -0.165X + 37.5$) between the GDL molar concentration (X) and the ink working time (Y) that, together with the experimental evidence here collected, can be useful to predict the behavior of the formulation over the processing time. The alginate-based 3D-printed scaffold formed by 6% w/v ALG, CaCO₃ 160 mM and 140 mM GDL

crosslinked for 3 days at 2 °C (3D-ALG) resulted to have the best overall characteristics, and for this reason, they were chosen for further characterization and EPI loading. In particular, we decided to set the curing time of 3 days at 2 °C to ensure the maximum crosslinking rate, avoid hydrogel dehydration during the crosslink time, and retain the 3D-printed structure. EPI-loaded hydrogels were prepared to the final concentrations of 0.6 and 1 µg per hydrogel (named ALG_EPI_0.6 and ALG_EPI_1, respectively) as described in the experimental section. As no differences in scaffold production were experimentally evidenced after drug addition, we conclude that EPI did not affect the processing parameters described above.

Table 1. Alginate-based formulations tested for 3D printing at various concentrations of GDL.

(GDL) mM	Ink Working Time * (Min)	Number of Layers	Curing at 2 °C	Detachment from Plate	Self Cross-Linking
180	8	2	1	no	yes
160	11	3	1	no	yes
			2; 3; 4	yes	
			5	difficult	
140	14	4	1	no	no
			2	no	yes
			3	yes	yes
			4	yes	yes
			5	difficult	yes
120	18	6	1	no	no
			2	no	
			3	yes	
			4; 5	yes	

* Ink working time is intended as the time occurring from the preparation of the ALG/CaCO₃/GDL mixture to the printable end point.

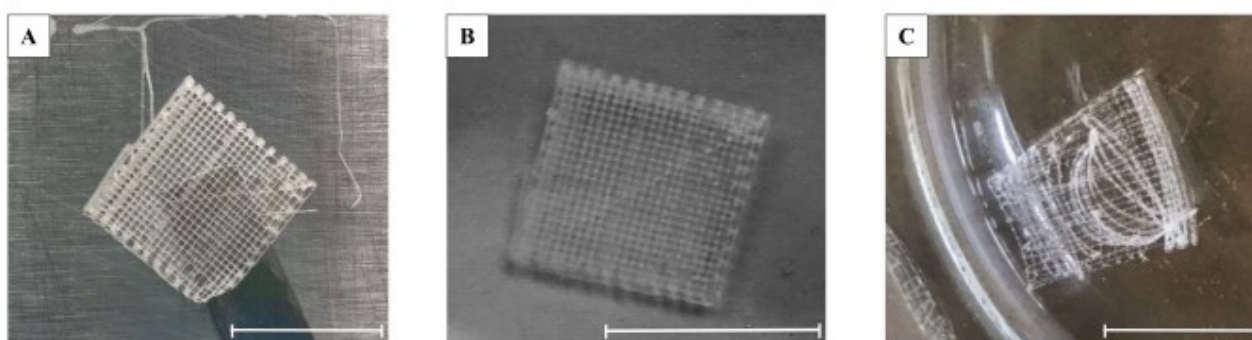


Figure 1. Alginate-based 3D-printed scaffold. (A) 3-layers-160 mM scaffold detached correctly from the plate; (B) 4-layers-140 mM self-crosslinked scaffold in water retained the 3D structure; (C) 6-layers-120 mM scaffold in water flaked off; the crosslinking reaction was not successful.

2.2. ATR FT-IR Scaffold Characterization

The ATR FT-IR spectrum of raw alginate powder was compared with the spectra of self-crosslinked 3D-printed hydrogel (3D-ALG) in order to chemically characterize the new formulation developed (Figure 2c). In the alginate powder spectrum, the wide peak between 3000 and 3600 cm^{-1} corresponds to the $-\text{OH}$ stretching vibration. Stretching vibrations of aliphatic $\text{C}-\text{H}$ were observed at 2986–2898 cm^{-1} . Bands at 1601 and 1412 cm^{-1} can be attributed to asymmetric and symmetric COO^- stretching vibrations of carboxylate salt groups. The bands at 1032 and 947 cm^{-1} were attributed to the $\text{C}-\text{O}$ stretching vibration of a pyranosyl ring and the $\text{C}-\text{O}$ stretching with contributions from $\text{C}-\text{C}-\text{H}$ and $\text{C}-\text{O}-\text{H}$ deformation. Finally, at 886 cm^{-1} the vibration of a mannuronic acid functional group was shown [27,32]. Comparing this spectrum with 3D-ALG, the latter presents general bands broadening and peak shifts. In 3D-ALG (Figure 2a), the asymmetric stretching vibration of carboxylate ion shifted to significantly lower wave numbers (i.e., from 1601 to 1586 and from 1412 to 1404 cm^{-1}), whereas the vibration of the mannuronic-acid functional group shifted from 886 cm^{-1} to 874 cm^{-1} . This behavior can be attributable to the presence of calcium ions and to their cross-linking effects involving the carboxylate groups that are diagnostics of the changes in the structure of alginate. The ATR FT-IR spectrum of a similar alginate hydrogel obtained by immersion in a CaCl_2 solution was even acquired as a reference to compare the alginate intermolecular structures obtained after 3D printing. Both the spectra obtained were superimposable, and no significant differences in the vibration wave numbers were observed (data not shown). This finding suggests that the CaCO_3/GDL cross-linking system produced a final alginate structure very similar to that obtained by conventional cross-linking in a free Ca^{2+} ion solution. Figure 2b shows the spectrum of ALG_EPI_1 hydrogel. No significant differences between the spectra of ALG and ALG_EPI_1 were observed in terms of peak wave numbers. However, in the ALG_EPI_1 spectrum, the bands at 1587 cm^{-1} and 1404 cm^{-1} were more intense compared to the control scaffold. The EPI ATR FT-IR spectrum (Figure 2d) exhibited two intense bands at 1574 cm^{-1} and 1404 cm^{-1} that overlap with the bands of alginate scaffold. Even the band at 1030 cm^{-1} was more intense in the ALG_EPI spectrum compared to the ALG spectrum. On the contrary, the vibration of the mannuronic-acid functional group at 874 cm^{-1} resulted in more intensity in the 3D-ALG. These findings suggest the presence of EPI in the scaffold and its effect of the ATR FT-IR spectrum even if the drug was present at the amount of 1 $\mu\text{g}/\text{scaffold}$.

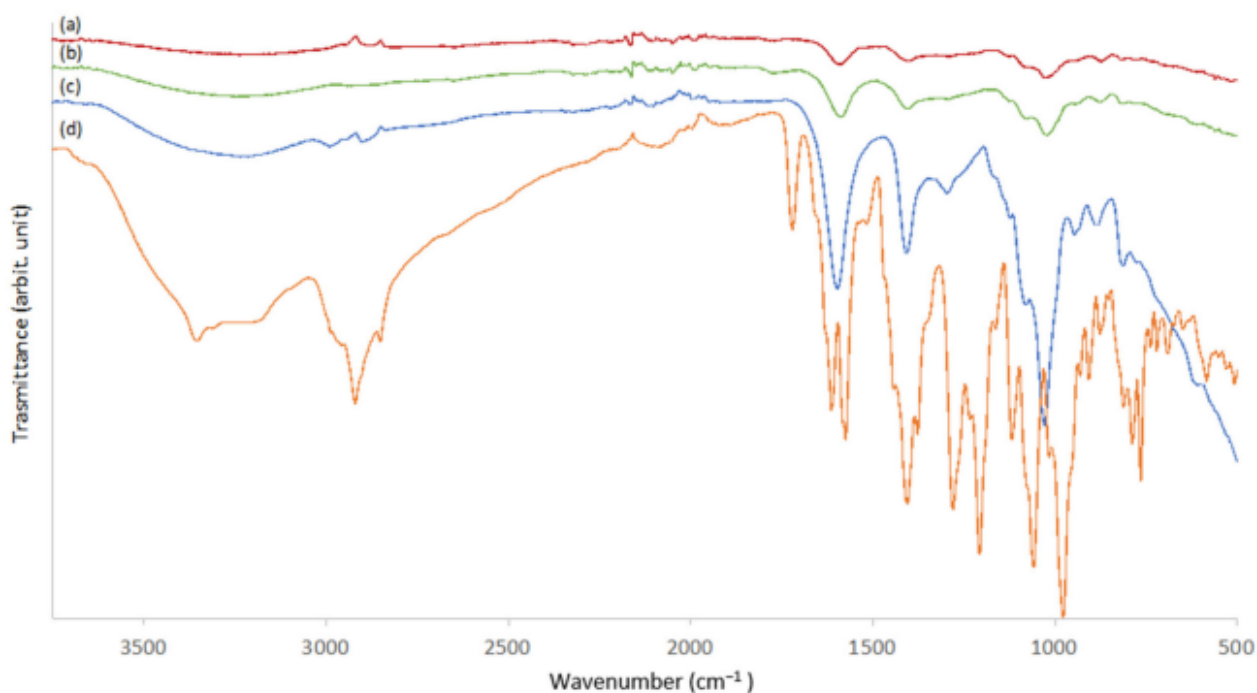


Figure 2. ATR FT-IR spectra of the 3D-printed samples. (a) 3D-ALG, (b) ALG_EPI_1, (c) ALG, (d) EPI.

2.3. Swelling Behavior and Drug Release from 3D-Printed Hydrogel

The release of drugs from hydrophilic crosslinked networks strongly depends on the swelling behavior. The ability of a hydrogel to absorb and retain biological fluids is strictly related to the physical structure and the characteristics of the fluid used to simulate the physiological condition of use. The presence of a crosslinked network generally modifies the swelling of a hydrogel, reducing the swelling ratio as a consequence of a more rigid structure. The rate and duration of swelling for 3D-ALG were studied over a five-minute period using sodium acetate buffer (pH 5.5) or PBS solution (pH 7.4) at 37 °C to verify if the slightly alkaline or acidic physiological condition may affect the swelling behavior. Figure 3 shows a similar trend in both pH conditions, with a rapid initial increase in water uptake in the first 60 s of the experiment followed by an equilibrium state until the end. In the first minute after the immersion, the swelling kinetics were faster for the hydrogel placed at pH 7.4, then the behavior at the two pH levels became more and more uniform, overlapping between 60 and 300 s and stabilizing on a rehydration percentage of about 900%. In the further step, the release of EPI from the 3D-printed hydrogel was followed to evaluate the ability of the system to release an hydrophilic molecule. The EPI actual amount in the hydrogel was experimentally quantified by using the HPLC-UV/VIS method specifically developed, resulting in an actual loading of $72.3 \pm 7.5\%$. This value, evaluating the process production in its totality, can be considered acceptable; thus, the process

developed to obtain a self-crosslinking alginate 3D-printed hydrogel allowed to successfully functionalize scaffolds with water-soluble drugs such as EPI.

The release profile of EPI from the ALG_EPI_1 scaffold in PBS at pH 7.4 and 37 °C is reported in Figure 4. Usually, there is a direct correlation between the drug release from biodegradable polymeric matrices and the water-uptake kinetics (i.e., swelling), as the force that drives the drug release of hydrophilic molecules is the entrance of water into the matrix [18,33]. In this case, the drug-release experiment showed a slow release of EPI up to 40 min, a much longer time compared with the 5 min required to reach the maximum hydration state of the hydrogel. This difference could be explained if we also consider the internal structure of the hydrogel. A crosslinked polymeric network consists of a series of open spaces (meshes) between polymer chains that allow for liquid and small-solute diffusion. The mesh size depends on the polymer and crosslinker concentrations, as well as external stimuli such as temperature and pH [34]. In our case, we reasonably believe that the 40 min needed to reach 100% of the drug release are due to the complex internal structure/interactions of the ALG_EPI_1 scaffold that slow down the diffusion of active molecules. Finally, it is worth to notice that no drug-degradation phenomena were detected under pH and temperature conditions used in accordance with the existing literature on EPI [35].

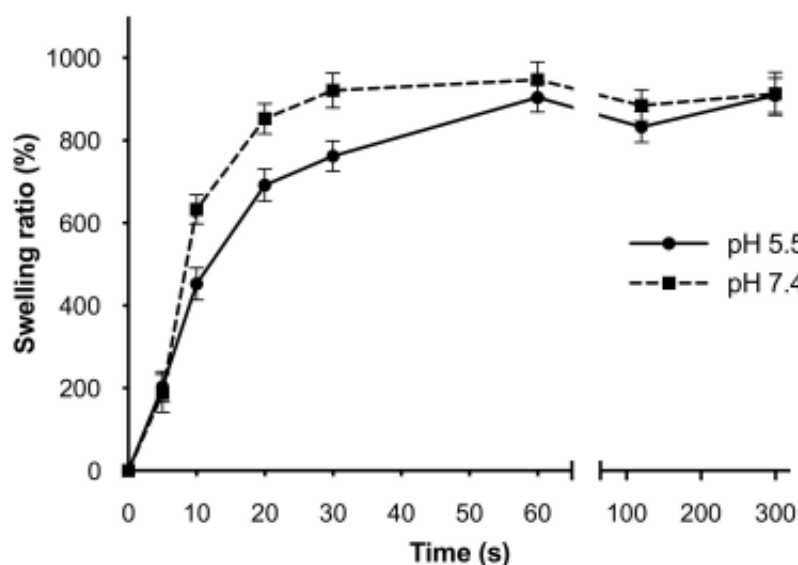


Figure 3. Swelling profile of the 3D-ALG at different pH. Swelling profiles at pH 7.4 (dotted line) and pH 5.5 (continuous line) were shown; results are reported as mean $\pm$ standard deviation of three independent measurements.

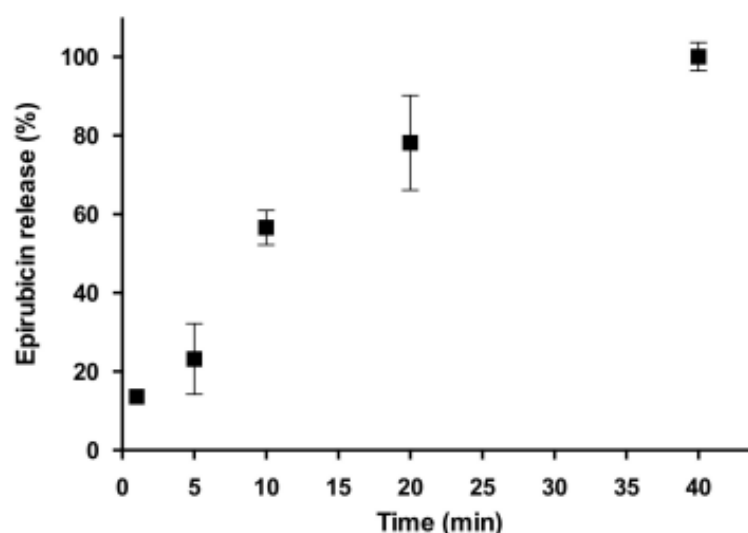


Figure 4. EPI-release profile from ALG_EPI_1 in PBS pH 7.4. Results are reported as mean $\pm$ standard deviation of three independent measurements.

2.4. Cytotoxic Activity towards MCF-7 Breast-Cancer Cells

The cytotoxic activity of both unloaded ALG and loaded ALG_EPI_1 hydrogel was tested on MCF-7 cells using resazurin assay. MCF-7 cells are often used as in vitro breast cancer models, and they proved to be particularly sensitive to the action of EPI [36]. According to the recommended guidelines for the evaluation of in vitro cytotoxicity for medical devices and delivery systems (ISO 10993-5:2009), a biomaterial can be deemed non-cytotoxic if the cell viability after exposure does not fall below 70% [37]. The biocompatibility test carried out at both 4 h and 24 h and reported in Figure 5 showed a cell viability significantly lower if compared to the control on the nude plate (CTRL). However, these values (87–89%) fall within the biocompatibility threshold defined by the recommended guidelines, allowing us to consider the delivery platform as biocompatible. Once loaded with EPI, the cytotoxic behavior changed significantly. After just 4 h of exposition to the drug-loaded scaffold, even ALG_EPI_0.6 (with the lowest drug amount, 0.6 μ g) significantly reduced cell viability compared to the CTRL. However, as expected, ALG_EPI_1 (loaded with 1 μ g of EPI) gave the best results in terms of cytotoxicity, with a reduction in the cell viability by about 19% compared to CTRL after 24 h. These observations suggest the efficacy of the developed 3D-printed hydrogel to perform cytotoxic effects on MCF-7 cells with an effect directly dependent on the drug concentration

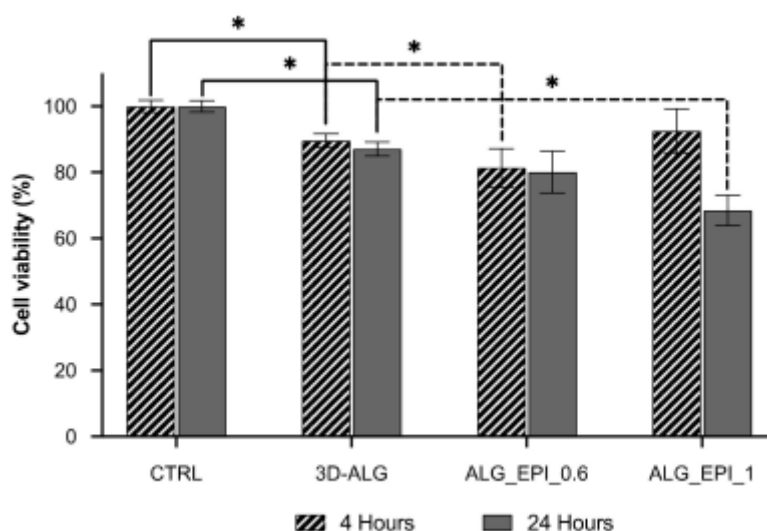


Figure 5. Effect of self-crosslinking 3D-printed hydrogel on MCF-7 cells viability. Cell viability was determined by resazurin assay at 4 h (diagonal lines) and 24 h (grey fill) on the nude plate (CTRL), 3D-ALG, and hydrogels containing 0.6 (ALG_EPI_0.6) and 1 μ g (ALG_EPI_1) of the drug, respectively. Results are reported as mean $\pm$ standard deviation of three independent measurements. Statistically significant values were denoted by * ($p < 0.05$; One-way ANOVA test).

2.5. Proteomic Analysis

In an endeavor to further investigate the effect of ALG_EPI_1 on the MCF-7 cell line, the main proteins involved in the processes of apoptosis were analyzed by LC-MS/MS SRM target analysis. In particular, we studied the anti-apoptotic Bcl-2 and Bcl-xL class proteins; the pro-apoptotic p53, BAX, and BAD proteins; the executioner caspase 3 (CASP-3); the initiation caspase 9 (CASP-9); and also the activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1), which are all proteins involved in the MCF-7 autophagy program as a mechanism of protection from cell death induced by EPI [38]. The presence/absence of such proteins and their relative quantification was performed after 4 h and 24 h of exposition both on the ALG and ALG_EPI_1 using the cells cultured on the nude plate as the control group (CTRL). The results of this analysis are summarized in Figure 6. Bcl-2 and Bcl-xL are two proteins involved in the anti-apoptotic process. Bcl-xL was expressed at similar levels in all samples. It worth to notice that the Bcl-2 expression rose significantly ($p < 0,05$) from 4 to 24 h in the CTRL and 3D-ALG hydrogel, confirming the biocompatibility of these two systems. Whereas in the ALG-EPI_1, no increase was observed over this time range. Regarding pro-apoptotic proteins, their expression varies over time and between treatments. The expression of the p53 protein increased over time and was more expressed in the ALG_EPI_1 samples with respect to CTRL and 3D-ALG, suggesting that the loaded drug started to induce DNA damage [39]. BAD, CASP-3, and BAX protein expressions were comparable between the hydrogel tested, increasing over

time. Interestingly, the CASP-9 expression level was similar between the CTRL and 3D-ALG and significantly increased in the drug-treated samples at 24 h. CASP-9 is an initiating caspase upstream of CASP-3 [40], unlike CASP-3, and differs significantly in expression from the 3D-ALG ($p < 0.05$), strengthening the thesis of ALG_EPI-mediated apoptosis induction that is only in the early phase. Finally, AMBRA1 expression, a marker of the autophagy cell process, increased over time in all samples. CTRL and 3D-ALG treatments showed a quite similar effect on MCF-7 cells at both times, whereas at 24 h, AMBRA1 expression significantly increased ($p < 0.05$) in ALG_EPI_1, confirming the cytotoxic effect of the hydrogel also evidenced by the resazurin assay. MCF-7 cancer cells, if treated with drugs that induce cell death such as EPI, can trigger the autophagy process as a defense mechanism [20]. These outcomes confirm some of those obtained from the viability assays and reveal that ALG_EPI_1 could be used as drug-delivery systems to induce target cells in the early stage of apoptosis.

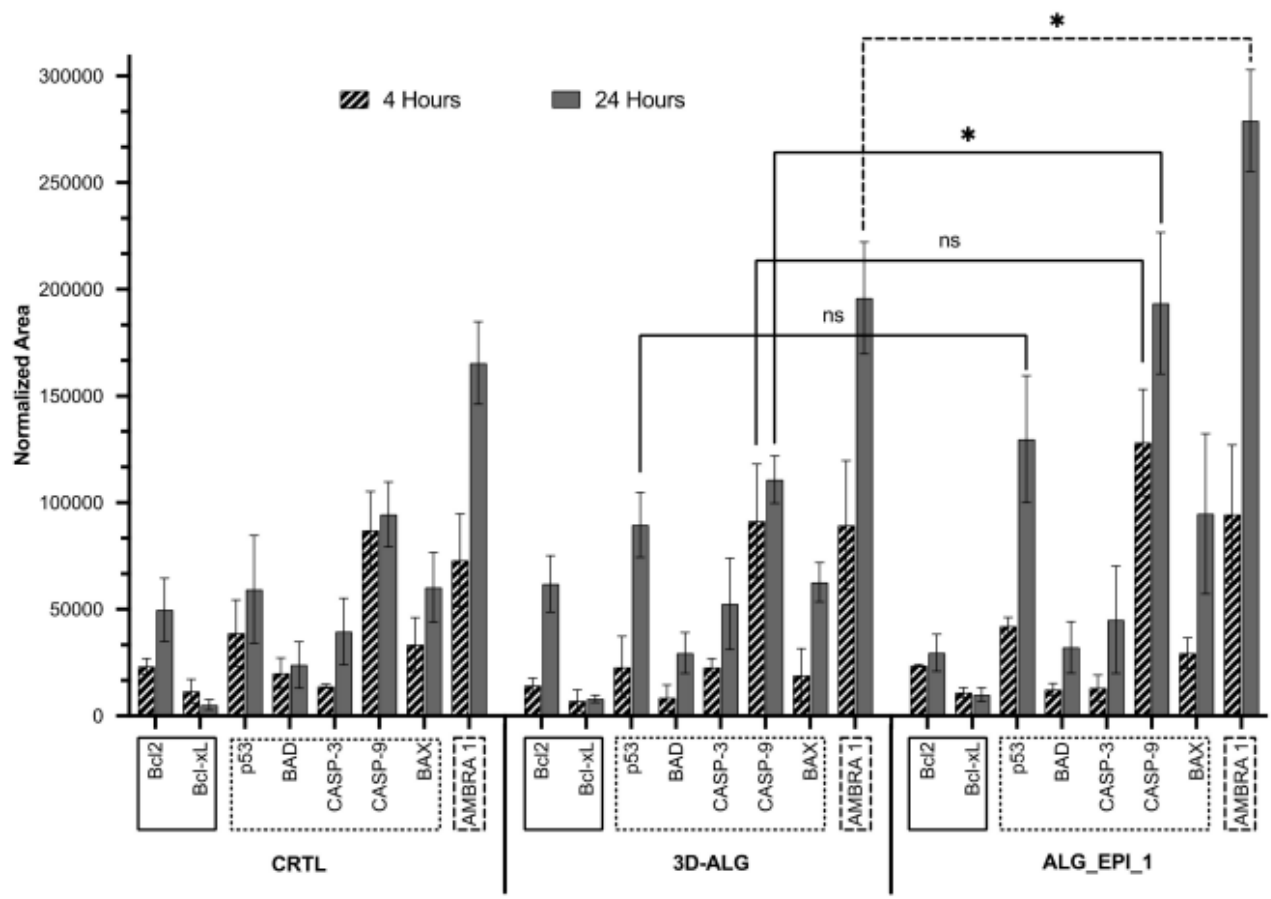


Figure 6. LC-MS/MS SRM-targeted protein analysis. Proteins' expression at 4 h (diagonal lines) and 24 h (grey fill) for CTRL, 3D-ALG, and ALG_EPI_1 (1 μ g drug loaded) treatment conditions were shown. Proteins are grouped according to their biological function: anti-apoptotic (continuous line), pro-apoptotic (dotted line), and involved in autophagy (dashed line). Results are reported as mean $\pm$ standard deviation of five total replicates (three biological replicates and two instrumental

replicates); statistically significant values were denoted by (* $p < 0.05$; one-way ANOVA test), whereas not-significant ones were denoted as ns ($p > 0.05$; one-way ANOVA test).

3. Materials and Methods

3.1. Reagents

Sodium alginate (Ph.Eur. grade; molecular weight by gel-filtration chromatography (GFC) 180–300 kDa; slowly soluble in water) was from Carlo Erba (Milan, Italy). Urea, tribasic sodium citrate dihydrate, and calcium carbonate (CaCO_3) were from Carlo Erba (Milan, Italy). Trypsin from bovine pancreas, bovine serum albumin (BSA), glucono- δ -lactone (GDL), ethylene diamino tetraacetic acid (EDTA), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (ABC), sodium acetate, formic acid (FA), and acetonitrile (ACN) were from Sigma-Aldrich® (Darmstadt, Germany). Dulbecco's Modified Eagle's Medium (DMEM), Streptomycin, and Penicillin was from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Fetal Serum Bovine (FBS) and Dulbecco's phosphate buffered saline (PBS) were from Euroclone (Milan, Italy). Epirubicin-hydrochloride (EPI) was obtained from Cayman Chemical (Ann Arbor, MI, USA). Protein assay dye reagent concentrate was from Bio-Rad. Resazurin sodium salt was from Alfa Aesar (Ward Hill, MA, USA). All solvents were of reagent grade with the highest purity available. Deionized ultra-filtered water was used throughout this study.

3.2. Ink Preparation for 3D Printing

Sodium alginate (ALG) (6% w/v) and calcium carbonate (CaCO_3) (80 and 160 mM) powders were dispersed in ultrapure water in a beaker and magnetically stirred for 48 h until a homogeneous dispersion was achieved. One mL of the ALG/ CaCO_3 dispersion was first sonicated in an ultrasonic bath (Branson 2510; OPTO-LAB, Modena, Italy) for 30 min at room temperature and then carefully and quickly mixed with 1 mL of a freshly prepared GDL water solution. Different GDL concentrations (120, 140, 160 and 180 mM: $\text{pH} = 4.5 \pm 1$) were tested. It is worth to notice that once the two solutions were mixed, the final concentration of ALG was 3%, and the concentrations of CaCO_3 and GDL were halved. For drug-loaded hydrogels, an EPI water solution was added drop by drop into ALG/ CaCO_3 dispersion to achieve a final concentration of 6 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$.

3.3. Scaffold Design

Three-dimensional models of the hydrogels were drawn using the Computer-Aided Design (CAD) software as previously reported [31,41]. Briefly, solidworks TM (Dassault systems, Waltham, MA, USA) was used for this purpose, allowing the creation of a “.stl” file (STereo Lithography interface

format). This file was further processed by Slic3r (RepRap), a slicing program that generates the machine code (GCode) file for the 3D printer. The three-dimensional models were built by overlapping two figures presenting parallel strands and rotating them by 90°. In total, 1 to 4 layers were printed, forming an orthogonal grid with fixed distances between filaments of 200 µm and creating a three-dimensional “gauze” shaped like a net of 1.6 cm × 1.6 cm in size.

3.4. 3D Printer and Scaffold Manufacturing

A custom-built low-temperature manufacturing system was designed and developed by combining Peltier cells and liquid/air exchangers with a commercial Direct Ink Writing (DIW) 3D printer previously described [31,41]. The ink formulation formed by ALG, CaCO₃, and GDL was loaded into a 5 mL syringe and extruded through a 26 G transversely cut needle (inner diameter 0.260 mm) by the application of controlled pressure on the syringe through a mechanically assisted piston. The deposition took place through a robotic arm that moves along three axes (x, y, z) following the directives imposed by the GCode file on a stainless-steel Peltier cell (−14 °C). For each ink formulation, a 4-layer square hydrogel was printed in triplicate using approximately 100 µL of the solution, resulting in a scaffold loaded with 0.6 µg or 1 µg of EPI. Once the deposition was completed, the scaffold was further frozen at −20 °C for 10 min and then left at 2 °C for a time that range from 1 to 5 days to complete the self-gelation process. After the gelation time, the hydrogels were brought to RT and detached from the steel plate. Once detached from the plates, the hydrogels obtained were stored at 4 °C until use.

3.5. Swelling Test

The hydrogels’ swelling behavior was evaluated by measuring the fluid uptake as a function of time. After printing and crosslinking (3 days at 2 °C), the 3D-ALG hydrogels were dried in vacuum oven at 30 °C for 24 h, and the initial weight of each sample was accurately recorded using an analytical scale. The fluid uptake was evaluated by placing the samples in sodium acetate buffer (20 mM; pH 5.5) or in Phosphate Buffer Saline solution (PBS, NaCl 120 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, pH 7.4) in a thermostatic bath at 37 °C. At specific times (5, 10, 20, 30, 60, 120, and 300 s) the samples were taken out, excess fluid was carefully removed using tissue paper, and after being weighed were re-immersed in the solutions. The percentage swelling ratio (SR%) at each time point was calculated using Equation:

$$SR\% = [(W - W_0)/W_0] \times 100$$

where W is the mass of the swollen sample, and W₀ is the mass of the initial dry sample.

3.6. Attenuated Total Reflection (ATR) Fourier Transform Infrared (FT-IR) Spectroscopy

ATR FT-IR spectra were obtained using a Perkin–Elmer spectrometer (Norwalk, CT, USA). The apparatus operates with a single reflection at an incident angle of 45°. The analysis was carried out on hydrogels and raw materials at room temperature and ambient humidity. For each spectrum, 32 scans were acquired between 4000 and 400 cm⁻¹ with a spectral resolution of 2 cm⁻¹.

3.7. Evaluation of the Epirubicin-HCl Loading

The EPI actual loading in hydrogels was evaluated by dissolving a single 4-layers scaffold, previously dried and weighted to calculate the theoretical EPI content from ink composition, in 400 µL of a solution of sodium citrate (55 mM) and EDTA (50 mM) at pH 7.4 under magnetic stirring. The EPI loaded content (EPI%) was evaluated by liquid chromatography-ultraviolet/visible spectrophotometry (HPLC-UV/VIS) using Equation:

$$\text{EPI}\% = M/M_0 \times 100$$

where M is the amount of EPI experimentally determined, and M₀ is the amount of EPI calculated theoretically from the composition % of the initial dried scaffolds. EPI analysis was carried out by RP-HPLC using an LC Agilent 1200 system equipped with a UV/VIS detector (Agilent Technologies, Santa Clara, CA, USA). The analysis was performed at 25 °C on a Luna C18 column (50 mm × 2.1 mm, 5 µm particles) (Phenomenex, CA, USA). The mobile phase consisted of aqueous formic acid 0.1% solution (v/v) and acetonitrile at the ratio 50:50. The elution was isocratic at a flow rate of 200 µL/min; the detector wavelength was fixed at 479 nm; and the injection volume was 10 µL in all the experiments. Each sample tested was injected in duplicate.

3.8. In Vitro Drug Release Studies

The in vitro release profile of EPI from ALG_EPI_1 was evaluated in PBS at pH 7.4. Three hydrogels were first weighed and then incubated in 1.4 mL of PBS and placed in a thermostatic bath at 37 °C. At scheduled time intervals up to 24 h, 30 µL of the release medium was withdrawn and replaced with the same volume of fresh medium. The collected samples were analyzed for EPI content by high-performance liquid-chromatograph tandem mass spectrometry (LC-MS/MS) using an LC Agilent HP 1260 (Agilent Technologies, Santa Clara, CA, USA) equipped with a 200-vial capacity sample tray and coupled to a QTRAP 4000 triple quadrupole mass spectrometer (ABSCIEX, CA, USA), interfaced with a pneumatically assisted electrospray source (ESI). A C18-reversed phase column (Atlantis dC18, 2.1 × 100 mm, 100 Å, 3 µm) (Waters, Milford, MA, USA) kept at a temperature of 25 °C was used for chromatographic separation. The mobile phase consisted of an aqueous formic acid 0.1% solution (v/v) and acetonitrile at the ratio 50:50. The elution was isocratic

at a flow rate of 200 $\mu\text{L}/\text{min}$. For the analysis, 10 μL of each sample was injected in duplicate. The sheath gas (nitrogen, 99.999% purity) and the auxiliary gas (nitrogen, 99.998% purity) were delivered at flow rates of 45 and 5 arbitrary unit, respectively. Optimized conditions of the interface were as follows: ESI voltage 5.5 kV, declustering potential (DP) 80 eV, and capillary temperature 350 $^{\circ}\text{C}$. MS experiments were performed under selective ion monitoring (SIM) conditions. The EPI SRM transition monitored was 566/545 m/z .

3.9. MCF-7 Cells Culture

Biological investigation on ALG_EPI_1 was performed on the MCF-7 breast-cancer cell line. MCF-7 cells were cultured at 37 $^{\circ}\text{C}$ with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 IU/mL streptomycin. Cultures were maintained in humidified atmosphere of 95% air and 5% CO_2 at 37 $^{\circ}\text{C}$. To test the 3D-printed hydrogels, MCF-7 were seeded in 12-well plates at a density of 5.5×10^5 cells/well and maintained in 2 mL of cell culture media. After 48 h, the medium was changed with a serum-free one, and a scaffold was placed in each well for the time of the experiment.

3.10. Cytotoxic Activity towards MCF-7 Breast-Cancer Cells

The cytotoxicity of the self-crosslinked hydrogels was assessed after 4 h and 24 h using the resazurin assay. ALG_EPI_1 and control hydrogels (3D-ALG) were sterilized under UV light for 30 min each side and directly placed in each well. Three replicates were performed for each grown condition (nude plate-CTRL, 3D-ALG, and ALG_EPI_1). At each time point (4 and 24 h), the hydrogels were removed, and the cell media was aspirated; cells were gently washed in PBS to remove eventual debris, and 1.2 mL resazurin solution (10 $\mu\text{g}/\text{mL}$) was added to each well. Plates were incubated for 2 h at dark at 37 $^{\circ}\text{C}$ in a wet atmosphere with 5% CO_2 and 95% air. Fluorescence was recorded at 540 nm excitation and 590 nm emission by a Spark[®] microplate reader (Tecan "Spark 10M"; Software Sparkcontrol Method Editor Tecan, Switzerland). The fluorescence values of the control plate were considered as 100% of viability.

3.11. Proteomic Analysis

MCF-7 seeded in 12-well plates were cultured with the 3D-ALG or ALG_EPI_1 for 4 h and 24 h in triplicate. After this time, the hydrogels were gently removed, and the cell culture mediums were taken from each culture well and stored for further analysis. Adherent cells were washed with PBS, detached using trypsin solution (150 $\mu\text{L}/\text{well}$), and collected in a small tube after centrifugation. Cell pellets were further washed with fresh PBS repeated 3 times and stored at -80°C until use. Total

proteins were extracted from cell pellets using an 8 M urea lysis buffer in 50 mM ammonium bicarbonate (pH 8). Lysates were vortexed and put on a stirring plate for 15 min then sonicated for 30 min at 4 °C. Extracts were centrifuged at 4 °C for 10 min at 15,000 rpm, and the supernatants thus obtained were concentrated with buffer exchange procedure using 50 mM ammonium bicarbonate in Amicon Ultra 0.5 mL centrifugal filters with a 3-kDa Molecular weight cut-off. The ensuing protein extracts were assayed for total protein content using the Bradford method. Briefly, 10 µL of each protein extract were added to 500 µL of a diluted Bradford reagent solution (1:5 with water) and incubated for 5 min in the dark at room temperature. After incubation, the ABS of the samples were recorded at a wavelength of 595 nm. The calibration curve was prepared using a BSA standard (1 mg/mL) diluted to a range of 10–400 µg/mL ($y = 0.001x$ $R^2 = 0.995$). The extract was further processed, and the proteins were reduced and alkylated with DTT (100 mM for 30 min at 37 °C), followed by IAA (200 mM for 30 min in the dark), and the addition of DTT (100 mM for 15 min at room temperature). Finally, samples were digested by incubation with trypsin (1:50 enzyme/substrate ratio) at 37 °C overnight and, the digestion stopped with 1 µL of FA added to each sample. All samples were dried under nitrogen flow and stored at –20 °C until use. The tryptic peptides were reconstituted in 100 µL of 0.1% (v/v) formic acid aqueous solution and 50% (v/v) acetonitrile before being analyzed with LC-MS/MS to identify and relative quantify pro and anti-apoptotic protein biomarkers using the selected reaction monitoring (SRM) approach. Samples were analyzed as biological (n = 3) and technical replicates (n = 2) using the same instrumentation reported in the previous section. An Xbridge Peptide BEH C18 (250 × 2.1 mm, 5 µm) column (Waters, Milford, MA, USA) equipped with a pre-filtering column was used for peptide separation. The injection volume was 15 µL. An elution system based on a solvent gradient (solution A: 0.1% aqueous formic acid (v/v); solution B: acetonitrile) was delivered at 0.2 mL/min. The gradient was set as follows: 0 min 2% solvent B, 4 min 2% solvent B, 150 min 90% solvent B, 155 min 90% solvent B, and 170 min 2% solvent B. As for semiquantitative analysis, experiments were performed under positive ion-SRM conditions using nitrogen as collision gas (pressure of 2.1×10^{-3} mbar in the collision cell; collision energy 40 V) and a 20 ms-dual time for each transition monitored. The SRM transitions monitored were reported in Table S1 (Supplementary Materials). The analytes were relatively quantified among samples by normalization with total protein content obtained by Bradford assay.

3.12. Bioinformatic Analysis and Mass Spectrometry Data Processing

A panel of eight anti and pro-apoptotic proteins (Bcl-2; Bcl-xL; p53; BAD; CASP-3; CASP-9; BAX; AMBRA1) were considered for the assay. FASTA protein sequences were obtained from Uniprot Human proteome database and SRM transitions for each protein were simulated by Skyline (v. 20.0,

SCIEX) (Redwood City, CA, USA), setting trypsin as digestion mode with no missed cleavage and carbamidomethylation of cysteins as structural modification (Table S1). The uniqueness of candidate peptide sequences was assessed by BLASTp tool (basic local alignment search tool; www.ncbi.nlm.nih.gov (accessed on 25 December 2021) link NCBI BLAST) search (algorithm: blastp; ATRIX PA 30; GAP COASTS: existence 10, extension 1; DATABASE: non redundant protein sequences) from NCBI (National Center for Biotechnology Information) (Bethesda, MA, USA). LC-MS/MS data were analyzed using the Analyst v 1.4 software, and integrations of the peaks areas were obtained by the MultiQuant program (version 2.1, ABSCIEX). 3.13. Statistical Analysis Statistical analyses were undertaken using GraphPad Prism®, version 6.00 (GraphPad Software, La Jolla California USA) using one- way ANOVA test. All experiments were performed in triplicate, and the results were expressed as the mean $\pm$ standard deviation (SD). A value of $p < 0.05$ was considered statistically significant.

4. Conclusions

A new alginate-based self-crosslinking formulation for 3D printing was successfully developed. Hydrogels were produced by an extrusion 3D printer with a one-step procedure that, by starting the crosslink during the extrusion, avoids the post-printing immersion in a crosslinking bath. The loading test carried out using a model water-soluble drug as EPI confirmed the potential of this procedure for the preparation of 3D-printed hydrogels intended as drug delivery systems. The 3D-printed hydrogels have good handling properties and a swelling behavior typical of hydrogels independently from the pH of the testing solution. The production procedure adopted allows an easy EPI loading, allowing to prepare hydrogels with an accurate drug title and a fast release up to 40 min. In vitro tests on MCF-7 breast cancer cells confirm the biocompatibility of the 3D-ALG hydrogel, with an increase in the cytotoxic effects at 24 h if functionalized with 1 μ g of EPI. The cytotoxic potential against MCF-7 was validated by the expression by cells of some apoptosis marker proteins studied in a target MS analysis. Hence, the use of internal gelation can be a promising approach in 3D printing of alginate, allowing the preparation of new functionalized biomaterial for medical applications.

Supplementary Materials: The following supporting information can be downloaded as following, Table S1: SRM transitions with their m/z values selected for LC-MS/MS analysis.

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L.E.; visualization, F.Q.; supervision, L.E.; project administration, L.E. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples of the compounds are available from the authors upon reasonable request.

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Supplementary Materials

Table S1. SRM transitions with their m/z values selected for LC-MS/MS analysis. Sequences chosen for semiquantitative evaluation are in bold.

PROTEIN ID	PROTEIN NAME	PEPTIDE SEQUENCE	PRECURSOR (m/z)	PRODUCT (m/z)
P10415	Apoptosis regulator Bcl-2 (Bcl-2)	DFAEMSSQLHLPFTAR	976,0	1009,4
		EMSPLVDNIALWMTEYLN R	1148,1	1369,7
Q07817	Bcl-2-like protein 1 (Bcl-xL)	EMQVLVSR	481,3	488,2
		WFLTGMTVAGVVLLGSLFS R	1077,6	1218,7
P04637	Cellular tumor antigen p53 (p53)	C[+57]SDSDGLAPPQHL R	833,4	931,5
		KPLDGEYFTLQIR	790,4	803,4
		QSQHMTEVVR	607,8	871,4
Q92934	Bcl2-associated agonist of cell death (BAD)	MSDEFVDSFK	602,8	742,4
		SAPPNLWAAQ R	605,8	631,3
P42574	Caspase-3 (CASP-3)	DGSWFIQSLC[+57]AML K	828,4	950,5
		VATEFESFSFDATFHAK	967,5	998,4
P55211	Caspase-9 (CASP-9)	QLIIDLETR	550,8	583,3
		FSSLHFMVEVK	662,3	850,4
		LSKPTLENLTPVVL RPEIR	1088,1	1097,6
Q07812	Apoptosis regulator BAX (BAX)	VVALFYFASK	572,8	615,3
		MGGEAPELALDPVPQDASTK	1013,5	446,2
Q9C0C7	Activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1)	SLGGPLSSHPSR	597,8	315,2
		LMAEGGMTAVVQ R	681,8	862,4

CHAPTER IX: *Lactobacillus delbrueckii* subs. *bulgaricus* derivatives for 3D printed alginate/hyaluronic acid self-crosslinking hydrogels: manufacturing and wound healing potential

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Abstract

Derivatives [i.e. proteins and exopolysaccharides (EPS)] from *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB) were extracted, characterized and for the first time used in the production of novel self-crosslinking 3D printed alginate/hyaluronic acid (ALG/HA) hydrogels, as high-value functional biomaterials with therapeutic potentials in regenerative medicine applications. Cytotoxicity, proliferation, and migration on human fibroblast of the derivatives coming from two different LB strains, LB1865 and LB1932, were tested *in-vitro* and compared. EPS received particular attention as showing relevant dose-dependent cytocompatibility against the human fibroblast. The derivatives showed an ability to increase cell proliferation and migration quantifiable between 10 and 20% if compared to controls, with higher values for the derivatives obtained from the LB1932 strain. Liquid chromatography-mass spectrometry targeted protein biomarker analysis explained at the molecular level the accentuated effects of LB1932 derivatives by a decrease in matrix-degrading and proapoptotic proteins, associated with an increase in collagen and antiapoptotic proteins production. It was possible to conclude that the potential of LB1932 enriched hydrogel was found to be of benefit compared to control dressings, serving the more promising results as potential for *in vivo* skin wound healing tests.

Keywords: 3D printing, alginate, hyaluronic acid, self-crosslinking bioinks, *L. bulgaricus*, EPS, wound healing.

1. Introduction

Tissue engineering is an interdisciplinary field of study, which aims to develop active materials capable of recreating, repairing, or engineering tissues and organs [1]. In this context, the development and application of innovative biomaterials as advanced therapeutic solutions are constantly evolving [2]. One of the application fields of tissue engineering deals with wound healing, a complex biological process including inflammation, proliferation, and tissue remodeling. The wound healing process consists in the activation of numerous and still poorly understood intracellular and intercellular pathways able to restore the complex physiological integrity and functionality of these multilayer tissues [3]. Wound healing is a global and serious medical issue with several concerns related to the increasing incidence of obesity and type II diabetes, an ageing population (principally in developed countries with low birth rates) and the requirement for more effective and cost-effective dressings [4]. Thus, new approaches are always required to induce and favor wound repair. Among the most promising strategies, the use of bacteria, both through oral and topical administration, has been recently considered especially for the good achievements in cutaneous wounds healing [5–11]. Selected bacteria demonstrated stimulatory effects on wound repair activating lymphocytes and macrophages, favoring immune system activities, inhibiting inflammation and promoting cell proliferation. Species of lactic acid bacteria (LAB) have been identified as promoters of the wound healing process. LAB have a long history of safe exploitation by humans, being used for centuries in food production and preservation and as probiotic agents to promote human health [12,13]. LAB have been classified as food grade microorganisms [generally recognized as safe (GRAS) organisms by the US Food and Drug Administration (FDA)] and fulfill criteria of the qualified presumption of safety (QPS) according to the European Food Safety Authority [14]. Due to their metabolisms and their health improving effects, LAB plays important roles in food, dairy, agricultural, pharmaceutical, and medical applications [15]. Their potential benefit in tissue regeneration processes has been demonstrated using both whole living bacteria and/or some of their derivatives. For example, *Lactobacillus bulgaricus* (LB) and *Lactiplantibacillus plantarum* whole cells improved diabetic wound healing via regulation of inflammatory factors [8]; soluble fraction from lysates of *Streptococcus thermophilus*, *L. plantarum*, and *L. acidophilus* promoted the *in vitro* re-epithelialization of HaCaT scratched monolayer through a mechanism involving nitric oxide synthase 2 [16]. Furthermore, exopolysaccharides (EPS) produced by some lactobacilli could favor fibroblast regenerative processes [17], by inducing fibroblasts migration and exhibiting an anti-elastase and anti-collagenase potential on human fibroblast. EPS could also contain residues of D-glucuronic acid, a fundamental component of the hyaluronic acid normally presents in the extracellular matrix (ECM), involved in the mechanoperception of cells [17].

In the last few years, many efforts have been spent to develop advanced therapies able to mimic the ECM and thus cell adhesion, migration, and proliferation. Three-dimensional (3D) scaffolds have been successfully involved in wound healing as supports for cell growth, maintenance and differentiation thanks to their suitable structural, physical and biochemical characteristics [1,2]. Nowadays 3D printing can be considered the main technology utilized in scaffold production for tissue engineering purposes [18]. Numerous advantages over traditional manufacturing methods, such as the possibility to design complex 3D multi-layered structures with tunable features and most importantly, device personalization have been well demonstrated [19,20]. A computer-aided design (CAD) allows to obtain 3D objects with high repeatability and precise control of complex microstructures [21,22]. These are key points in the development of advanced release systems with *ad hoc* features such as personalized locally controlled drug release [23]. Among the materials used as inks for 3D printing, natural polymers are certainly some of the most studied, as they can promote adhesion and provide support for cell growth. In particular, natural-based 3D printed hydrogels presents favorable biomimetic environments for living cells, such as high-water content, tunable mechanical properties and degradation rates, porous structure and the possibility to incorporate bioactive molecules or living cells [24–27]. Moreover, they are also biodegradable and therefore can be progressively degraded and replaced by a new extracellular matrix in tissue regeneration processes [28,29]. One of the most used biopolymers to prepare hydrogels for biomedical applications is alginate (ALG), due to its characteristics of biodegradability, non-toxicity and non-immunogenicity [30]. ALG is a natural polysaccharide produced by some bacteria as part of their cell wall and, above all, by brown algae, from which it is obtained in the form of sodium, calcium, or magnesium salts. The most used technique for the production of ALG hydrogels is the addition of divalent soluble cations (such as Ca^{2+} , Ba^{2+} , Zn^{2+} or Sr^{2+}) to an ALG solution, even though often leads to hydrogels with poor structural homogeneity and poor mechanical properties [31]. The internal gelation of the ALG through calcium carbonate and δ -glucono-lactone (CaCO_3 – GDL) system can be a useful crosslinking technique to obtain homogeneous hydrogels thanks to a slow and controlled release of calcium ions. The homogeneous hydrogels thus obtained, usually present a well-defined porosity, uniform mechanical properties and the possibility of including water-soluble active ingredients in the formulation [31,32]. ALG can be manufactured as hydrogels, film membranes, sponges, and 3D printed structures, alone or in combination with other biopolymers, such as hyaluronic acid (HA). To date, the use of self-crosslinking alginate-based inks for 3D printing is still in its infancy [33], but the ability of the internal gelation to prepare 3D homogeneous hydrogels open many possibilities [34]. HA is a natural nonsulfated linear glycosaminoglycan, made by repeating units of (β ,1-4)-d-glucuronic acid and (β ,1-3)-N-acetyl-d-glucosamine. HA offers various advantages for applications

in tissue regeneration; it is biodegradable and non-immunogenic, it is relatively easy to produce and modify, it has a strong hydrophilic character, and it is not adhesive [35]. High molecular weight HA (>100 kDa) is a polymer widely represented in a large variety of tissues, including dermis, epidermis, and nerve tissue, constituting one of the main components of the extracellular matrix. It contributes to the hydrodynamic regulation of tissues, cell compression and processes such as inflammation and tissue regeneration. In the early stages of wound healing, HA provides temporary support for the transport of nutrients and waste products, while promoting the migration and proliferation of keratinocytes. Its hydrophilic character also guarantees the tissue correct hydration [31]. Moreover, evidence of HA mediated modulation of fibroblast gene expression involved in repair and remodeling of extracellular matrix have been reported [36,37]. In this whole frame, since the wound healing process involves many variables and collective biological mechanisms, a reliable understanding of the therapeutic efficacy of natural derivatives is still a big challenge. Despite the wide availability of scientific literature, up to nowadays, the potential of lactobacillus derivatives for developing active polysaccharide-based hydrogels to be applied in tissue regeneration still requires deeper investigation. This work aims to add insights into this field, through different steps: 1) to perform the extraction and extensive analytical characterization of *L. bulgaricus* derivatives coming from two different strains (LB1865 and LB1932; where LB1932 is a well-known producer of cell-bound EPS); 2) to develop innovative self-crosslinking inks for 3D printing extrusion technology based on ALG, ALG/HA and ALG/HA/LB derivatives mixtures; 3) to manufacture novel 3D printed active ALG/HA hydrogels accurately containing well-characterized LB hydrophilic derivatives to be applied in regenerative medicine applications; 4) to perform a series of *in-vitro* tests on human fibroblasts to investigate their potential capability in wound healing.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Sodium alginate (Ph. Eur. grade; molecular weight by gel filtration chromatography (GFC) 180–300 kDa; slowly soluble in water), urea, and calcium carbonate (CaCO_3) were purchased from Carlo Erba (Carlo Erba Spa, Milan, Italy). Hyaluronic acid [Halien, High MW 100-1000 kDa (GFC)], was from ACME (ACME S.r.l., Cavriago, Italy). Trypsin from bovine pancreas, bovine serum albumin (BSA), glucono- δ -lactone (GDL), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (ABC), formic acid (FA), and acetonitrile (ACN) were purchased from Merck (Merck KGaA, Darmstadt, Germany). Dulbecco's modified eagle's medium (DMEM), L-glutamine, streptomycin, penicillin, Na-pyruvate, and the non-essential amino acids (NEAA) were obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Fetal serum bovine (FBS) and Dulbecco's

phosphate buffered saline (DPBS) were from Euroclone (Milan, Italy). Protein assay dye reagent concentrate was from Bio-Rad. Resazurin sodium salt was from Alfa Aesar (Ward Hill, MA, USA). All solvents were of reagent grade with the highest purity available. Deionized ultra-filtered water was used throughout this study.

2.1.2. Bacterial lysates preparation and quantification

The strains *L. bulgaricus* (LB) 1865 and 1932, belonging to the microbial culture collection of the University of Parma (UPCC), were maintained as stock cultures at -80°C in MRS broth (Oxoid, Basingstoke, UK) supplemented with 20% (v/v) glycerol until use. LB 1932 was chosen as a well-known EPS producer. Bacteria were then recovered in MRS broth by two sub-culturing steps (2% v/v) at 42 °C under anaerobiosis obtained with AnaeroGen gas packs (ThermoFisher Scientific Italia, Rodano, MI, Italy). Before use, the cultures were harvested by centrifugation (10000 g, 10 minutes at 4°C), saving the media for later analysis. The collected bacteria were washed twice in sterilized water to remove any media residues and resuspended to the original density. The bacterial lysate was obtained using a sonicator (Sonoplus HD3100, Bandelin, Berlin, Germany) by performing 5 cycles of treatment consisting of 1 minute of sonication at the power of 53 W then 1 minute of pause. During the entire treatment, the bacteria suspension was kept in an ice bath to avoid any overheating. LB derivatives used in this work and their abbreviations were reported in Table 1. The obtained bacterial lysates were named respectively L18 and L19.

Table 1

Abbreviations of the *L. bulgaricus* derivatives used.

<i>L. bulgaricus</i>	Strain 1865	Strain 1932
Raw lysate	L18	L19
Soluble Fraction	SF18	SF19
Cell-bound EPS	c-EPS18	c-EPS19
Released-EPS	r-EPS18	r-EPS19
Proteins extracted from Soluble Fraction's Pellet	P18	P19

2.1.3. Preparation and quantification of lysates soluble fraction

Raw lysates L18 and L19 were used to prepare the corresponding soluble fraction (SF). Samples were centrifugated at 6000 g for 20 minutes and the supernatants were filtered using a 0.22 µm pore filter (Sartorius, Germany) to remove any whole bacteria remaining. SF18 and SF19 were thus obtained

and the remained pellets, after being solubilized, were assayed for protein content using the procedure described below.

2.2. LB proteins

2.2.1. Protein extraction, quantification, and preparation for LC-MS/MS analysis

Total proteins were extracted from 1mL of each raw lysate (L18 and L19), the soluble fraction (SF18 and SF19) and the pellet (P18 and P19) by adding 8M urea. Samples were vortexed and put on a stirring plate at 500 g for 15 minutes, then sonicated for 30 minutes at 4°C. Extracts were centrifuged at 4°C for 10 minutes at 10000 g and the supernatants were concentrated with a buffer exchange procedure using 50 mM ammonium bicarbonate (Amicon® Ultra 0.5 mL centrifugal filters; 3-kDa MW cut-off). Protein extracts were assayed for total protein content according to the Bradford procedure. Briefly, 10 µL of each sample were added to 500 µL of a diluted Bradford reagent solution (1:5 with water) and incubated for 5 min in the dark at room temperature. After incubation, the sample absorbances were recorded at a wavelength of 595 nm. A calibration curve was prepared by using BSA standard solutions in the 10-400 µg/mL range ($y = 0.0011 (\pm 3.3022e^{-5}) x$; $r^2 = 0.995$).

The extracts were further processed for liquid chromatography-mass spectrometry (LC-MS) analysis. Proteins were reduced and alkylated with DTT (100 mM for 30 min at 37° C), followed by IAA (200 mM for 30 min in the dark), and the addition of DTT (100 mM for 15 min at room temperature). Finally, samples were digested by incubation with trypsin (1:50 enzyme/substrate ratio) at 37° C overnight, then stopping the digestion with 1 µL of FA. All samples were dried under nitrogen flow and reconstituted with 50 µL of a water/acetonitrile/formic acid aqueous solution (49.95/49.95/0.1, v/v) for qualitative mass spectrometry analysis. Each sample was extracted in duplicate (n=2 biological replicates).

2.2.2. LC-LTQ-Orbitrap XL analysis

Peptide separation of the tryptic digests was carried out by using an Xbridge Peptide BEH C18 (250 x 2.1 mm, 5 µm) column (Waters, Milford, MA, USA) equipped with a pre-filtering column. Eluting mixtures included solvent A (0.1% aqueous formic acid, v/v) and B (0.05% formic acid in acetonitrile, v/v) delivered under gradient elution at the flow rate of 200 µL/min. The gradient was set as follows: 0 min 2% solvent B, 4 min 2% solvent B, 150 min 90% solvent B, 155 min 90% solvent B, 170 min 2% solvent B. The mobile phase was delivered by a Dionex Ultimate 3000 liquid chromatographic (LC) system (Dionex Corporation, San José, CA, USA) equipped with a 100-vial capacity sample tray. The volume of the injected samples was 5 µL. Qualitative protein analysis was obtained by mass spectrometry using an LTQ linear ion trap-Orbitrap XL instrument (ThermoScientific Corporation, San José, CA, USA), equipped with an electrospray source (ESI) interface and controlled by Xcalibur

software. Optimized conditions of the interface were: capillary voltage 3 kV, capillary voltage 13 V, capillary temperature 275° C, tube lens 100 V, sheath gas flow, auxiliary gas flow, and sweep gas flow delivered at 40, 10, and 5 arbitrary units, respectively. In the first scan event (full scan), the m/z window was 250-2000 with a resolution of 3000. The four highest m/z ratios over the threshold of 1000 counts were selected for collision-induced dissociation (CID) in the ion trap, with a normalized collision energy of 35 % in the collision cell. Each sample was analyzed twice (n=2 instrumental replicates).

2.3. *LB lipids*

2.3.1. Extraction and preparation for mass spectrometry analysis

Total lipids were extracted from 1mL of each raw lysate (L18 and L19) re-adapting the procedure proposed by Bligh & Dyer [38]. A mixture of chloroform: methanol (2:1 v/v) was added to each lysate in a 1: 1 ratio (v/v), the mix was vortexed, centrifuged at 5000 g for 5 minutes and the organic phase was collected; the procedure was repeated twice. The organic phases thus obtained were combined, dried under a nitrogen flow, and reconstituted with 200 µL of hexane for target mass spectrometry analysis. Each lysate was extracted in duplicate (n=2 biological replicates).

2.3.2. LC-MS/MS SRM analysis

Lipid target analysis was carried out by liquid chromatography with tandem mass spectrometry in selective reaction monitoring mode (LC-MS/MS SRM). Each solution was eluted by using an Atlantis dC18 column (150 x 2.1 mm, 5µm; Waters, Milford, MA, USA) equipped with a C18 pre-filtering column. An elution system based on a solvent gradient (solution A: acetonitrile; solution B: methanol) was delivered at 0.2 mL/min. The gradient was set as follows: 0 min 50% solvent B, 5 min 80% solvent B, 8 min 95% solvent B, 17 min 50% solvent B until 25 min. The mobile phase was delivered by the Agilent HP 1200 chromatographic system (Agilent Technologies, CA, USA) equipped with a 100-vial capacity sample tray. The injection volume was 10 µL and each sample was analyzed twice (n=2 instrumental replicates). A QTRAP 4000 triple quadrupole mass spectrometer (ABSCIEX, Foster City, USA), equipped with a pneumatically assisted ESI interface was used. The sheath gas (nitrogen, 99.999% purity) and the auxiliary gas (nitrogen, 99.998% purity) were delivered at flow rates of 45 and 5 arbitrary units, respectively. Optimized conditions of the interface were as follows: ESI voltage 5.5 kV, capillary voltage 50 V, capillary temperature 350 °C. Experiments were performed under positive and negative ion-SRM conditions using nitrogen as collision gas (pressure of $2,1 \times 10^{-3}$ mbar) and a 50 ms-dual time for each transition monitored. The SRM transitions monitored were reported in Table S1 and S2 (Supplementary Materials). Each sample was analyzed

twice (n=2 instrumental replicates). The analytes were relatively quantified among samples by normalization with total protein content.

2.4. *LB exopolysaccharides*

2.4.1. *Extraction*

EPS were extracted from both *L. bulgaricus* strains and the relative culture medium re-adapting the procedure proposed by Kun Wang *et al.* [39]. Cell-bound EPS (c-ESP) were extracted from the raw lysates (c-EPS18 and c-EPS19), while the released EPS (r-EPS) were extracted from the culture medium (r-EPS18 and rEPS19). EPS were precipitated in ethanol (1:3 v/v ratio) overnight at 4°C after gently mixing. The solution was centrifugated at 5000 g for 45 minutes and the supernatant thus obtained was eliminated. The pellet was dispersed again in a volume of ultrapure water equal to the initial volume of the sample. This procedure was repeated twice. To remove proteins, acetone precipitation was carried out by incubating the samples overnight at 4°C and then centrifuging at 9000 g for 2 minutes. The supernatant, containing EPS, was collected and dried under nitrogen flow.

2.4.2. *ATR FT-IR spectroscopy*

FTIR spectra were performed in the attenuated total reflection mode (ATR), using a Perkin-Elmer spectrometer (Norwalk, CT, USA). The apparatus works with a single reflection at an incident angle of 45°. The analysis was carried out on r-EPS and c-EPS from both *L. bulgaricus* strains at room temperature and ambient humidity. For each spectrum, 32 scans were acquired between 4000 and 400 cm⁻¹ with a spectral resolution of 2 cm⁻¹.

2.5. *ALG/HA self-crosslinking inks for 3D printing*

2.5.1. *Ink preparation for 3D printing*

Sodium alginate (6% w/v), hyaluronic acid (10-20-30-40 % of ALG weight) and calcium carbonate (160mM) powders were dispersed in ultrapure water in a beaker and magnetically stirred for 48 h until a homogeneous dispersion was achieved. 1 mL of the ALG/HA/CaCO₃ dispersion was first sonicated in an ultrasonic bath (Branson 2510; OPTO-LAB, Modena, Italy) for 30 min at room temperature and then carefully and quickly mixed with 1 mL of a freshly prepared GDL water solution as previously reported [33]. Different GDL concentrations (80, 100, 120 and 140 mM: pH= 4.5 ± 1) were tested. For hydrogels containing *L. bulgaricus* derivatives, the final inks were prepared by using GDL solubilized in the LB derivative solutions.

2.5.2. *3D printing and hydrogel production*

A custom-built low-temperature manufacturing system was designed and developed by combining a Peltier cell with a homemade Fuse Deposition Manufacturing (FDM) 3D printer, as previously described [33,40]. The ink formulation based on ALG, HA, CaCO₃ and GDL was loaded into a 5 mL

syringe and extruded through a 26 G needle (inner diameter 0.260 mm) by application of controlled pressure to the syringe through a mechanically assisted piston (see Table S3 and Table S4 for ink composition). The deposition took place through a robotic arm that moves along three axes (x, y, z) following the directives imposed by the g.code file on a stainless-steel cold Peltier cell (-14°C). For each ink formulation, a square hydrogel (grid 1.6 cm × 1.6 cm in size of parallel overlapped filaments with a distance of 200 µm) was printed similarly as previously reported [33]. Once terminated the deposition, the hydrogel was post-printing processed at -2°C to complete the self-gelation process (from 4 up to 7 days, as a function of the number of layers and composition). After the gelation time, the hydrogels were brought to RT to allow the detachment from the steel plate and stored at 4°C until use.

2.5.3. Scanning Electron Microscopy (SEM) Analysis

SEM morphological characterization was conducted on the 3D printed alginate-based hydrogels. The hydrogels were dehydrated by immersion twice for 10 min in increasing grades of ethanol from 50° to absolute. Anhydrous hydrogels were obtained by the critical point drying technique (Balserz Union, Lake Butler, FL, USA) to avoid structure deformations. The anhydrous hydrogels were then fixed on support using double-sided carbon tape, sputter coated with gold (thickness 60 nm) (E5100, Polaron, Quorum Technologies Ltd., Leves, UK) and observed by SEM (Philips 501, Philips, Eindhoven, The Netherlands).

2.6. In vitro-fibroblast cell culture studies

Primary human skin fibroblasts, coded as C86, were derived from a forearm biopsy of a healthy donor after the signature of an informed consent. Cells were maintained in a standard culture medium: formed by DMEM supplemented with 1% (v/v) of L-Glutamine, 1% of Streptomycin and penicillin, 1% of Na-pyruvate, 1% NEAA and 10% FBS. All the LB derivatives were dried under nitrogen flow, sterilized under a UV lamp for 2 hours and reconstituted in the assay medium. Table 2 summarized the *in vitro* experiments performed on fibroblast cells.

Table 2

Scheme of the *in vitro* tests, time points, LB derivatives and concentrations evaluated on fibroblast cells.

Test	Time (h)	SF* (µg/mL)	r-EPS** (µg/mL)	P*** (µg/mL)
Cytotoxicity test 1 (LB derivatives dissolved in medium; resazurin assay)	24/72	50	2000	50
Cytotoxicity test 2	24/72	25	20	25

(LB derivatives dissolved in medium; resazurin assay)				
Scratch test 1 (<i>In vitro</i> wound healing model)	0/8/24/48	25	20	25
Scratch test 2 (<i>In vitro</i> wound healing model)	0/8/24/48	50	40	50
Proliferation test on hydrogel-loaded treatments (resazurin assay)	24/72	3.4 µg/well	3 µg/well	****
Proliferation test for protein biomarkers evaluation by target mass spectrometry analysis	48	25	20	25
Viability test in parallel to mass spectrometry analysis. (Treatments dissolved in medium; resazurin assay)	48	25	20	25

* Soluble Fraction of raw lysates of LB1865 and LB1932 strains.

** r-EPS from LB1865 and LB1932 strains.

***Protein extracted from the pellet of raw lysates of LB1865 and LB1932 strains.

**** Not tested.

2.6.1. Cytotoxicity test of *L. bulgaricus* derivatives

The cytotoxicity of *L. bulgaricus* derivatives was tested on C86 fibroblast cells seeded in a 96-well plate at 4.000 cells/well (150 µL standard medium). After 24 h of incubation at 37°C and 5% CO₂, the standard medium was removed and replaced with a medium enriched with *L. bulgaricus* derivatives. The cytotoxicity of these derivatives was assessed after 24h and 72h using the resazurin assay. The treatment concentrations evaluated in the various tests are summarized in Table 2. Six replicates were performed for each of the six conditions and the control (Ctrl). At each time point, the cell media enriched with *L. bulgaricus* derivatives were aspirated, the cells were gently washed in PBS to remove eventual debris and 110 µL resazurin solution (10 µg/mL) was added to each well. Plates were incubated for 2h at dark at 37 °C in a wet atmosphere with 5% CO₂ and 95% air. Fluorescence was recorded at 540 nm excitation and 590 nm emission by a Spark® 10M microplate reader (Tecan, Switzerland). The fluorescence values of the control wells were considered as 100% of viability.

2.6.2. *In vitro* wound healing model

A scratch test was performed to evaluate the migration of fibroblast cells treated with *L. bulgaricus* derivatives, as an *in vitro* wound healing model. Fibroblast cells were cultured in a 48 well-plate (20.000 cells/well, 400 µL standard medium) for 48h in standard conditions to reach ~90% confluence. After this time, the standard medium was removed, and the cell monolayers were scratched using a 200 µL sterile pipet tip to create a uniform cell-free gap area with reproducible width of wounding. Debris was removed from the culture by gently washing each well with sterile PBS. The cells were then incubated with the medium enriched with the *L. bulgaricus* derivatives

(SF18 and 19; r-EPS 18 and 19; P18 and 19, according to the scheme reported in Table 2), at 37°C in a 5% CO₂ humidified atmosphere. Four replicates were made for each treatment condition, the positive control (standard medium with 10% FBS; Ctrl⁺) and the negative control (standard medium with 2% FBS; Ctrl⁻) (n=4). Two independent scratch tests were performed. Cell migration was monitored by microscopy using an inverted light microscope (Leica DMi1, Leica Microsystems, Milan, Italy) and images were captured (45× magnification) at different time points (0, 8, 24, 48 h) after the injury.

At each time point, the gap was photographed at two different points for each well. To calculate the % wound closure, the images collected were analyzed quantitatively using the public domain ImageJ software v. 1.53e (NIH, Bethesda, MD, USA). The quantification of relative wound closure was evaluated according to the following equation:

$$Woundclosure(\%) = \frac{A_0 - A_t}{A_0} \times 100$$

where A_t is the wound area at time t and A₀ is its initial area.

2.6.3. Cell proliferation test on 3D hydrogels functionalized with *L. bulgaricus* derivatives

The resazurin assay was performed to estimate cell proliferation on 3D-printed hydrogels containing SF19 and r-EPS19. After the first 24 h of cell culture in a standard medium supplemented with 10% FBS, the latter was replaced by a standard medium with 2% FBS, in which the functionalized hydrogels were inserted to the bottom of the well. Before use, the 3D hydrogels were brought to dryness, punched (6mm diameter) and sterilized under a UV lamp for 2h. LB derivative quantities present in each hydrogel piece are indicated in Table 2. Each treatment was tested in quadruplicate (n=4).

2.6.4. Study of cell mechanisms through protein biomarkers

Fibroblast cells were seeded in 6-well plates (170.000 cells/well, 2mL standard medium) for proteomic analysis and in 96-well plates (5.000 cells/well, 150 µl standard medium) to assess cell viability (incubation time 24 h, 37 °C, 5% CO₂). Then, the medium was replaced by a serum-free one, enriched with *L. bulgaricus* derivatives (SF18 and 19; r-EPS 18 and 19; P18 and 19, Table 2, n=6) for 48h.

For proteomic analysis, adherent cells were washed with PBS, detached using trypsin solution (250 µL/well) and collected in a small tube after centrifugation. Cell pellets were further washed with fresh PBS 3 times and stored at -20° C until protein extraction. Total proteins were extracted and quantified, and samples were prepared for LC-MS/MS analysis following the protocol described in paragraph 2.2.1.

The tryptic peptides were reconstituted in 100 μ L of 0.1% (v/v) formic acid aqueous solution and 50% (v/v) acetonitrile before LC-MS/MS to identify and monitor the distribution of protein regeneration biomarkers using the SRM approach. Samples were analyzed as biological (n = 2) and technical replicates (n = 2) using the same LC separation reported in the previous section (paragraph 2.3.2). As for semiquantitative analysis, experiments were performed under positive ion-SRM conditions using nitrogen as collision gas (pressure of 2.1×10^{-3} mbar in the collision cell; declustering potential 80V, collision energy 40V) and a 20 ms-dual time for each transition monitored. The SRM transitions monitored were reported in Table S5. Resazurin assay was performed to evaluate cell viability.

2.7. Data evaluation

2.7.1. Bioinformatic analysis and mass spectrometry data processing

LC-LTQ-Orbitrap XL raw data were processed using the Proteome Discoverer software (v. 1.6, Thermo Fisher Scientific) with SEQUEST as the search algorithm. Raw files were processed using precursor ion tolerance at 0.1 amu and fragment tolerance at 0.5 amu. Processed files were then searched against the *L. bulgaricus* database to obtain the total number of proteins identified in samples. LC-LTQ-Orbitrap XL raw data were processed also using MaxQuant software (v. 1.6.2.10) in label-free mode using FASTA sequences of *L. delbrueckii bulgaricus* ATCC 11842 (taxid: 390333) proteome downloaded from Uniprot. The software was set up to recognize the specific digestion of trypsin, allowing for a maximum of 2 missing cuts, setting N-terminal acetylation and methionine oxidation as variable modifications and the carbamylation of cysteines as a fixed modification. Then, data were reprocessed by Perseus software (v.1.6.15.0) to qualitatively compare the proteins extracted from the two different strains. Only identified by site, reverse and potential contaminants filters were applied, to exclude proteins not correctly identified and potential contaminants. A panel of 14 proteins involved in tissue regeneration were considered for the target mass spectrometry assay. FASTA protein sequences were obtained from Uniprot Human proteome database and SRM transitions for each protein were simulated by Skyline (v. 20.0, SCIEX, Redwood City, CA, USA), setting trypsin as digestion mode with no missed cleavage and carbamidomethylation of cysteines as structural modification (Table S5). The uniqueness of candidate peptide sequences was assessed by BLASTp tool (basic local alignment search tool; www.ncbi.nlm.nih.gov link NCBI BLAST) search (algorithm: blastp; ATRIX PA 30; GAP COASTS: existence 10, extension 1; DATABASE: non-redundant protein sequences) from NCBI (National Center for Biotechnology Information) (Bethesda, MA, USA). All LC-MS/MS SRM data (target proteins and lipids analysis) were analyzed using the Analyst v 1.4 software, and integrations

of the peaks' areas were obtained by the MultiQuant program (version 2.1, ABSCIEX). For the protein biomarkers study, Perseus software was used to cluster the samples: for all data relating to the abundance of each protein considered in each sample, the Z-score was calculated. For each measure group relating to a single protein abundance in the various samples, the mean and the standard deviation were calculated, then the mean was subtracted from each value of the group, and the result was divided by the standard deviation. Data thus transformed were used for the construction of a heatmap through clustering based on the calculation of Euclidean distances.

2.7.2. Statistical analysis

Statistical analyses were performed applying the t-Student test at the 95% significant level using the Microsoft Excel software. All experiments were performed at least in triplicate and the results were expressed as the mean $\pm$ standard deviation (SD).

3. Results and Discussion

*3.1. Characterization of *L. bulgaricus* derivatives*

To increase the understanding of the potential therapeutic efficacy of some LB derivatives in wound healing, the first part of the study was focused on the isolation of the main classes of chemical compounds (i.e., proteins, lipids, and EPS) expressed by both LB strains (LB1865 and LB1932) and on the qualitative/quantitative characterization of these extracts. The knowledge of the exact composition of bioactive components in these extracts is pivotally related to their applications in regenerative medicine, as the activity of such complex extracts on cell behavior is strictly connected to their chemical composition. The bioactivity can be related to a predominant defined class of compounds or be synergistic. Finally, the exact composition of bioactive components in these kinds of extracts can be essential for further development steps, such as standardization and certification of quality processes.

3.1.1. Quali- and quantitative analysis of LB proteins

In this investigation, proteins found in the raw lysate coming from the two strains (LB1865 and LB1932) were considered at first. The percentage of the total protein content, quantified both in the soluble fraction and in the pellet (isolated by centrifugation) of the raw lysate, exhibited highly significant quantitative differences between the two strains. The soluble fraction (supernatant of the raw lysate, SF18) contained 50% w/v of proteins and its corresponding pellet (P18) the 30% w/w. In the case of the LB1932 strain, only 5% w/v of proteins was present in the supernatant, while most of the proteins precipitated in the pellet (P19: 88% w/w). This difference could be largely explained by considering that compared to LB1865, LB1932 is a well-known producer of cell-bound EPS that

could link proteins and/or lipids (as biofilm) to lysate fragments and increase their precipitation during the centrifugation step. Furthermore, these differences could also be related to a different EPS structure of the polymeric chain (linear or branched) or composition, which could lead to different functions and binding abilities [41].

Proteins extracted from both lysates were thus characterized by liquid chromatography-high resolution mass spectrometry. Among the almost 4000 identified proteins, only 22% of them were common to the two strains. Mainly, these common proteins were identified as those involved in basic metabolic and energy processes, or ribosomal and chaperonin proteins.

3.1.2. Lipid characterization by LC-MS/MS SRM analysis

Lipids extracted from raw lysates were characterized by LC-MS/MS SRM analysis. The detected lipids were grouped into lipid classes and their relative abundances measured and compared between strains (Table S6). Among the free fatty acids, palmitic acid (C16:0) and oleic acid (C18: 1), were the most abundant ones. For both strains, it was possible to identify, at low intensity and without differences in relative abundance, the C19:0 cyc fatty acid, a characteristic compound of the lactobacilli membrane with a saturated hydrocarbon chain with 19 carbon atoms bringing a cyclopropane ring. This molecule has an important role in maintaining membrane fluidity, and its production increases under stress conditions such as variations in osmolarity, the presence of ethanol and acidity [42]. Triacylglycerols (TAG) data were comparable between the two strains. Similar results were obtained for the lysyl-phosphatidylglycerides (LPG) data, considered characteristic of the membranes of Gram-positive bacteria [43], with the exception of LPG 17:1, LPG 16: 1 and LPG 20: 4 which were detected only in LB1865. Finally, as for the phosphatidylglycerides (PG) class, the PGs 14: 0-18: 2, 16: 0-18: 0 and 16: 0-18: 1 had been identified exclusively in LB18, while the PGs 14: 0- 18: 1 and 16: 0-18: 2 were detected with higher intensities in LB19.

3.1.3. EPS characterization by ATR FT-IR spectroscopy

The last characterization step involved the extraction and characterization of the extracellular microbial exopolysaccharides. Cell bound and released EPS (c-EPS and r-EPS) were examined. Generally, LB1932 strain was able to express r-EPS up to 90% more than the LB1865 strain.

The ATR FT-IR spectrum of r-EPS from LB18 or LB19 were then acquired and compared (Fig. S1). Spectra did not show significant differences between r-EPS18 and r-EPS19. In both spectra there is the stretching of the O-H bond (r-EPS18: 3284 cm^{-1} ; r-EPS19: 3271 cm^{-1}), that of the C-H bond (r-EPS18: 2929 cm^{-1} ; r-EPS19: 290026 cm^{-1}); peak at 1637 cm^{-1} is indicative of protein presence and is associated with the NH bond bending, the peaks at 1453 cm^{-1} (r-EPS18) 1457 cm^{-1} (r-EPS19) indicate the symmetrical stretching of the COO- group, while the peaks at 1242 cm^{-1} and at 1216 cm^{-1} for r-EPS18 and for r-EPS19 respectively, suggest the possible presence of sulfated groups [44]. Finally,

some peaks related to glycosidic bonds (r-EPS18: 1043 cm^{-1} ; r-EPS19: 1019 cm^{-1}), confirm the presence of carbohydrates [17]. The same profile was obtained for c-EPS18 and c-EPS19 (spectra not shown).

3.2. Optimization of the Self-Crosslinking Formulation for 3D Printing

The second part of this work was focused on the development of ALG and ALG/HA-based self-crosslinking bioinks to support the 3D printing manufacturing of hydrogels incorporating accurate amounts of hydrophilic bioactive substances. These bioinks should allow the 3D printing process via room-temperature extrusion from a mechanically driven syringe and be able to retain the hydrogel 3D structure when extruded on a -10°C stainless steel plate. The cross-linking mechanism should then occur after printing to prepare hydrogel with a well-defined 3D structure and mechanical properties suitable for use as wound dressing [33]. A formulation previously developed by our research group (3% ALG (w/v), 80 mM CaCO_3 and 140 mM GDL) [17], was used as a starting point and optimized as a function of different combinations of ALG / CaCO_3 / GDL and content of LB derivatives (raw lysate or soluble fraction). Parameters, such as the pH of the bioinks, the number of printable layers, the maximum ink extrusion time, the curing time at 2°C necessary to complete the hydrogel self-crosslinking, and the capacity of the 3D printed hydrogel to retain its structure in water were evaluated (Table S3). The monitoring of these parameters was crucial to print structured hydrogels with proper mechanical characteristics. The internal gelation process is pH dependent, as the acidification of the alginate-based suspension containing calcium carbonate, due to the GDL hydrolysis, drives the release of Ca^{2+} which become available for the formation of the "egg-box" structure [45]. An increase in the concentration of GDL in solution leads to a faster cross-linking reaction, reducing both the ink homogeneity and maximum extrusion time, due to the increased viscosity. A summary of the different combinations tested to prepare 3D-printed functionalized hydrogels containing LB derivatives is reported in Table S3. In general, the addition of raw lysates or their soluble fractions to ALG-based formulations seemed to have an accelerating effect on the ink cross-linking kinetics, reducing the printability of the solution which can be attenuated by reducing the concentrations of GDL. Several combinations can allow 3D manufacturing of functionalized hydrogels, but the best results in terms of number of printable layers and stability were obtained with the following combination: 3% ALG (w/v), 80 mM CaCO_3 , 80 mM GDL aqueous solution (final volume 0.5 mL) and 0.5 mL of LB sample extract (final volume of the ink: 1 mL). To confirm the quality of the 3D constructs, ALG hydrogels were characterized morphologically by SEM (Fig. S2) to evaluate the 3D macrostructure. The ALG hydrogel presented a well-defined 3D macrostructure with homogeneous surface and filament inner pore distribution (pore size distribution between 3 and 52 μm) (Fig. S2 A-C). Since HA is a hydrophilic natural polymer extensively used in wound healing

and regenerative medicine [20,31,46,47], to further improve the properties of the 3D printed hydrogel, this polysaccharide was added to the optimized ALG ink formulation. One of the main characteristics of HA is the capacity to attract water molecules, therefore, in combination with ALG, it increases the hydrophilic character of the resulting hydrogels, promoting cell adhesion [48]. ALG/HA ink formulations were prepared by keeping constant the final concentrations of ALG (3% w/v) and CaCO_3 (80 mM) but testing different concentrations of HA and GDL as summarized in Table S4. During the preparation of ALG/ CaCO_3 /HA suspensions, an increase in the viscosity of the formulation proportional to the increase in the quantity of HA was observed. It was already reported that an increase in viscosity hinders the molecular movement of ALG chains increasing the time required for gel formation [31]. In our case, this translates into a increase in the extrudability time of the solution and in a rise of the curing times of the ink. The preliminary tests reported in Table S4 allowed us to select the ink formulation based on ALG (3% w/v), HA (40% w/w), GDL (80 mM) for further test with the LB derivatives. 80mM GDL was the best compromise for inks containing raw lysates, whereas 140 mM GDL allowed for the use of the LB soluble fractions (SF). The use of EPS was carefully tested and excellent printability was obtained under the same conditions used for the soluble fraction. The SEM analysis of ALG/HA hydrogels allowed to evidence that HA did not interfered with the quality of the final morphological structure, both in terms of macrostructure and homogeneous surface and filament inner pore distribution (pore size distribution between 5 and 48 μm) (data not shown). All these data showed that the production of a variety of self cross-linking ink formulations possibly used for manufacturing hydrogels with the minimum requirements described above is allowed, supporting the new critical need to develop advanced inks.

3.3. Effect of lysates' derivatives on in vitro-fibroblast cell cultures

3.3.1. Cytotoxicity test of *L. bulgaricus* derivatives

The biocompatibility of LB derivatives was evaluated over fibroblast cells and the results were summarized respectively in Fig. 1A and 1B. In this first test, a dramatic cytotoxic effect was recorded for r-EPS within 24 hours (Fig. 1A) for both LB strains at the concentration tested (2000 $\mu\text{g/mL}$). The properties of EPS strictly depend on several factors (i.e. structure, size, branching, composition, positive and negative charges, viscosity, purity, etc.) [49,50]. In our studies, the ATR-FT-IR experiments described above suggested the presence, in the EPS extracts, of sulfate groups and proteins. The EPS-fibroblast physico-chemical interaction behavior is difficult to elucidate, but this partially purified extract strongly affected different cell communication mechanisms including apoptosis. A dose-dependent activity was demonstrated as no signs of toxicity were observed by lowering r-EPS concentration at 20 $\mu\text{g/mL}$ (Fig. 1B). The soluble fraction of the two LB strains tested at the same protein concentration level (50 $\mu\text{g/mL}$) showed a different opposite behavior. In

particular, the LB1865 strain improved ($p>0.05$) cell proliferation, whereas the LB1932 strain decreased cell activity up to 60% in 72 hours. Finally, at 72h, treatments with the protein extracted from the pellets (P18 and P19), after an initial reduction in cell proliferation, allowed for significant cell growth.

This test was followed by a second test, (Fig. 1B) in which lower concentrations of the same treatments [SF (25 $\mu\text{g/mL}$); P (5 $\mu\text{g/mL}$)] were evaluated. At these concentrations, none of the treatments showed significant cytotoxic effects.

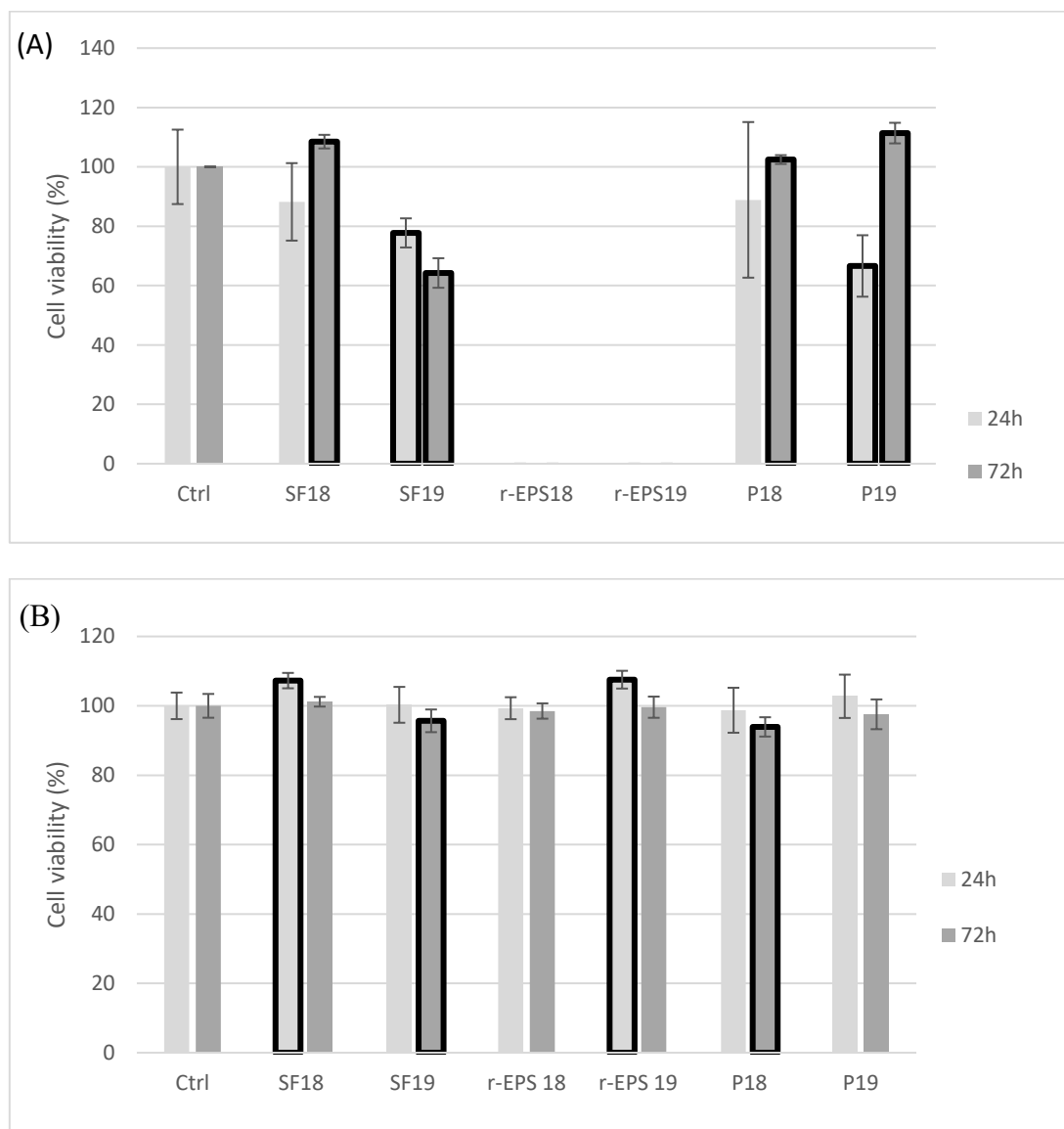


Fig. 1. Effect of *L. bulgaricus* derivatives on fibroblast cells viability at 24h and 72h. (A) test 1; treatments SF (50 $\mu\text{g/mL}$), r-EPS (2000 $\mu\text{g/mL}$), P (50 $\mu\text{g/mL}$). (B) test 2; SF (25 $\mu\text{g/mL}$), r-EPS (20 $\mu\text{g/mL}$), P (25 $\mu\text{g/mL}$). Results are reported as mean $\pm$ standard deviation of six independent measurements. The results are expressed as a percentage of viability compared with the control (Ctrl)

for samples treated with various *L. bulgaricus* derivatives; statistically significant differences ($p < 0.05$, T-student test) between the samples and the relative control are outlined in black.

3.3.2. Evaluation of fibroblast migration treated with *L. bulgaricus* derivatives

The ability of LB derivatives to stimulate the migration of fibroblasts, showing thus a regenerative potential, was evaluated by scratch test. To this aim an artificial discontinuity was created on the cell monolayer at confluence, a fundamental condition to avoid confounding overlap between the effects of cell proliferation and migration [51,52]. As previously reported in Table 2, two scratch tests were performed. The first test (Test 1) highlighted that the scratch area decreases over time (from 0 to 48h) until they become almost non-detectable at 48h, with improved migration effects for the derivatives of the LB1932 strain (Fig. 2). A further scratch test (Test 2) was performed by doubling LB derivative concentrations, and the results were compared by normalizing the re-epithelialization data obtained for the two tests on the respective negative control (Ctrl-). Fig. 3 shows the comparison at 48h, the last and significant time point. Scratch test 1 gave the best re-epithelialization rates in all samples ($p < 0.05$, T-student test) allowing us to conclude that the best effects, for stimulating cell migration, were those obtained under the conditions of Test 1.

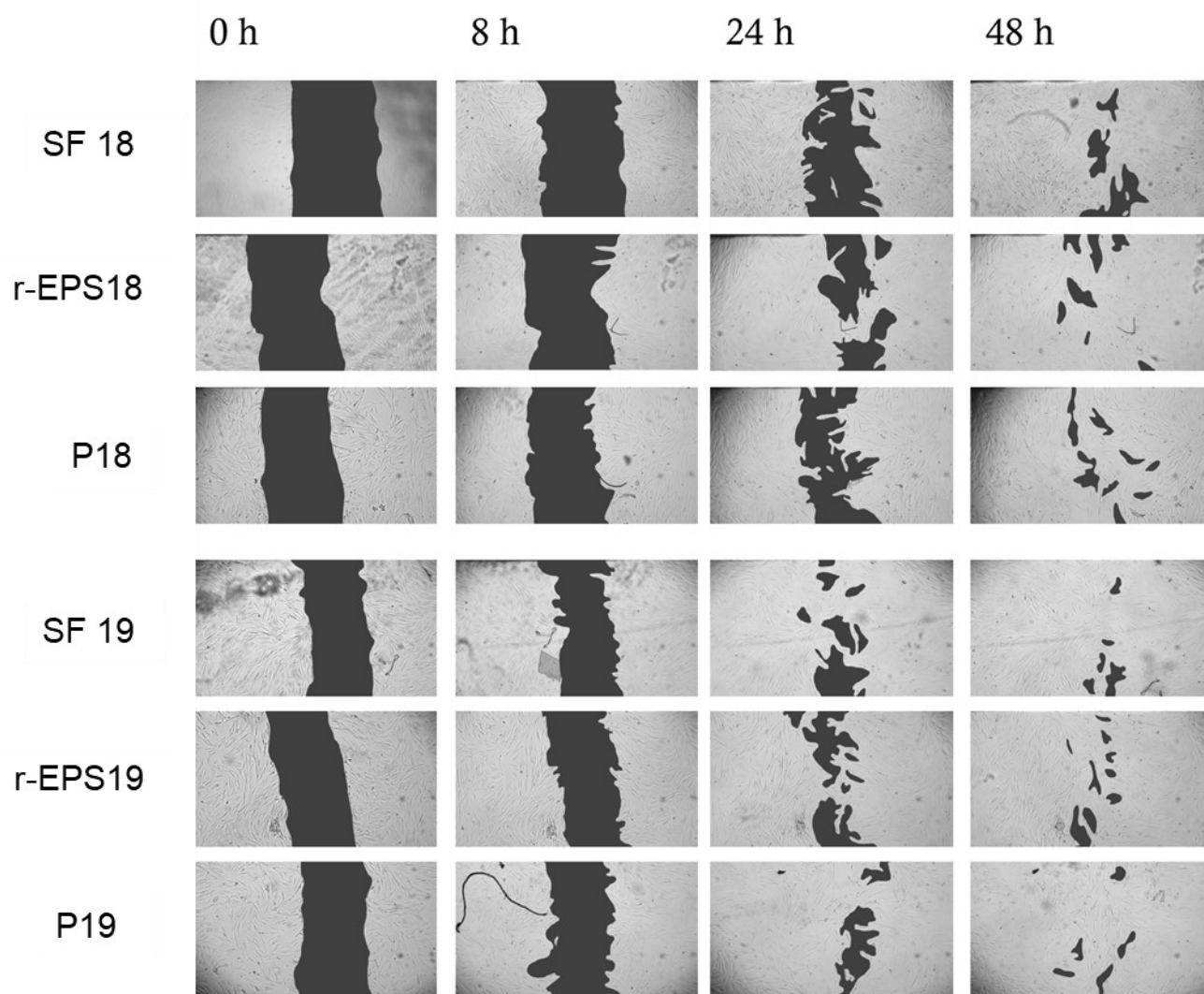


Fig. 2. Images from the first scratch test for the various treatments and over time (from 0 to 48h). The photographs, taken under a microscope, were used to calculate the scratch area (highlighted in black) using the ImageJ software.

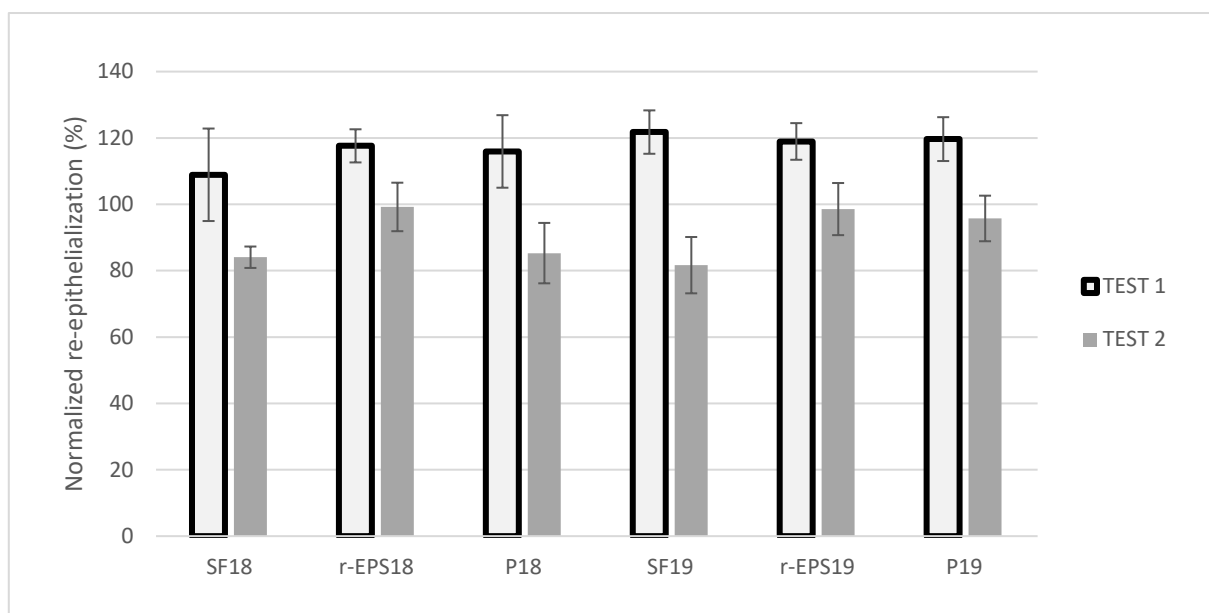


Fig. 3. Comparison between scratch test 1 and scratch test 2 at 48h. Results are reported as mean $\pm$ standard deviation of four independent measurements. The results are reported as the mean percentage of normalized re-epithelialization on the respective negative control; statistically significant differences ($p < 0.05$, T-student test) between tests 1 and 2 are outlined in black.

3.3.3. Cell proliferation test on 3D hydrogels functionalized with *L. bulgaricus* derivatives

Based on the collected results, cell proliferation tests on functionalized hydrogels were carried out by selecting the soluble fraction and the r-EPS derivatives deriving from the LB1932 strain, as they provided the best performance. The use of proteins extracted from the pellet was excluded because the related cultures showed the presence of aggregates in the medium upon microscopic observation (data not shown).

Treatments selected above were used to produce self-crosslinking 3D hydrogels as described in paragraph 2.6.3. To evaluate cell proliferation, viability measurements were performed, using resazurin assay, at 24 and 72 h on fibroblast cells grown in the presence of hydrogels.

In a first step, cell viability data obtained for the samples grown on nude wells (Ctrl) were compared with those grown in the presence of the unloaded hydrogel (Ctrl Hydrogels); a statistically significant reduction of cell viability for Ctrl Hydrogels, at 24 and 72h ($p < 0.05$, T-student test), was detected compared to those grown on the nude wells. However, Ctrl Hydrogels allow cell viability of 84-86% at 24 h and 72 h respectively if compared to the Ctrl samples. According to guidelines for the evaluation of in vitro cytotoxicity for medical devices and delivery systems (ISO 10993-5 : 2009), these data confirmed the biocompatibility of Ctrl Hydrogels that showed vitality values above the 70% threshold [53].

In a second step, functionalized hydrogels were considered (Fig. 4). Fibroblasts incubated in the presence of functionalized hydrogels showed an increase in cell viability compared to the Ctrl

Hydrogels indicating an increase in their proliferation. This increase was higher at 24 h and comparable at 72 h for both treatments. In detail, it is more pronounced in the SF19 hydrogels samples, where it settled around 20%, compared to the r-EPS19 hydrogels samples, where it settled around 10%.

In conclusion, in these experimental conditions, the hydrogels functionalized with the soluble fraction or with r-EPS of LB1932 strain have a positive effect on fibroblasts growth.

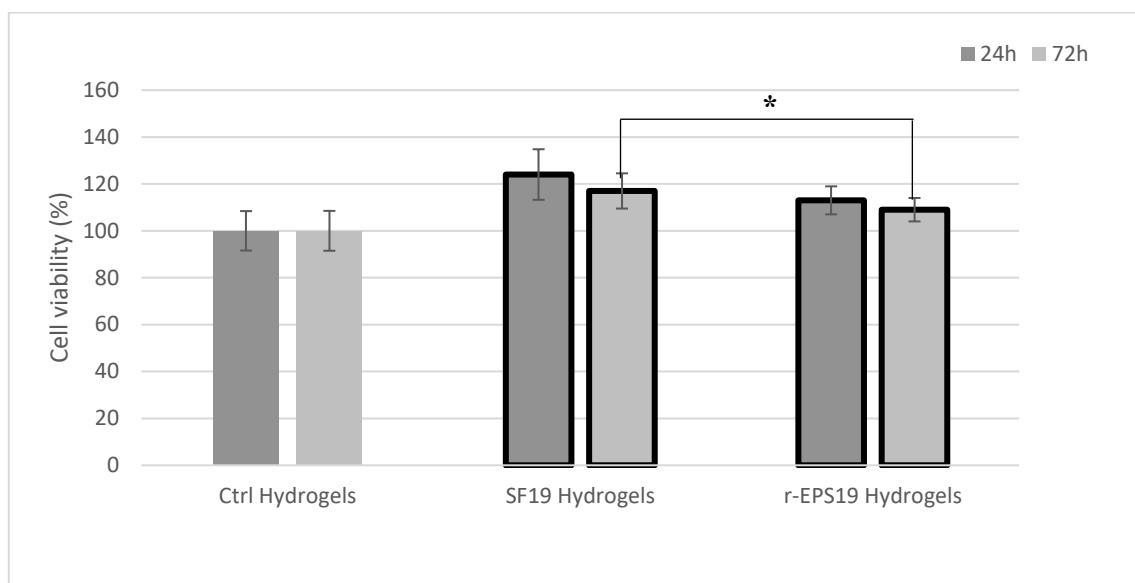


Fig. 4. Cell proliferation test with hydrogels. Cell viability for incubated samples with SF19 and r-EPS19 functionalized hydrogels is expressed as a percentage with respect to the mean of the control samples (Ctrl hydrogels, untreated); the results are reported as mean $\pm$ standard deviation of four independent measurements. Statistically significant differences ($p < 0.05$, T-student test) between Ctrl scaffolds are outlined in black, while asterisk (*) denotes the statistically significant difference ($p < 0.05$, T-student test) between functionalized hydrogels at 72h.

3.3.4. Regeneration protein biomarkers study

In an endeavor to further investigate the effect of *L. bulgaricus* derivatives on fibroblast cells, a set of protein biomarkers involved in extracellular matrix modelling, proliferation, wound healing, and apoptosis processes were analyzed by LC-MS/MS SRM target analysis. Initially, in parallel to the cell cultures intended for proteomic analysis, a viability test with resazurin assay was performed as a control on the culture conditions used, confirming, as expected, the non-cytotoxicity of the treatments, at the concentrations used (Table 2), also in combination with serum-free medium.

Thus, the proteomic analysis was carried out and after data transformation, as described in paragraph 2.7.1, clusters were constructed and the resulting heatmap is shown in Fig. 5. Samples SF18 and r-EPS 18 cluster with the controls (C) in the Ctrl cluster, indicating that these two treatments are

considered to not have relevant effects. Together with the control, they outline the basic cellular situation in the absence of treatments.

Another cluster (cluster 2) is formed by samples r-EPS 19 and PP19 with sample PP18, while sample SF19 stands alone (cluster 1). This suggests that the treatment with the soluble fraction of LB1932 strain has unique effects compared to the others. Biomarkers analyzed tend to organize in two large clusters (clusters A and B), showing an opposite behavior in samples clustered with or without controls. Within these two macro groups, some subgroups showed some differences between cluster 1 and cluster 2, as reported in Table S7.

Taking the Ctrl cluster as a reference, we observed that for both cluster 1 and cluster 2, proteins in cluster A are generally under-expressed (green), while those belonging to cluster B, are over-expressed (red). Considering the biomarkers involved in the proliferation process, we noted that in cluster A there is TNF-alpha, a mitogenic factor stimulating the proliferation of fibroblasts and the production of proteases [54], while in cluster B, the IL-1R-1 interleukins receptor [55] and PDGF subunit A, a powerful mitogen that favors fibroblasts proliferation by autocrine stimulation mechanism [54], are present. As for biomarkers involved in the extracellular matrix, cluster A contained the ECM degradation protein MMP3 [56,57] and the factor IGF1, which promotes cell growth and collagenase synthesis [55]; cluster B, on the other hand, included collagen I, ECM components produced by fibroblasts [58], and the TGF-beta-1 factor, which stimulates chemotaxis and proliferation of fibroblasts as well as the deposition of collagen [55]. All the factors involved in the healing process such as HGF that accelerates re-epithelialization once secreted by fibroblasts [57], vimentin that is involved in feral healing [59] and Engrailed-1 which is associated with scars formation [60], are present in the cluster A. As regards apoptotic process biomarkers, cluster A contained only the proapoptotic factor CASP-3, while cluster B included the proapoptotic factor Thy-1 and the antiapoptotic factors AKT and FAK [61,62]. Therefore, treatment clusters (clusters 1 and 2) compared to the control cluster (cluster Ctrl) showed a reduction expression of proteins associated with ECM degradation (MMP3 and IGF1), proapoptotic factors (CASP-3) and biomarkers associated with the healing process (HGF, vimentin and Engrailed-1), and an increase in expression of proteins involved in ECM deposition (collagen I and TGF-beta-1) and in apoptosis process (Thy-1, AKT and FAK), of which the majority (AKT and FAK) have anti-apoptotic significance. Treatment clusters also appear to be characterized by an increase in two proliferative factors (IL-1R-1 and PDGF subunit A) compared to a decrease in the third factor considered for this category (TNF-alpha). These differences largely coincide with the divergence in behavior between samples LB18 and LB19. The only exception is constituted by sample 18C, which was included in cluster 2. Focusing on the differences between cluster 1 and cluster 2, FADK subgroup was weakly under expressed in cluster

2 and strongly over expressed in cluster 1, while CASP-3 subgroup was strongly over expressed in cluster 1 but weakly under expressed in cluster 2. Cluster 1 was further characterized by a weak under-expression of Thy-1 and Collagen I and a weak over-expression of vimentin and AKT. This suggests an accentuation of the trend just described for the treatments (cluster 1 and 2) compared to cluster Ctrl, with intensified anti-apoptotic effect. In cluster 1 a decrease in collagen deposition could be probably associated with the increased IGF1 and MMP3 expression. A slight increase in vimentin expression, compared to cluster 2, together with the slight increase in TGF-beta-1 expression and the strong FAK increase, could suggest an augmented tendency to cell migration, with a possible regenerative significance.

Therefore, overall, these findings confirmed the difference in behavior between treatments derived from LB1865 and LB1932 strains. The derivatives of LB18, in fact, (except 18C) clustered with controls, and showed, compared to LB19 derivatives, an upregulation of factors involved in the degradation of the ECM, the proapoptotic factor CASP-3 and of factors associated with healing and scar formation, included in cluster A. Moreover, the downregulation of factors involved in the deposition of the ECM, antiapoptotic factors and factors associated with cell adhesion and migration processes, included in cluster B were observed for LB18 derivatives. The distribution of biomarkers associated with the proliferation process between clusters A and B, the first being upregulated and the second downregulated in the Ctrl cluster, suggests an overall balance of their effects and can explain the absence of substantial differences in viability detected by resazurin assay at 24 and 72 h. The derivatives of strain LB1932, on the other hand, outline an opposite situation compared to that described for those of LB1865, suggesting a generally favored antiapoptotic molecular structure, predisposed to ECM deposition and cell migration. These results confirmed the choice of samples FS19 and r-EPS 19 as the most suitable for stimulating the regenerative effects observed in the scratch test.

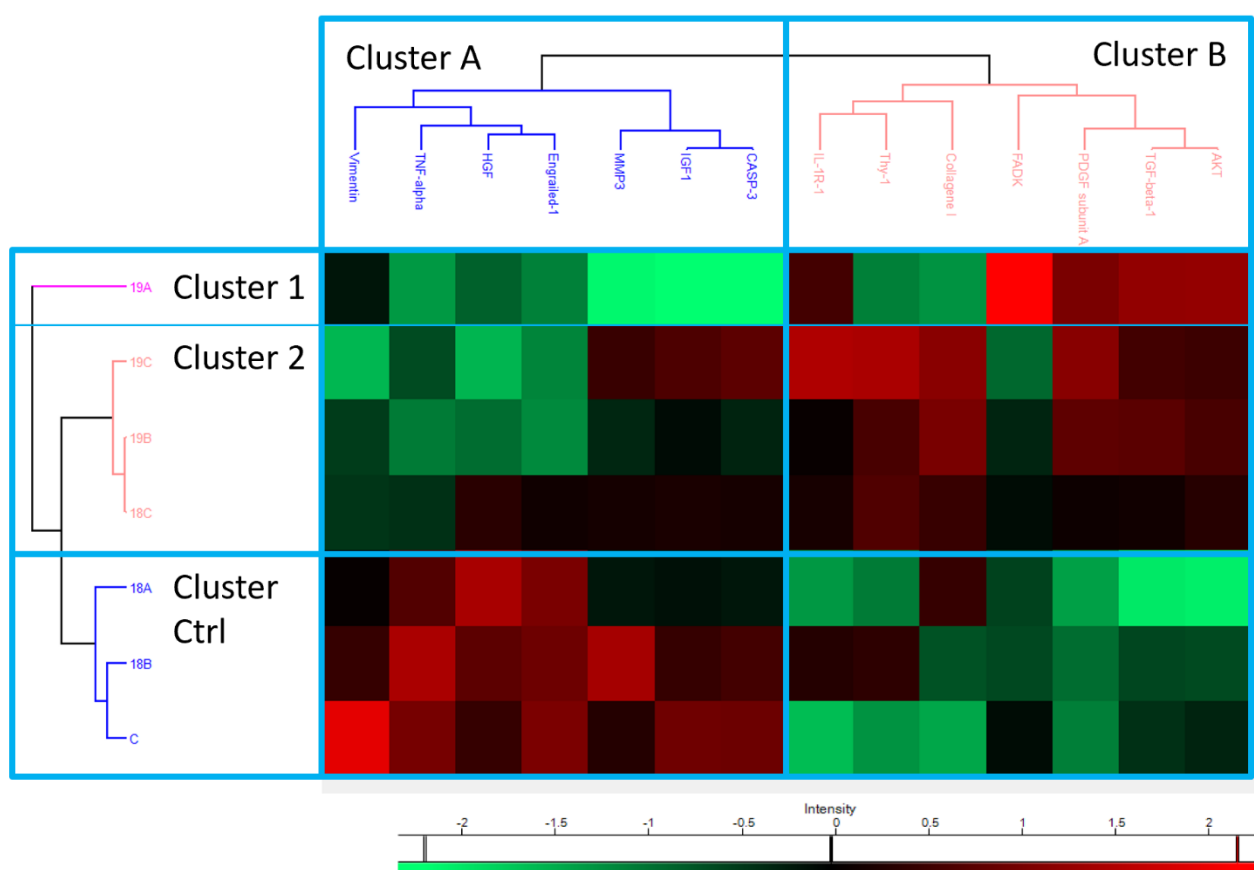


Fig. 5. Heatmap derived from data clustering on protein abundance derived from LC-MS / MS SRM analysis. Sample clusters: Cluster 1 (19A), Cluster 2 (19C, 19B, 18C), Cluster Ctrl (18A, 18B, C). Protein biomarkers clusters: Cluster A (Vimentin, TNF-alpha, HGF, Engrailed-1, MMP3, IGF1, CASP-3), Cluster B (IL-1R-1, Thy-1, Collagen I, FADK, PDGF subunit-I, TGF-beta-1, AKT). Downregulated proteins are represented in green, upregulated ones in red.

4. Conclusions

The potential use of *Lactobacillus delbrueckii* subsp. *bulgaricus* derivatives, characterized in terms of proteins, lipids, and EPS composition, in wound healing applications has been successfully investigated. The *L. delbrueckii bulgaricus* protein and EPS-based derivatives extracted from two strains, LB1865 and LB1932, and suitably dosed after cytotoxicity studies, were used to formulate and manufacture, ALG and ALG/HA acid self-cross-linking 3D printed hydrogels as novel active dressing able to promote *in-vitro* fibroblast proliferation and migration. Our tests highlighted differences between the derivatives of the two strains. In particular, the proteins of the soluble fraction and the r-EPS derived from LB1932 strain were able to stimulate cell migration with an increase of regenerative potential ranging from 10 to 20%. This evidence was explained at the molecular level by a decrease in matrix-degrading and proapoptotic proteins, associated with an increase in collagen and antiapoptotic proteins production. In the specific case of SF19 treatments, these effects seemed to be accentuated, associated with a decrease in collagen deposition and an increase in factors

involved in cell migration. All these findings suggest a potential use of *L. bulgaricus* derivatives, including proteins and EPS, embedded in 3D-printed hydrogels as advanced therapeutic solutions for successful applications in regenerative medicine, laying the basis for future investigations on this current topic. It is opinion of the authors that a deeper investigation especially focused on the characterization of EPS produced by these different LB strains could allow to add significative insights into their biological properties and potential use, including antimicrobial, anti-inflammatory, antioxidant activities and personalized dosages.

Supplementary Materials: Table S1, S2, S3, S4, S5, S6; Fig. 1,2.

CRedit authorship contribution statement: Giulia Remaggi: investigation, methodology, data curation, writing - original draft. Benedetta Bottari: supervision; writing, reviewing, and editing. Elena Bancalari: investigation, data curation, reviewing and editing. Ovidio Catanzano: methodology, writing - reviewing and editing. Erasmo Neviani: critical reviewing and supervision. Lisa Elviri: conceptualization, methodology, funding acquisition; project administration; supervision; writing - reviewing and editing.

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Supplementary Materials

Table S1

Lipid SRM transitions with their m/z values monitored in ESI + and selected for LC-MS/MS analysis.

Lipid class	Lipid code	Precursor m/z	Product m/z	Dwell time	CE (V)
Diacylglycerols	DG12:0-12:0	474.4	257.2	50	20
	DG14:0-12:0	502.4	257.2	50	20
	DG12:0-14:1	500.4	257.2	50	20
	DG12:0-16:0	530.5	257.2	50	20
	DG12:0-16:1	528.5	257.2	50	20
	DG12:00-18:00	558.5	257.2	50	20
	DG12:00-18:1	556.5	257.2	50	20
	DG12:0-18:2	554.5	257.2	50	20
	DG12:0-20:3	580.5	257.2	50	20
	DG12:0-20:4	578.5	257.2	50	20
	DG14:0-14:0	530.5	285.3	50	20
	DG14:0-14:1	528.5	285.3	50	20
	DG14:0-16:0	558.5	285.3	50	20
	DG14:0-16:1	556.5	285.3	50	20
	DG14:0-18:1	584.5	285.3	50	20
	DG14:0-18:2	582.5	285.3	50	20
	DG14:0-20:3	608.5	285.3	50	20
	DG14:0-20:4	606.5	285.3	50	20
	DG16:0-16:0	586.5	313.3	50	20

	DG16:0-16:1	584.5	313.5	50	20
	DG16:0-18:0	614.6	313.3	50	20
	DG16:0-18:1	612.6	313.3	50	20
	DG16:0-18:2	610.5	313.3	50	20
	DG18:1-18:1	638.6	339.3	50	20
	DG18:1-18:2	636.6	337.3	50	20
	DG18:1-20:3	662.6	339.3	50	20
	DG18:1-20:4	660.6	339.3	50	20
	DG18:2-18:2	634.5	337.3	50	20
	DG18:2-20:3	660.6	337.3	50	20
	DG18:2-20:4	658.5	337.3	50	20
	DG20:3-20:3	686.6	363.3	50	20
	DG20:3-20:4	684.6	363.3	50	20
	DG20:4-20:4	682.5	361.3	50	20
Diglycosyl glycerides	Diglycosyl glycerides 34 : 1	941.8	243.1	50	20
	Diglycosyl glycerides 35 : 2	953.8	243.1	50	20
	Diglycosyl glycerides 36 : 2	967.8	243.1	50	20
Lisyl Phosphatidyl Glycerides	LPGC12:0	429.2	257.2	50	20
	LPGC14:0	457.3	285.2	50	20
	LPGC14:1	455.2	283.2	50	20
	LPGC 16:0	485.3	313.3	50	20
	LPGC16:1	483.3	311.3	50	20
	LPGC17:1	497.3	352.2	50	20
	LPGC18:0	513.3	341.3	50	20
	LPGC18:1	511.3	339.3	50	20
	LPGC18:2	509.3	337.3	50	20
	LPGC20:3	535.3	363.3	50	20
	LPGC20:4	533.3	362.3	50	20
Triacylglycerols	C12:0-tag 36:0	656.6	656.6	50	10
	C14:0-TAG 42:0	740.7	740.7	50	10
	C16:1-TAG 48:3	818.7	818.7	50	10
	C16:1-TAG 48:2	820.7	820.7	50	10
	C16:1-TAG48:1	822.8	822.8	50	10
	C16:0-TAG48:0	824.8	824.8	50	10
	C17:0-TAG51:0	866.8	866.8	50	10
	C18:1-TAG54:3	902.8	902.8	50	10
	C18:1-TAG54:2	904.8	904.8	50	10
	C18:1-TAG54:1	906.8	906.8	50	10
	C18:0-TAG54:0	908.9	908.9	50	10
	C18:2-TAG54:6	896.8	896.8	50	10
	C18:2-TAG54:5	898.8	898.8	50	10
	C18:2-TAG54:4	900.8	900.8	50	10

Table S2Lipid SRM transitions with their *m/z* values monitored in ESI - and selected for LC-MS/MS analysis.

Lipid class	Lipid code	Precursor <i>m/z</i>	Product <i>m/z</i>	Dwell time	CE (V)
Fatty acids	C12:0	199.2	199.2	50	10
	C14:0	227.2	227.2	50	10
	C14:1	225.2	225.2	50	10
	C15:0	241.382	241.382	50	10
	C15:0 branched	241.382	241.382	50	10
	C 16:0	255.2	255.2	50	10
	C16:1	253.2	253.2	50	10
	C17:0	269.2	269.2	50	10
	C17:0 branched	270.442	270.442	50	10
	C17:1	266.392	266.392	50	10
	C18:0	283.3	283.3	50	10
	C18:1	281.2	281.2	50	10
	C18:2	279.2	279.2	50	10
	C19:0 cyc	295.47	295.47	50	10
	C20:3	305.2	305.2	50	10
	C20:4	303.2	303.2	50	10
	C18-oxo	297.444	297.444	50	10
	C17:1	267.418	267.418	50	10
Phosphatidylglycerides	PG 12:0-12:0	609.4	199.3	50	40
	PG 12:0-14:0	637.4	199.3	50	40
	PG 12:0-14:0	637.4	227.3	50	40
	PG 12:0-16:0	665.4	199.3	50	40
	PG 12:0-16:0	665.4	255.3	50	40
	PG 12:0-16:1	663.4	199.3	50	40
	PG 12:0-16:1	663.4	253.3	50	40
	PG 12:0-18:1	691.5	199.3	50	40
	PG 12:0-18:1	691.5	281.3	50	40
	PG 14:0-18:1	719.5	227.3	50	40
	PG 14:0-18:1	719.5	281.3	50	40
	PG 16:0-18:1	747.5	255.3	50	40
	PG 16:0-18:1	747.5	281.3	50	40
	PG 12:0-18:0	693.5	199.3	50	40
	PG 12:0-18:0	693.5	283.3	50	40
	PG 14:0-18:0	721.5	227.3	50	40
	PG 14:0-18:0	721.5	283.3	50	40
	PG 16:0-18:0	749.5	255.3	50	40
	PG 16:0-18:0	749.5	283.3	50	40
	PG 12:0-18:2	689.4	199.3	50	40
	PG 12:0-18:2	689.4	279.3	50	40
	PG 14:0-18:2	717.5	227.3	50	40
	PG 14:0-18:2	717.5	279.3	50	40
	PG 16:0-18:2	745.5	255.3	50	40
	PG 16:0-18:2	745.5	279.3	50	40

Table S3

3D printing tests of hydrogels containing bacterial lysate. Formulations provided the best results are highlighted in light-blue; components volumes refer to 1 mL of activated formulation. N/D is not detected.

Sample Type	Sample Volume (μl)	ALG (%w/v)	[CaCO ₃] mM	[GDL] mM	H ₂ O (μl)	pH	Number of hydrogel printed layers	Printing time (min)	Curing time (days at 2°C)	Self cross-linking
L19	500	3	80	140	0	N/D	0	N/D	N/D	N/D
L19	250	3	80	140	250	N/D	2	12	3	no
L19	125	3	80	140	375	4-5	2,5	14	3	yes
L18	250	3	80	120	250	5-6	3	14	3	yes
L19	250	3	80	120	250	5-6	3,5	15	5	yes
L18	375	3	80	120	125	5-6	4	11	5	yes
L19	375	3	80	120	125	7	3,5	13,26	5	yes
L19	375	2,5	80	140	125	6	1	8	5	yes
L19	500	2,5	80	140	0	7	1,5	9,5	5	yes
L18	500	2,5	80	140	0	7	1,5	8	5	yes
L19	375	2,5	80	120	125	5-6	0,5	7,5	5	yes
L19	500	2,5	80	120	0	8	1	6,3	5	yes
L18	375	2,5	66,5	120	125	5-6	1	8	6	yes
L18	375	2,5	66,5	140	125	6	1	7	6	yes
L18	375	3	80	100	125	5-6	4	15	6	yes
L18	500	3	80	100	0	5-6	4	16	6	yes
L18	500	3	80	80	0	6	6	20	6	yes
L19	500	3	80	80	0	6	3,5	14	6	yes
SF18	500	3	80	80	0	5	3	12,5	6	yes
SF19	500	3	80	80	0	6	2	12	6	no

Table S4

3D printing tests of hydrogels containing (ALG 3% w/v), CaCO₃ (80mM), HA and GDL at various concentrations. Formulations provided the best results are highlighted in light-blue; components volumes refer to 1 mL of activated formulation. N/D is not detected.

HA* %	[GDL]** mM	pH (ALG+HA+CaCO ₃)	Number of hydrogel printed layers	Printing time (min)	Curing time (days at 2°C)	Self cross- linking
10	140	8/9	4	13	4	yes
10	120	8/9	4	16	4	yes
10	100	8/9	5	15	4	yes
10	80	8/9	6	18	4	yes
20	140	8/9	4	13	4	yes
20	120	8/9	4	15	4	yes
20	100	8/9	5	15	4	yes
20	80	8/9	6	20	4	No
30	140	N/D	4	15	4	yes
30	120	N/D	N/D	N/D	N/D	N/D
30	100	N/D	7	23	4	partial
30	80	N/D	8,5	25	4	partial
40	140	7/8	5	18	4	partial
40	120	7/8	5	22	4	yes
40	100	7/8	8	25	4	partial
40	80	7/8	16	35	4	partial
40	140	7	10	>30	6	yes
40	140	7	12	>30	6	yes
40	100	7	12	>30	6	yes
40	100	7	15	>30	6	partial

* HA concentration is expressed in terms of weight percentage referred to ALG (w/w).

**Solubilized in 500 µl of water.

Table S5

Proteins SRM transitions with their m/z values monitored for LC-MS/MS analysis and the cellular location; sequences chose for semi quantitative evaluation are in light-blue.

Protein	Location	Peptide Modified Sequence	Precursor m/z	Product m/z	Dual time	Fragment Ion
AKT	cytoplasm, nucleus, cell membrane	DEVAHTLTENR	6433	870	20	y7
		FYGAEIVSALDYLHSEK	971	1005	20	y8
		RPHFPQFSYSASGTA	827	998	20	b8
		VTMNEFEYLK	637	699	20	y5
CASP-3	cytoplasm	IPVEADFLYAYSTAPGYYSWR	1235	9288	20	y7
		LEFMHILTR	580	639	20	y5
		VATEFESFSFDATFHAK	967	998	20	b9
		VATEFESFSFDATFHAK	967	1171	20	y10
Collagen I	extracellular matrix	EGAPGAEGSPGR	543	258	20	b3
		GANGAPGIAGAPGFPGAR	769	837	20	b11
		GSAGPPGATGFPGAAGR	714	753	20	b10
		GSPGEAGRPGEAGLPGAK	804	896	20	y10
		NSVAYMDQQTGNLK	7855	903	20	y8
		NSVAYMDQQTGNLK	7855	788	20	y7
		STGGISVPGPMGPSGR	777	12823	20	b15
Engrailed-1	nucleus	EQPPPQLLVAAAAAR	7311	355	20	b3
		YPEHGNPAILLMGSANGGPVVK	11111	698	20	b6
		YSDRPSSGPR	561	522	20	b4
FAK	plasma membrane, cytoskeleton, nucleus	FLQEALTMR	5555	589	20	b5
		GIGQVLPTLMEER	790	568	20	b6
		GIGQVLPTLMEER	790	11488	20	b11
		LAQQYVMTSLQQEYK	915	935	20	b8
		LLNSDLGELINK	6655	7866	20	y7
		LVNGTSQSFIIRPQK	8444	9344	20	b9
		QMLTAAHALAVDAK	720	824	20	b8
		VFHYFESNSEPTTWASIR	1143	1338	20	b11
HGF	extracellular region or secreted	DYEAWLGIHDVHGR	834	1004	20	y9
		EFGHEFDLYENK	764	781	20	y6
		NYMGNLSQTR	592	780	20	b7
IGF1	extracellular region or secreted	GFYFNKPTGYGSSSR	834	955	20	b8
		GFYFNKPTGYGSSSR	834	1012	20	b9
		YQPPSTNK	468	573	20	b5
IL-1R-1	extracellular region or secreted, cellular membrane	HQLLSPATK	498	729	20	y7
		STLITVLNISEIESR	838	956	20	b9
		TPVSTEQASR	538	590	20	y5
		VLPEVLEK	464	651	20	b6
		VLPEVLEK	464	488	20	y4
		WSGDFTQGPQSAK	705	715	20	y7
MMP3	extracellular matrix	DAVDSAVEK	467	648	20	y6
		GIHTLGFPPTVR	648	726	20	b7
		IVNYTPDLPK	580	916	20	b8
		LDSDTLEVMR	590	748	20	y6

		LYEGEADIMISFAVR	857	1123	20	y10
		YLENYYDLK	611	701	20	y5
PDGF subunit A	extracellular region or secreted	AHGVHATK	411	555	20	y5
		TVIYEIPR	496	606	20	b5
		TVIYEIPR	496	677	20	y5
TGF-beta-1	extracellular region or secreted	AQHLQSSR	464	477	20	y4
		DNTLQVDINGFTTGR	826	865	20	y8
		LLAPSDSPEWLSFDVTGVV R	1095	298	20	b3
		QSTHSIYMFNTSELR	981	1014	20	y8
		TIDMELVK	475	488	20	y4
		VEQHVELYQK	637	835	20	b7
		VEQHVELYQK	637	680	20	y5
Thy-1	plasma membrane	HENTSSSIQYEFSLTR	998	743	20	b7
		HVLFGTVGVPEHTYR	856	958	20	y8
		VLYLSAFTSK	565	576	20	b5
		VLYLSAFTSK	565	640	20	y6
TNF-alpha	plasma membrane, extracellular region or secreted	DLSLISPLAQAVR	692	754	20	y7
		DLSLISPLAQAVR	692	629	20	b6
		ETPEGAEAKPWYEPIYLGGVFQLEK	1426	913	20	b9
		TPSDKPVAHVVANPQAEGQLQWLNR	1378	1317	20	b13
Vimentin	nucleus, cytoplasm and cytosol, plasma membrane, cytoskeleton	DGQVINETSQHHDDLE	919	1081	20	y9
		ETNLDSLPLVDTHSK	835	773	20	b7
		ETNLDSLPLVDTHSK	835	984	20	b9
		ETNLDSLPLVDTHSK	835	1097	20	y10

Table S6

Lipids identified in LB18 and LB19 samples. Molecules are grouped on lipid class and the relative abundance ($\pm$ SD) are reported; N/D is not detected value.

Lipid Class	Lipid Molecule	Relative abundance $\pm$ SD in LB18		Relative abundance $\pm$ SD in LB19	
Free Fatty Acids	C16:0	7,60E+07	1,59E+07	8,25E+07	7,93E+06
	C14:0	7,63E+06	3,14E+06	1,01E+07	2,44E+06
	C16:1	1,41E+07	5,33E+06	7,65E+06	1,18E+06
	C17:0	5,52E+06	1,01E+06	4,80E+06	9,97E+05
	C17:0 branched	9,06E+05	2,62E+05	8,49E+05	9,43E+04
	C17:1	7,96E+06	1,15E+06	1,09E+07	6,47E+06
	C18:0	1,92E+07	2,11E+06	2,16E+07	3,49E+06
	C18:1	6,68E+07	1,66E+07	5,87E+07	7,37E+06
	C18:2	1,34E+07	3,75E+06	1,12E+07	3,21E+06
	C18-oxo	1,49E+07	6,10E+06	9,05E+06	8,59E+05
	C19:0 cyc	2,43E+06	4,82E+05	4,69E+06	2,57E+06
	C20:3	1,07E+06	4,22E+05	9,39E+05	1,80E+05
	C20:4	1,72E+07	7,56E+06	1,39E+07	4,09E+06
Triacylglycerols (TAG)	C12:0-TAG 36:0	3,73E+05	2,10E+04	4,78E+05	3,12E+04
	C14:0-TAG 42:0	1,56E+05	1,11E+04	2,19E+05	1,34E+04
	C16:0-TAG 48:0	1,07E+05	9,22E+03	1,16E+05	8,82E+03
	C16:1-TAG 48:2	2,97E+05	3,31E+04	2,81E+05	5,41E+04
	C16:1-TAG 48:3	5,72E+05	5,56E+04	1,13E+06	7,64E+04
	C16:1-TAG 48:1	9,42E+04	8,21E+04	7,12E+04	8,21E+04
	C17:0-TAG 51:0	6,13E+04	5,41E+03	7,68E+04	4,13E+03
	C18:0-TAG 54:0	4,73E+04	2,20E+03	6,39E+04	3,12E+03
	C18:1-TAG 54:1	6,26E+04	2,56E+03	1,13E+05	5,62E+04
	C18:1-TAG 54:2	5,50E+04	4,10E+03	6,81E+04	6,30E+03
	C18:1-TAG 54:3	5,62E+04	1,41E+03	7,56E+04	2,31E+03
	C18:2-TAG 54:4	6,72E+04	8,31E+03	7,02E+04	6,67E+03
	C18:2-TAG 54:5	6,86E+04	5,12E+03	1,13E+05	1,51E+04
	C18:2-TAG 54:6	5,93E+04	4,67E+03	8,00E+04	7,10E+03
Lysylphosphatidylglycerides (LPG)	LPG C14:1	1,92E+04	4,02E+03	1,20E+04	3,31E+03
	LPG C16:1	1,31E+04	2,11E+03	N/D	N/D
	LPG C17:1	3,09E+03	9,93E+02	N/D	N/D
	LPG C18:0	7,05E+03	3,47E+02	1,02E+03	2,09E+02

	LPG C18:1	1,07E+04	4,45E+03	6,66E+03	1,42E+03
	LPG C18:2	1,13E+04	4,86E+03	7,45E+03	3,46E+03
	LPG C20:4	8,76E+03	3,29E+02	N/D	N/D
Phosphatidylglycerides (PG)	PG 14:0-18:0	2,83E+04	1,23E+03	8,33E+03	4,92E+03
	PG 12:0-14:0	7,85E+03	3,94E+03	8,26E+03	1,86E+03
	PG 12:0-16:0	2,32E+04	9,05E+03	1,37E+04	1,50E+03
	PG 12:0-16:1	6,02E+03	2,39E+03	3,43E+03	1,33E+03
	PG 12:0-18:0	1,84E+04	3,65E+03	1,93E+04	8,48E+03
	PG 12:0-18:1	1,57E+04	6,71E+03	N/D	N/D
	PG 12:0-18:2	4,26E+03	1,30E+02	2,79E+03	3,17E+02
	PG 14:0-18:0	6,17E+03	2,19E+03	8,33E+03	4,92E+03
	PG 14:0-18:1	1,82E+05	9,88E+04	3,26E+05	1,02E+04
	PG 14:0-18:2	2,92E+04	1,31E+03	N/D	N/D
	PG 16:0-18:0	8,13E+04	4,36E+04	N/D	N/D
	PG 16:0-18:1	2,25E+05	1,70E+05	N/D	N/D
	PG 16:0-18:2	4,03E+04	1,65E+04	1,85E+05	8,10E+03

Table S7

Summary differences between clusters A and B identified by LC-MS/MS SRM analysis on fibroblast cells grown with *L. bulgaricus* derivatives. Cluster A is in light-blue while cluster B is in light-orange; for each cluster the most expressed proteins are listed.

Cluster A		Cluster B	
Biomarkers involved in ECM modelling			
IGF1	Growth factor that acts on fibroblasts, promotes the synthesis of collagenase	Collagen I	Extracellular matrix component
MMP3	Endopeptidase, degrades components of the extracellular matrix (including collagen)	TGF-beta-1	Stimulates chemotaxis and fibroblast proliferation, as well as collagen deposition
Biomarkers involved in proliferation process			
TNF-alpha	Mitogenic factor, stimulates fibroblast proliferation	IL-1R-1	Stimulation of this receptor increases the expression of the proliferative WNT gene
		PDGF subunit A	It stimulates the proliferation of fibroblasts with an autocrine stimulation mechanism
Biomarkers involved in healing process			
HGF	Accelerates re-epithelialization in rats, secreted by activated fibroblasts		
Vimentin	Involved in wound healing, associated with fibroblasts growth		
Engrailed-1	Factor associated with scar formation		
Biomarkers involved in apoptosis process			
CASP-3	Factor involved in apoptosis process	AKT	Factor involved in resistance to apoptosis
		Thy-1	Factor involved in proliferation suppression and apoptosis
		FADK	Antiapoptotic factor and involved in cell adhesion and migration processes



Fig. S1. ATR FT-IR spectra of r-EPS from the growth medium of LB18 (red) and LB19 (purple).

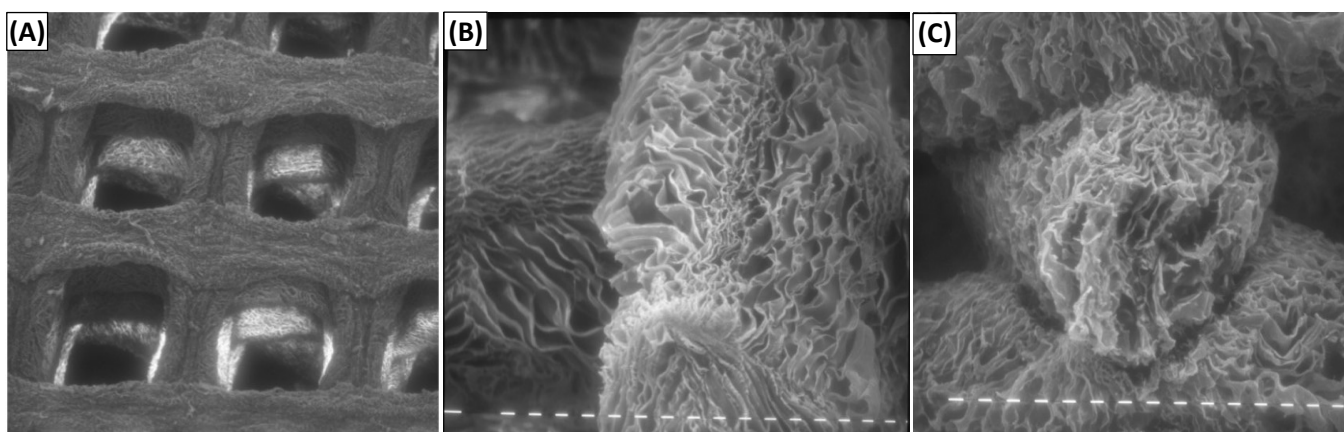


Fig. S2. SEM pictures of 3D ALG hydrogels (3% ALG (w/v), 80 mM CaCO_3 , 80 mM GDL (aqueous solution). Calcium alginate hydrogel (magnification 40 X) (A); calcium alginate hydrogel surface (B) and cross section (C) (magnification 640 X).

CHAPTER X: Conclusions and Future Perspectives

LC-MS is among the most versatile powerful analytical technique used for applications in various sector of life sciences.

Here, targeted and untargeted LC-MS/MS approaches have been successfully developed, validated and applied for the study of protein biomarkers of biomedical or clinical interest and metabolite profiles.

Going into details, LC-MS has been used for the characterization of protein and hormonal profile of rat thyroid cell models within the European Horizon 2020 SCRENEED project. This project deals with the development of *in vitro* 3D assays to screen the effect of low dose of EDs on thyroid function; overcoming current tests limitations. Our unit has set up two rat thyroid cell models, the first exploits the FRTL-5 cell line, whose protein profile has been demonstrated to be comparable with that of primary adult rat thyrocytes; the second cell model, submitted to a patent and positively evaluated by the JRC, concerns rat thyroid organoids used for the screening of EDs at low concentrations, whose proteomic analysis has allowed to obtain the first reference molecular panels, introducing groundwork for a new rapid and reproducible diagnostic *in vitro* assay.

In the context of regenerative medicines of natural scaffolds involved in engineering of human organs, the decellularization effects on pig matrices has been investigated by targeted and untargeted mass spectrometry. Based on the detergent involved (SDS or TX100) in decellularization, porcine liver, kidney, pancreas, and skin, showed relevant quantitative differences on key extracellular and/or basal membrane proteins providing hints for optimized preparation of these 3D matrices to be involved as tools for eventual recellularization of 3D bioartificial human organs.

LC-ESI-MS/MS effective new methods have been developed for the detection of active compounds in food (IGF-1 in milk) and in biological matrices (24-, 25- and 27-OHC in serum and brain). By operating under SRM conditions IGF-1 protein has been selective detected in all kinds of milk samples at the levels of interest with a simple and reliable procedure, providing an accurate detection method that does not required expensive high resolution MS instruments, thus being useful and easily applicable for routine quantitative analysis in food control. Using the same analytical instrumentation, 24-, 25- and 27-OHC were quantified in murine serum, while only 24- and 27-OHC were detectable in the murine brain, all at comparable levels between experimental groups; suggesting that recovery of lipid homeostasis appears to be only minimally involved in the effects caused by the deletion of the possible new drug target, PCSK9, implicated in neurodegenerative diseases as Alzheimer's disease.

The other main topic covered in this PhD thesis involves the 3D printing of polysaccharide biomaterials. Here, a homemade 3D printer based on an extrusion system has been adopted to fabricate alginate-based 3D hydrogel scaffolds with tunable and controlled properties for applications in regenerative medicine and drug delivery. Two crosslinking techniques have been opportunely exploited, the external gelation in aqueous solutions with calcium ions and the internal gelation using, calcium carbonate and d-glucono- δ -lactone.

Alginate hydrogels were successfully produced by extrusion-based 3D printing for applications in regenerative medicine and drug delivery. In this frame targeted LC-MS has been involved to study the effect of these biomaterials in contact with cells in *in vitro* experiment. 3D printed silver nanoparticles-alginate-nanocrystalline cellulose hydrogels were explored as new active materials for clinical applications due to the antimicrobial activity provided efficacy towards both Gram⁺ and Gram⁻ bacteria. In addition, targeted MS analysis of protein biomarkers highlighted silver nanoparticles effects on HEPG2 cells used as *in vitro* cell model. A LC-MS targeted approach was also exploited to investigate the effects of cell-biomaterial interaction on the profile of selected lipid classes in the context of human fibroblasts grown on alginate 2D films or 3D printed hydrogels. Thus, interesting variations in the cell membrane lipid profile were detected based on the biomaterial's architecture (2D vs 3D) used as substrate for cell cultures. In these two applications 3D printed alginate hydrogels, were crosslinked using the commonly adopted technique of immersion in calcium or iron water solution, a procedure that could be limiting for functionalization with hydrophilic molecules. Thus, internal gelation for alginate hydrogels 3D printing has been exploited and successfully adopted, by fine tuning of calcium carbonate-GDL ratio and scaffold production parameters. In this way 3D hydrogels were functionalized with water soluble actives as Epirubicin-HCl or *Lactobacillus delbrueckii* subs. *bulgaricus* derivatives for applications in drug delivery and wound healing respectively. Finally, by targeted MS proteomics, the cytotoxic potential of EPI loaded scaffold against MCF7 cancer cells was validated, whereas by using the same analytical technique, the potential regenerative effect of *Lactobacillus delbrueckii* subs. *bulgaricus* derivatives on human fibroblast was verified. Taking together, these evidence highlighted the use of internal gelation as promising approach in 3D printing of alginate for preparation of new functionalized biomaterial as advanced therapeutic solutions in biomedical applications.

The aforementioned results, underline how LC-MS in its targeted approach is an analytical technique useful to study protein and metabolic biomarkers; thanks to rather simple sample treatments and not too expensive (a triple quadrupole analyzer was used) instrumentations, targeted LC-MS has made it possible to obtain precise, repeatable and therefore useful and transferable information not only for biomedical research but also for routine applications. However, the continuous analytical

technologies improvement, suggests the imminent affirmation of DIA approach in MS, and the LC-ESI system miniaturization, allowing accurate analyzes using minimum sample quantities. This is accompanied by developments in the world of biomaterials and 3D-4D printing, towards the creation of smart hydrogels capable, for example, of fine tuning their properties over time based on specific stimuli.

Therefore, personalized precision medicine, increasingly within everyone's reach, based on multidisciplinary, multi-omics approaches could be the flagship of a near future of personalized therapies and new diagnostic tests.

APPENDIX: Dissemination of research activity

Publications

- Bergonzi, C.; Remaggi, G.; Graiff, C.; Bergamonti, L.; Potenza, M.; Ossiprandi, MC.; Zanolli, I.; Bernini, F.; Bettini, R.; Elviri, L. Three-Dimensional (3D) Printed silver nanoparticles/alginate/nanocrystalline cellulose hydrogels: study of the antimicrobial and cytotoxicity efficacy; *Nanomaterials*, 10, 844; 2020
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- Vilella, A.; Bodria, M.; Papotti, B.; Zanolli, I.; Zimetti, F.; Remaggi, G.; Elviri, L.; Poti, F.; Ferri, N.; Lupo, MG.; Panighel, G.; Daini, E.; Vandini, E.; Zoli, M.; Giuliani, D.; Berini, F.; PCSK9 ablation attenuates A β pathology, neuroinflammation and cognitive dysfunctions in 5XFAD mice; *Molecular Neurodegeneration* (prepared for submission); 2023

- Remaggi, G.; Bancalari, E.; Bottari, B.; Neviani, E.; Catanzano, O.; Elviri, L. *Lactobacillus delbrueckii* subs. *bulgaricus* derivatives for 3D printed alginate/ hyaluronic acid self-crosslinking hydrogels: manufacturing and wound healing potential; *International Journal of Biological Macromolecules* (submitted for publication); 2023
- Zaccarelli, A.; Remaggi, G.; Elviri, L. 3D printed biopolymers for medical applications and devices; *Elsevier* 2023, (chapter accepted for publication); 2022
- Remaggi, G.; Zaccarelli, A.; Elviri, L. Polysaccharide-based materials for the development of point-of-care devices; *Springer* 2023, (chapter accepted for publication); 2022

Congress/Conference Participations

Posters:

- Preparation of decellularized rat thyroids for tissue engineering applications: a mass spectrometry characterization; *Bioanalytical day: from molecules to devices-Parma (Italy)*; 2019
- 3D organoids for in vitro screening of Endocrine Disrupting Chemicals: a mass spectrometry study of matrix proteins using different decellularization detergents; *ULLA Summer School (online)*; 2021
- Development and optimization of alginate-based-self-crosslinking inks for 3D extrusion-based cryoprinting; *ULLA Summer School-Uppsala (Sweden)*; 2022
- An innovative European Joint Research Center-submitted *in vitro* assay for screening Endocrine Disruptors effects on thyroid cell function: Horizon Project SCREENED #825745; *Research Day DIMEC-Parma (Italy)*; 2022

Orals:

- Polymeric TiO₂-biomaterials with photocatalytic activities for drug degradation in aqueous environment; *SIFB (online)*; 2021
- Mass spectrometry characterization of FRTL5 thyroid cells used as model system for colonization of either decellularized or engineered 3D organoids; *Bioanalytical day (online)*; 2021
- Developments in the Horizon 2020 European SCREENED project: proteomic signature of FRTL5 thyroid cells for engineering 3D organoids; *Research Day DIMEC-Parma (Italy)*; 2021

- The Horizon European SCREENED project 825475: “High dimensional data” for endocrine disruptor assays on thyroid organoids; *Statistical Seminars-University of Bologna (Italy)*; 2022

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