PNC
Piano nazionale per gli investimenti complementari al PNRR
Ministero dell'Università e della Ricerca

FIT4MEDROB

D10.2.1

RTB2 - METHODS AND PROTOTYPES OF BIOHYBRID INTERFACES FOR TISSUE REGENERATION AND REPAIR #1

Piano Nazionale Complementare (PNC) – Decreto Direttoriale n. 931 del 6 giugno 2022 – Avviso per la concessione di finanziamenti destinati ad iniziative di ricerca per tecnologie e percorsi innovativi in ambito sanitario e assistenziale
Initiative identifier: PNC0000007
Start date: 01/12/2022
Duration: 44 months
Website: www.fit4medrob.it

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Due date of deliverable: 30/11/2023
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Version: 1.1

DISSEMINATION LEVEL OF DELIVERABLE		
PU	Public, fully open, e.g. web	**X**
CO	Confidential, restricted under conditions set out in Partners Agreement	

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History of Changes

VERSION	SUBMISSION DATE	CHANGES
1.0	30/11/2023	First version
1.1	20/09/2024	Renaming of the Deliverable in light of the upcoming reorganization of the Deliverables/Objectives Introduction modified following reviewers' suggestions.

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1 EXECUTIVE SUMMARY

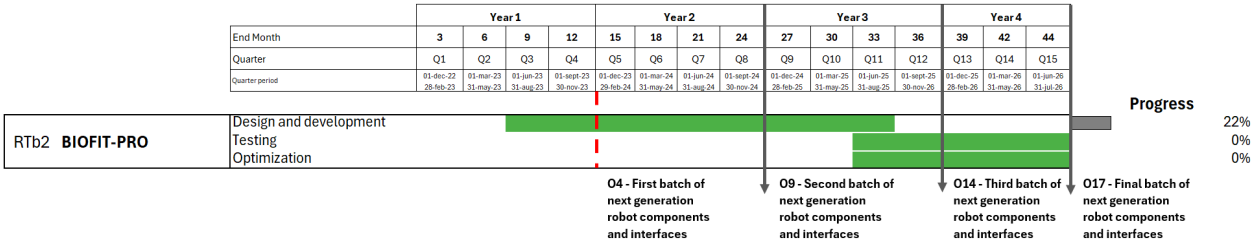
Mission 3 is devoted to support **frontier research topics** pertaining to physical and computational aspects of robot *bodies*, robot intelligence, and interfaces with the patient. Seven research topics (RTa1…RTa4, RTb1..RTb3) are articulated in 19 sub-projects, running in parallel and covering complementary enabling technologies in the field of robotics and biorobotics.

In view of laying the foundations for the next wave of healthcare and personal care robots, this piece of research aims at gaining significant breakthroughs in the fields of (bio)materials interacting with human tissues.

In this context, **Research Topic b2**, targets the clinical need of **Tissue regeneration and repair** and comprises of two complementary subprojects. More in detail the aim of RTb2 is to create and implement innovative approaches aimed at enhancing the integration of implants in intraosseous transcutaneous amputation prostheses (**BIOFIT-PRO**[1]) and guiding the process of tissue regeneration and repair (**NO-GAP**[2]). The resulting biomaterials and scaffolds will be tested in advanced 3D human in vitro models.

BIOFIT-PRO progress

The timeline of BIOFIT-PRO is represented by the green lines in the Gantt below.

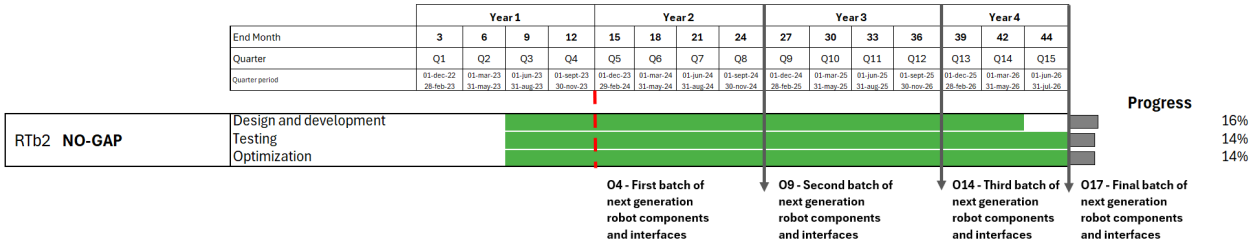


The "**Design and Developmen**t" phase comprises the design of stretchable and 3D-printable hydrogels for both the sealing and bioactive layers, the development of these hydrogels, bilayer assembly, mechanical and biological characterization, and in vitro testing (tasks 3.1, 3.2, 3.3, 3.4, 3.5). This phase progress is **22%**. Key accomplishments include definition of the best suited approach to promote the integration of soft tissue with intraosseous transcutaneous amputation prosthesis (lower limb amputation), definition of device configuration, materials and technologies to be adopted. The "**Testing**" (task 3.6), and the "**Optimization**" phase (task 3.7) have not yet began as reported in the Gantt chart.

There are no deviations on the original plan and the research is progressing as originally foreseen.

NO-GAP progress

The timeline of NOGAP is represented by the green lines in the Gantt below.



The "**Design and Development**" phase includes the selection of protocols for 3D culturing of motor neurons, the design of smart biomaterials to stimulate nerve growth, and the biofabrication of the nerve guide (tasks 4.1, 4.2,

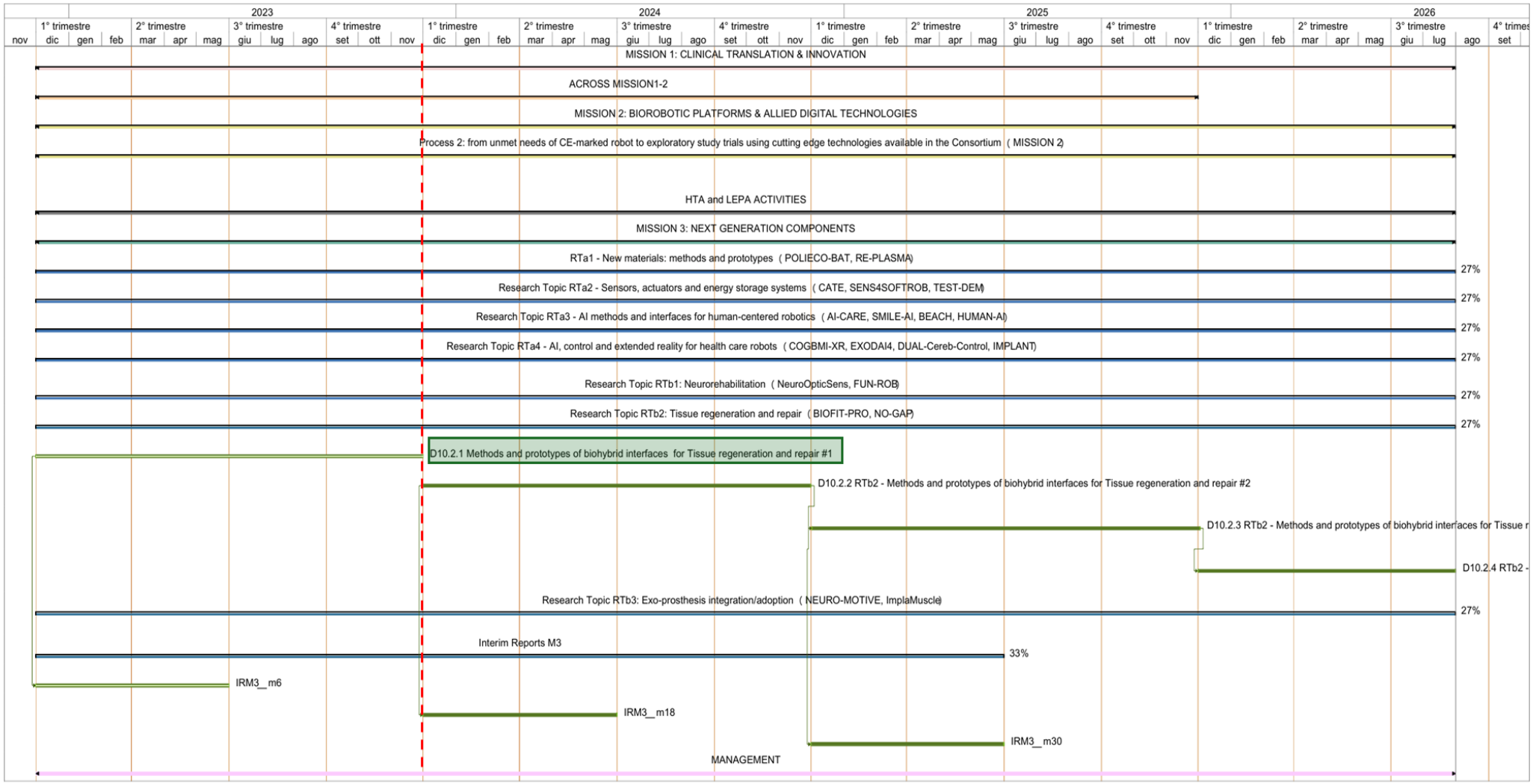
[1] **PIs**: F. Scalera (CNR). F. Gervaso, A. Polini (CNR); P. Netti, F. Urciuolo (UNINA); E. Gruppioni (INAIL).

[2] **PIs**: F. Gervaso (CNR) Polini (CNR); L. Pastorino (UNIGE); C. Chisari (UNIPI).

4.3). The progress of this phase is currently **16%**. Key accomplishments during this phase include the definition of the best suited approach to promote the regeneration of long-gap peripheral nerve injuries, the definition of the biomaterial to be used and the optimization of the protocol for its modification with peptidic sequencing promoting neurogenesis and characterization of the functionalized biomaterial. The progress of the "**Testing**" and "**Optimization**" phases (task 4.4) are currently **14%**. Key accomplishments include the design and microfabrication of a microfluidic platform to test in vitro the ability of nerve to regenerate under different (three) chemical stimuli.

There are no deviations on the original plan and the research is progressing as originally foreseen.

Overall, BIOFIT-PRO and NO-GAP will contribute to Fit4MedRob by developing advanced biohybrid interfaces for improved prosthetic integration and innovative nerve regeneration solutions. These technologies will reduce complications, promote tissue healing, and accelerate nerve repair, directly advancing Fit4MedRob's goal of integrating cutting-edge bioengineering solutions into clinical medical robotics applications.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MISSION 1: CLINICAL TRANSLATION & INNOVATION
ACROSS MISSION1-2
MISSION 2: BIOROBOTIC PLATFORMS & ALLIED DIGITAL TECHNOLOGIES
Process 2: from unmet needs of CE-marked robot to exploratory study trials using cutting edge technologies available in the Consortium (MISSION 2)
HTA and LEPA ACTIVITIES
MISSION 3: NEXT GENERATION COMPONENTS
RTa1 - New materials: methods and prototypes (POLIECO-BAT, RE-PLASMA)
27%
Research Topic RTa2 - Sensors, actuators and energy storage systems (CATE, SENS4SOFTROB, TEST-DEM)
27%
Research Topic RTa3 - AI methods and interfaces for human-centered robotics (AI-CARE, SMILE-AI, BEACH, HUMAN-AI)
27%
Research Topic RTa4 - AI, control and extended reality for health care robots (COGBMI-XR, EXODAI4, DUAL-Cereb-Control, IMPLANT)
27%
Research Topic RTb1: Neurorehabilitation (NeuroOpticSens, FUN-ROB)
27%
Research Topic RTb2: Tissue regeneration and repair (BIOFIT-PRO, NO-GAP)
27%
D10.2.1 Methods and prototypes of biohybrid interfaces for Tissue regeneration and repair #1
D10.2.2 RTb2 - Methods and prototypes of biohybrid interfaces for Tissue regeneration and repair #2
D10.2.3 RTb2 - Methods and prototypes of biohybrid interfaces for Tissue r
D10.2.4 RTb2 -
Research Topic RTb3: Exo-prosthesis integration/adoption (NEURO-MOTIVE, ImplaMuscle)
27%
Interim Reports M3
33%
IRM3__m6
IRM3__m18
IRM3__m30
MANAGEMENT

2 BIOFIT-PRO: BIOACTIVE SCAFFOLDS FOR SKIN-IMPLANTS INTEGRATION IN INTRAOSSEOUS TRANSCUTANEOUS AMPUTATION PROSTHESES

This Section provides an overview of **BIOFIT-PRO**, which is focused on the design, fabrication and validation of bioactive scaffolds for skin-implants integration in intraosseous transcutaneous amputation prostheses. During this reporting period, several meetings were held to align the clinical needs with the researchers' expertise. Considering the specific requirements highlighted by Dr Emanuele Gruppioni from **INAIL** regarding the integration of soft tissues in lower limb prostheses, a proposal for SubActivity#3 was formulated by **CNR NANOTEC** with the support of **UNINA** and under the advises of **INAIL** (**Figure 1**).

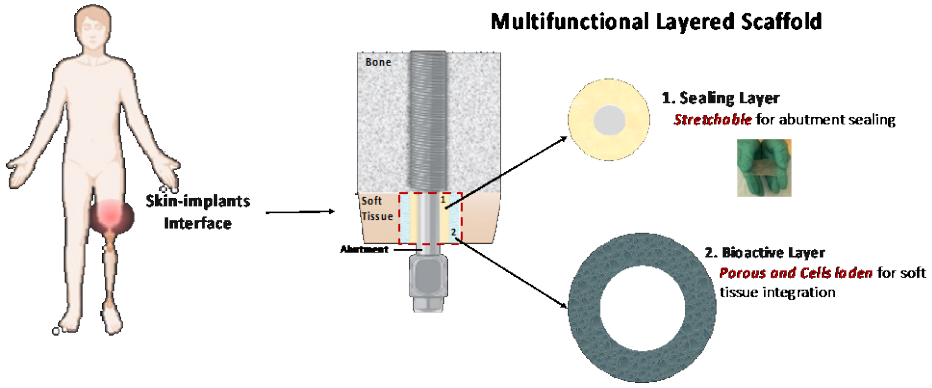

Fig. 1 - Graphical abstract of SubActivity#3.

The clinical need refers to the integration of soft tissue with the intraosseous transcutaneous amputation prosthesis, in cases of lower limb amputation. These prostheses consist of a titanium alloy implant that is osseointegrated to the bone and penetrates the skin, establishing a direct skeletal connection for artificial limbs. In normal conditions, the implant osseointegration process is highly effective, ensuring a strong connection between the implant and the surrounding bone tissue. However, this system creates a breach (the stoma) in the skins' protective barrier, essential to prevent infections. In addition, during the initial stages of rehabilitation, exudate tends to make soft tissues adhere to the implant's *abutment*, connecting element between the fixture and the prosthetic limb, leading to painful "tearing" of the tissues themselves. Even when the situation stabilizes, friction between the soft tissues and the abutment lead to frequently local inflammation. To address these issues, a comprehensive, multidisciplinary approach is crucial. Strategies are needed to prevent stoma infections, improve exudate management, and mitigate negative effects on soft tissue integration with the implant's abutment. Therefore, the primary focus of **SubActivity#3** is to pioneer innovative tissue engineering solutions that enhance the integration of soft tissue with the implant.

The main achievement of this reporting period is the definition of the work plan for SubActivity#3. To this aim, CNR NANOTEC proposed the development and characterization of cell-laden stretchable scaffolds with a dual function: providing a sealing against infections and promoting interaction between the abutment and soft tissue. Therefore, it has been considered to develop a Bilayer scaffold with a first layer adhering to the abutment (Sealing layer), stretchable and acting as a seal, and a second more porous layer (Bioactive layer), interacting with soft tissue. For the second layer, the option to load it with cells (like fibroblasts) or leave it cell-free will be considered. The research activity has been identified and organized in subtasks as reported below:

ST 3.1. Design of stretchable and 3D printable hydrogels for Sealing Layer and Bioactive Layer.

ST 3.2. Development of hydrogels for Sealing Layer and physicochemical characterization.

ST 3.3. Development of hydrogels for Bioactive Layer (cell-laden hydrogels and cell free). Physicochemical characterization and in vitro testing (including live/dead assay, proliferation test etc).

ST 3.4. Development of Microneedles Layer.

ST 3.5. Development of Bilayer, Mechanical and biological characterization.

ST 3.6. Coupling of Bilayer with an abutment-like support (Bilayer-abutment) and mechanical characterization.
ST 3.7. 3D human skin models for in vitro testing of Bilayer-Abutment.

As a result, the device configuration (as shown in **Figure 2**), materials, and technologies to be used have been identified in **Task 3.1 (M7-M12) (see also** full description in **Annex C)**. In the literature on soft tissue engineering, various approaches have been explored, including naturally derived engineered scaffolds such as collagen/gelatin and hyaluronic acid [17], as well as synthetic scaffolds like poly (lactic-co-glycolic acid), poly(ethylene glycol) diacrylates [18], and Polyvinyl Alcohol (PVA) [19].

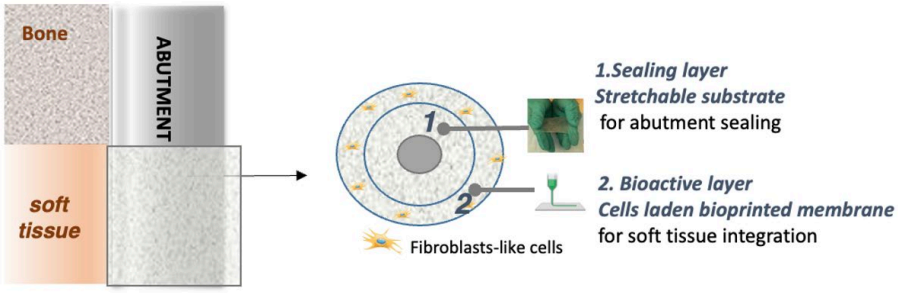

Fig. 2 - Schematic representation of a bilayer scaffold.

In the context of **Subactivity#3**, the potential of a combination of synthetic polymers like PVA and PEGDA, along with natural polymers such as GelMA [20], to develop an injectable and stretchable scaffold with robust mechanical properties will be explored. The developed hydrogels will be processed by 3D fabrication techniques (bioprinting, DLP 3D printing, freeze-drying,) to produce 3D structures showing morphology compatible with the ECM of human tissues and designed to be integrate to intraosseous transcutaneous amputation prostheses already available on the market. Cryopolymerization will be also evaluated as scaffold synthesis technique. Cryogels, infact, possess a macro-porous structure and more robust mechanical characteristics when compared to traditional hydrogels [21]. Cryogels are particularly advantageous as shape recovery material [22], much like other injectable hydrogels [23]. The combination of cryogels with 3D printing, has the potential to explore new possibilities in reconstructive soft tissue surgery.
The physicochemical, rheological with strain, frequency and time sweep tests, and mechanical properties with uniaxial tests of each layer will be analyzed. These characterizations, along with morphological observations by SEM, will allow us to modulate and optimize the mechanical properties and in vitro stability of the scaffolds. Biological characterization of cell-laden hydrogels and micro-macro-structured scaffolds (through live/dead assays, proliferation tests, etc.) will also be performed.
Once the two layers are characterized, they will be coupled onto abutment-like surfaces. Mechanical characterization, using a peel test, will be employed to assess the adhesion capacity.
An advanced *in vitro* testing platform to study the interaction between the *abutment,* scaffold and human tissue will be developed by **UNINA**. This *in vitro* platform will be constituted by a functional 3D full thickness human skin equivalent developed at UNINA: the epidermal layer consists of well-differentiated, properly stratified keratinocyte layers, while the dermis is formed by fibroblasts embedded in their own extracellular matrix. The group have already used this model to study the ECM remodeling after a mechanical damage demonstrating the possibility to study ECM remodeling and re-epithelization [24]. Indeed, the presence of such endogenous ECM allows to study tissue response at cellular and extracellular levels under different conditions.
In the 3D human skin equivalent scaffolds will be inserted coupled to an abutment-like support. After the insertion, it will be possible to study the host's response in terms of epidermis/scaffold interface and the formation of fibrotic tissue at interface dermis/scaffold. Moreover, the space between the scaffold and the 3D human skin can be filled with porous microparticles acting as promoters of neo-tissue ingrowth.

3 NOGAP: DEVELOPMENT OF A MULTI-FUNCTIONAL NERVE GUIDE FOR LONG GAP NERVE REGENERATION

Spontaneous axonal regeneration in peripheral nerve injuries has been seen in small gaps [25]. However, regenerated nerve function is restricted, particularly in long-gaps (> 30 mm). Among current treatments, autologous superficial cutaneous nerves are known as the gold standard for bridging the nerve gap but does not represent an acceptable solution. Therefore, long-gap peripheral nerve injuries (> 30 mm) still need a solution. In order to propose a successful solution that could speed up the peripheral nerve regeneration process, that represents a very challenging task in the neuro-rehabilitation field, a scientific discussion was started that involved all the partners of Activity 10. After several meetings, **CNR-NANOTEC (Francesca Gervaso, Alessandro Polini, Antonio Turco, Francesco Ferrara)** with the support of **UniGE (Laura Pastorino, Michela Chiappalone, Sergio Martinoia, Donatella Di Lisa, Andrea Andolfi)** and under the advice of **Azienda Ospedaliera Universitaria Pisana (Carmelo Chisari, Stefania Dalise)** defined a workplan that included different research aspects. More in detail, to significantly speed-up the process of axon regeneration, the solution we propose within subactivity#4 consists in combining several stimuli within a unique device. In particular, the partners involved in **SubActivity#4** will work synergically on the following main topics, as graphically represented in **Figure 3**:

- Design of smart biomaterials.
- Integration of iPS cells within the nerve guide.
- Enrichment with grow factors (e.g., NGF, BDNF) in a constant concentration or with a specific gradient along the nerve guide.

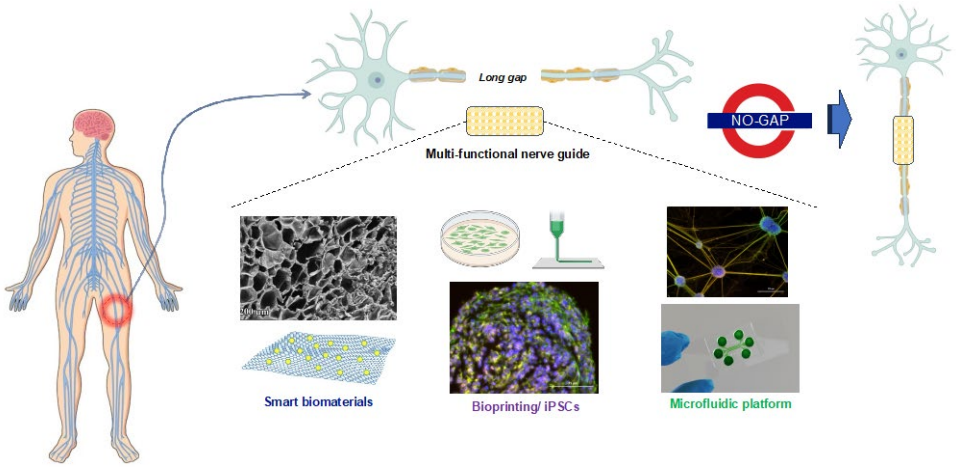

Fig. 3 - Graphical abstract of SubActivity#4.

To achieve the final goal of **SubActivity#4**, the research activity has been divided in subtasks (ST) listed below:

ST 4.1. Protocols for 3D culturing of motor neurons.
ST 4.2. Design of smart biomaterials for nerve growth stimulation.
ST 4.3. Nerve guide biofabrication.
ST 4.4. Advanced microfluidic *in vitro* models for nerve regeneration study.

STs 4.1-4.3 are focused on the development of the long-gap nerve regeneration guide, while ST4.4 aims at developing advanced *in vitro* models as platform to test the proposed solution preliminarily to animal testing.

3.1.1 Sub-task 4.1. Protocols for 3D culturing of motor neurons

The activity of **ST 4.1** focuses on the development of Extra Cellular Matrix (ECM)-like hydrogels for motor neurons 3D culture. Polysaccharide-based polymers, i.e., chitosan (Ch), have been selected as starting materials because of their higher affinity to physiological ECM, their availability and low cost. To develop hydrogels suitable for bioprinting, we focused on stimuli responsive materials, and, on thermoresponsive Ch-based hydrogels. We designed a new chitosan-based thermoresponsive hydrogel by functionalizing Ch chains with bioactive motifs

present in the ECM, specifically the pentapeptide epitope consisting of IKVAV of laminin which is found to be actively involved in different biological activities such as promoting cell adhesion, neurite outgrowth, angiogenesis, and collagenase IV production. The successful functionalisation of Ch polymeric chains with IKVAV was verified through various assays and analyses. The resulting hybrid hydrogel will be characterized in terms of morphology (scanning electron microscopy, SEM), swelling and stability/degradation tests in physiological conditions (gravimetric measurements). As next steps, biological tests will be performed to evaluate the IKVAV effect on cell process and behaviour.

3.1.2 Sub-task 4.4. Advanced microfluidic in vitro models for nerve regeneration study

ST 4.4. Advanced microfluidic *in vitro* models for nerve regeneration study.

The activity of **ST 4.4** focuses on development of microfluidic platforms to test the ability of nerve to regenerate both spontaneously and by means of different strategies, such as *(i)* nerve growth factor administration, *(ii)* iPSCs addition in the gap region, *(iii)* use of smart biomaterials. Microfluidic devices allow for precise control over factors affecting distinct neuronal regions, compartmentalized by ad hoc designed microchannels and microchambers, making the study of neuronal injury (applied mechanically or chemically) and axon degeneration feasible [Methods Mol Biol. 2020:2143:133-144.]. These devices present several advantages over the conventional Campenot chambers. Among them, the use of transparent materials (i.e., PDMS) guarantees high efficiency, good biological compatibility, and ability to integrate with other characterization assays, such as electrophysiology and high-resolution microscopy. Although in vivo models (e.g., sciatic nerve transection, optic nerve crush, and lateral fluid percussion) represent established methods for recapitulating neuron degeneration after trauma, they fail in applying insults locally (i.e., soma versus axon) and accurately assessing the biochemical events associated with degeneration in specific subcellular portions. In the first 6 months of activity a three channels device, able to allow the competitive administration of different stimuli, was designed and microfabricated, starting from a preliminary device developed in our group (ref. Sci Rep).

Microfluidic devices were designed and fabricated by optical lithography and polydimethylsiloxane (PDMS) replica molding [8], as described in detail in **Annex D**. Three different perfusable compartments (500 µm wide, 6 mm long), having distinct inlets and outlets but interconnected through a series of narrow microchannels (10 µm wide, 250 µm long), were considered for culturing/administrating different cell types and/or biological molecules and physical stimuli. The fabrication process consisted of two subsequent lithographic steps for obtaining a photoresist/silicon mold. First, SU-8 2005 was spin coated on silicon wafers and exposed in a mask aligner apparatus (SUSS MA6) in order to achieve a target thickness of 5 µm, corresponding to the height of the interconnecting microchannels in the final PDMS devices. Subsequently, a SU-8 2050 lithography process was optimized to build the remaining larger channels and inlets/outlets chambers aligned to the pristine narrow microchannels. The final molds were used for generating PDMS replicas by soft lithography. We treated PDMS replicas with oxygen plasma (200 sccm, 100 W, 30''), placed the patterned side on a glass microscopy slide to form covalent –O-Si-O-bonds between the two surfaces and finalized the bonding procedure by thermal treatment (30' at 75°C). This procedure led to the formation of an irreversible bonding between PDMS and glass surfaces. In the newly designed mold, the cross microchannel length was reduced to 50 µm, the minimum feature we could achieve with our approach. This was intended to have a shorter microchannel for axon extension without axon-Schwann cell interaction (in the microchannel there is indeed not enough space for such interaction) and favour such communication in the bigger channel. The devices made from such mold are currently under use in cell culture studies.

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Project identifier: PNC0000007

Start date: 01/12/2022

Duration: 44 months

Website: www.fit4medrob.it

ACTIVITY 10– BIOHYBRID INTERFACES AND BIOMATERIALS

SUBACTIVITY#3

RESEARCH TEAMS PERIODIC REPORT, M1-M12

PI: Francesca Scalera
Partner Acronym: CNR NANOTEC
Date: 15/10/2023

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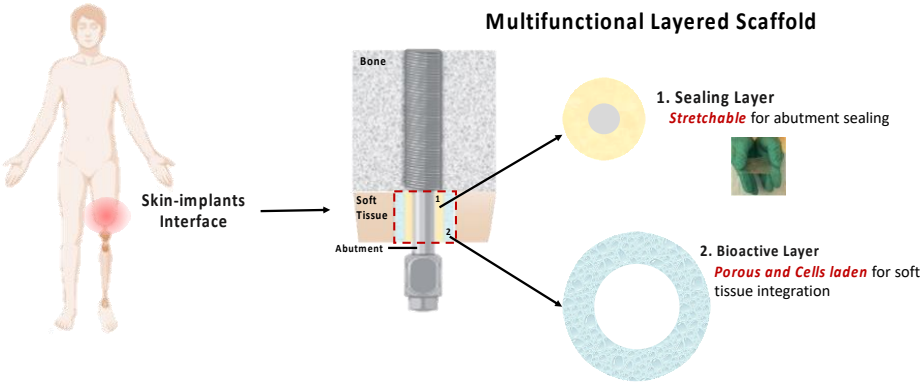
1 Deliverables worked on during this period

- D 10.1 – Subactivity#3: D 10.1 - BIOACTIVE SCAFFOLDS FOR SKIN-IMPLANTS INTEGRATION IN INTRAOSSEOUS TRANSCUTANEOUS AMPUTATION PROSTHESES (BIOFIT-PRO).



Graphical abstract of SubActivity#3.

2 SUBACTIVITY#3: BIOACTIVE SCAFFOLDS FOR SKIN-IMPLANTS INTEGRATION IN INTRAOSSEOUS TRANSCUTANEOUS AMPUTATION PROSTHESES

During this reporting period, several meetings were held to align the clinical needs with the researchers' expertise. Considering the specific requirements highlighted by Dr Emanuele Gruppioni from INAIL regarding the integration of soft tissues in lower limb prostheses, a proposal for subactivity#3 was formulated by **CNR NANOTEC** with the support of **UNINA** and under the advises of **INAIL**.

The clinical need refers to the integration of soft tissue with the intraosseous transcutaneous amputation prosthesis, in cases of lower limb amputation. These prostheses consist of a titanium alloy implant that is osseointegrated to the bone and penetrates the skin, establishing a direct skeletal connection for artificial limbs. In normal conditions, the implant osseointegration process is highly effective, ensuring a strong connection between the implant and the surrounding bone tissue. However, this system creates a breach (the stoma) in the skins' protective barrier, essential to prevent infections. In addition, during the initial stages of rehabilitation, exudate tends to make soft tissues adhere to the implant's *abutment*, connecting element between the fixture and the prosthetic limb, leading to painful "tearing" of the tissues themselves. Even when the situation stabilizes, friction between the soft tissues and the abutment lead to frequently local inflammation.

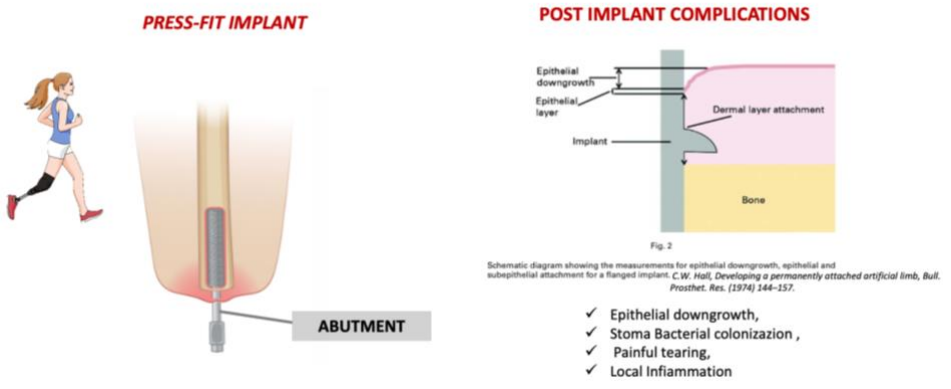


Figure 1. Implant soft tissue complications.

To address these issues, a comprehensive, multidisciplinary approach is crucial. Strategies are needed to prevent stoma infections, improve exudate management, and mitigate negative effects on soft tissue integration with the implant's abutment. Therefore, the primary focus of this research is to pioneer innovative tissue engineering solutions that enhance the integration of soft tissue with the implant.

2.1 ACHIEVEMENTS

To achieve this goal, CNR NANOTEC has proposed the development and characterization of cell-laden stretchable scaffolds with a dual function: providing a sealing against infections and promoting interaction between the abutment and soft tissue. Therefore, it has been considered to develop a Bilayer scaffold with a first layer adhering to the abutment (***Sealing layer***), stretchable and acting as a seal, and a second more porous layer (***Bioactive layer)***, interacting with soft tissue (Fig.2). For the second layer, the option to load it with cells (like fibroblasts) or leave it cell-free will be considered. The main activities have been identified and divided into subtasks, as reported below:

ST 3.1. Design of stretchable and 3D printable hydrogels for Sealing Layer and Bioactive Layer.
ST 3.2. Development of hydrogels for Sealing Layer and physicochemical characterization.
ST 3.3. Development of hydrogels for Bioactive Layer (cell-laden hydrogels and cell free). Physicochemical characterization and in vitro testing (including live/dead assay, proliferation test etc).
ST 3.4. Development of Microneedles Layer.
ST 3.5. Development of Bilayer, Mechanical and biological characterization.
ST 3.6. Coupling of Bilayer with an abutment-like support (Bilayer-abutment) and mechanical characterization.
ST 3.7. 3D human skin models for in vitro testing of Bilayer-Abutment.

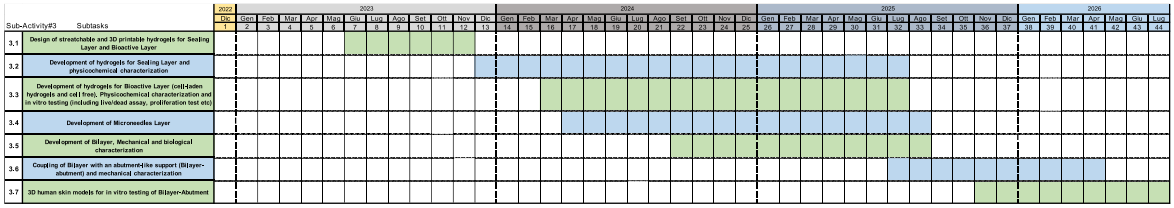


Figure 2. Gantt diagram of SubActivity#3.

As previously indicated, during the period from M7 to M12, focus of this deliverable, the device configuration (as shown in Figure 3), materials, and technologies to be used have been identified in ST 3.1.

In the literature on soft tissue engineering, various approaches have been explored, including naturally derived engineered scaffolds such as collagen/gelatin and hyaluronic acid [1], as well as synthetic scaffolds like poly (lactic-co-glycolic acid), poly(ethylene glycol) diacrylates[2], and Polyvinyl Alcohol (PVA) [3] .

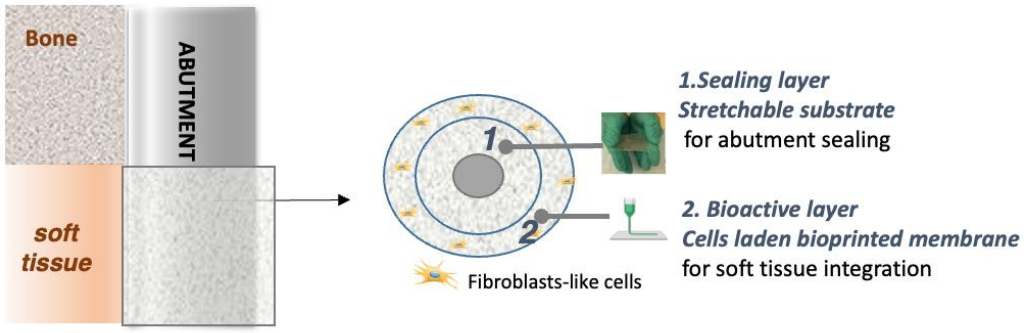


Figure 3. Schematic representation of Bilayer scaffold.

In the context of Subactivity#3, the potential of a combination of synthetic polymers like PVA and PEGDA, along with natural polymers such as GelMA[4], to develop an injectable and stretchable scaffold with robust mechanical properties will be explored. The developed hydrogels will be processed by 3D fabrication techniques (bioprinting, DLP 3D printing, freeze-drying,) to produce 3D structures showing morphology compatible with the ECM of human tissues and designed to be integrate to intraosseous transcutaneous amputation prostheses already available on the market. Cryopolymerization will be also evaluated as scaffold synthesis technique. Cryogels, infact, possess a macro-porous structure and more robust mechanical characteristics when compared to traditional hydrogels [5]. Cryogels are particularly advantageous as shape recovery material[6], much like other injectable hydrogels [7]. The combination of cryogels with 3D printing, has the potential to explore new possibilities in reconstructive soft tissue surgery.

The physicochemical, rheological with strain, frequency and time sweep tests, and mechanical properties with uniaxial tests of each layer will be analyzed. These characterizations, along with morphological observations by SEM, will allow us to modulate and optimize the mechanical properties and in vitro stability of the scaffolds. Biological characterization of cell-laden hydrogels and micro-macro-structured scaffolds (through live/dead assays, proliferation tests, etc.) will also be performed.

Once the two layers are characterized, they will be coupled onto abutment-like surfaces. Mechanical characterization, using a peel test, will be employed to assess the adhesion capacity.

An advanced *in vitro* testing platform to study the interaction between the *abutment,* scaffold and human tissue will be developed by **UNINA**. This *in vitro* platform will be constituted by a functional 3D full thickness human skin equivalent developed at UNINA: the epidermal layer consists of well-differentiated, properly stratified keratinocyte layers, while the dermis is formed by fibroblasts embedded in their own extracellular matrix. The group have already used this model to study the ECM remodeling after a mechanical damage demonstrating the possibility to study ECM remodeling and re-epithelization [8]. Indeed, the presence of such endogenous ECM allows to study tissue response at cellular and extracellular levels under different conditions.

In the 3D human skin equivalent will be inserted the scaffolds coupled to an abutment-like support. After the insertion, it will be possible to study the host's response in terms of epidermis/scaffold interface and the formation of fibrotic tissue at interface dermis/scaffold. Moreover, the space between the scaffold and the 3D human skin can be filled with porous microparticles acting as promoter of neo-tissue ingrowth.

3 DISSEMINATION ACTIVITIES (SCIENTIFIC PAPERS, POP MAGAZINES, TV, ETC.)

- Scalera F, Palazzo B, Scialla S, Gervaso F, **Multilayer scaffold for the repair of massive osteochondral lesions,** *in preparation.*

4 MEETINGS IN THIS PERIOD

List of meetings. Include date, place, participants. If minutes were prepared, please indicate so.

- February 9, 2023-Online meeting. Partecipants: All involved in Activity 10
- February 28, 2023-Online meeting. Partecipants: All involved in Activity 10
- March 8, 2023- Online meeting. Partecipants: F.Scalera, F.Gervaso, A.Polini, L.delMercato from CNR NANOTEC and E. Gruppioni from INAIL
- March 15, 2023 – Meeting in person at CNR NANOTEC Lecce with CNR NANOTEC Lecce researchers on research activity proposals on Fit4MedRob issues.
- May 15, 2023 – Online meeting. Partecipants: All involved in Activity 10
- July 24, 2023- Online meeting. Partecipants: F.Scalera (CNR NANOTEC Lecce) and F.Urciuolo (UNINA) on collaborations on Subactivity3

- September 21-221, 2023Meeting in person at CNR NANOTEC Lecce with all participants in Activity 10.
- October 4, 2023- Online meeting. Partecipants: F.Scalera (CNR NANOTEC Lecce) and F.Urciuolo (UNINA) Subactivity3 .

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Piano Nazionale Complementare (PNC) – Decreto Direttoriale n. 931 del 6 giugno 2022 – Avviso per la concessione di finanziamenti destinati ad iniziative di ricerca per tecnologie e percorsi innovativi in ambito sanitario e assistenziale

Project identifier: PNC0000007

Start date: 01/12/2022

Duration: 44 months

Website: www.fit4medrob.it

ACTIVITY 10– BIOHYBRID INTERFACES AND BIOMATERIALS

SUBACTIVITY#4

RESEARCH TEAMS PERIODIC REPORT, M1-M12

PI: Francesca Gervaso
Partner Acronym: CNR-NANOTEC
Date: 15/10/2023

TABLE OF CONTENTS

1 DELIVERABLES WORKED ON DURING THIS PERIOD

- D 10.1 – Subactivity#4: *Development of a multi-functional nerve guide for long gap nerve regeneration*

The following people have been involved in defining Subactivity#4:

- Francesca Gervaso (CNR)
- Alessandro Polini (CNR)
- Antonio Turco (CNR)
- Francesco Ferrara (CNR)
- Laura Pastorino (UNIGE)
- Michela Chiappalone (UNIGE)
- Sergio Martinoia (UNIGE)
- Donatella Di Lisa (UNIGE)
- Andrea Andolfi (UNIGE)
- Carmelo Chisari (Azienda Ospedaliera Universitaria Pisana)

2. SUBACTIVITY#4: DEVELOPMENT OF A MULTI-FUNCTIONAL NERVE GUIDE FOR LONG GAP NERVE REGENERATION

Spontaneous axonal regeneration in peripheral nerve injuries has been seen in small gaps [1]. However, regenerated nerve function is restricted, particularly in long-gaps (> 30 mm). Among current treatments, autologous superficial cutaneous nerves are known as the gold standard for bridging the nerve gap but does not represent an acceptable solution. Therefore, long-gap peripheral nerve injuries (> 30 mm) still need a solution.

The most recent and innovative studies present in literature include several advanced technological and biotechnological approaches such as *(i)* micropatterning, fiber orientation, *(ii)* addition of Schwann cells within the nerve guide, *(iii)* addition of grow factors (NGF, BDNF,..) in a constant concentration or with gradient along the nerve guide (building blocks, peptide grafting), *(iv)* use of conductive polymers and/or graphene-polymers composites, *(v)* modulation of macrophages phenotypes (M1, M2) [2-4]. In order to propose a successful solution that could speed up the peripheral nerve regeneration process, that represents a very challenging task in the neuro-rehabilitation field, a scientific discussion was started that involved all the partners of Activity 10. After several meetings, CNR-NANOTEC with the support of UniGE and under the advice of @Azienda Ospedaliera Universitaria Pisana defined a workplan that included different research aspects.

More in detail, to significantly speed-up the process of axon regeneration, the solution we propose within Sub-activity#4 consists in combining several stimuli within a unique device.

In particular, the partners involved in this sub-activity will work synergically on the following main topics, as graphically represented in **Fig. 1**:

- Design of smart biomaterials;
- Integration of iPS cells within the nerve guide;
- Enrichment with grow factors (e.g., NGF, BDNF) in a constant concentration or with a specific gradient along the nerve guide.

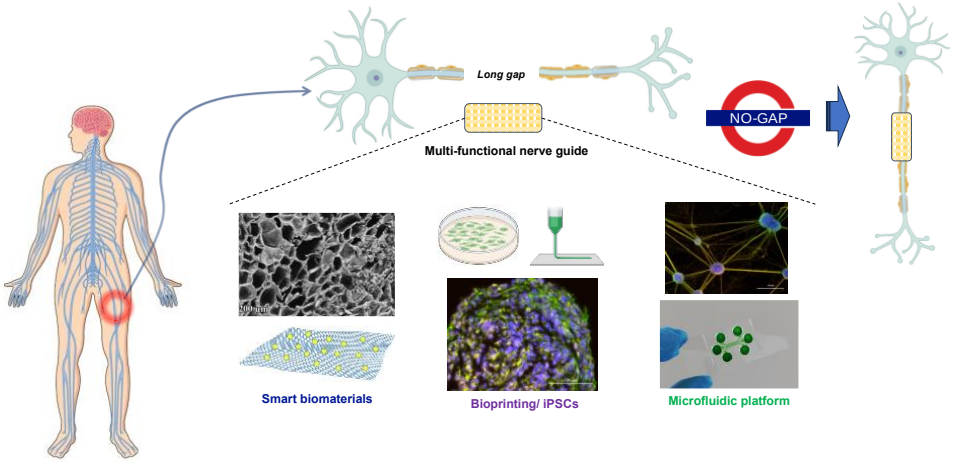

Fig. 1. Graphical Abstract of SubActivity#4.

2.1 ACHIEVEMENTS

In order to achieve the final goal of SubActivity#4, the research activity has been divided in subtasks (ST) listed below:

- **ST 4.1.** Protocols for 3D culturing of motor neurons.
- **ST 4.2.** Design of smart biomaterials for nerve growth stimulation.
- **ST 4.3.** Nerve guide biofabrication.
- **ST 4.4.** Advanced microfluidic *in vitro* models for nerve regeneration study.

STs 4.1-4.3 are focused on the development of the long-gap nerve regeneration guide, while ST4.4 aims at developing advanced *in vitro* models as platform to test the proposed solution preliminarily to animal testing. The workplan of the above reported STs is reported in the GANTT of **Figure 2**.

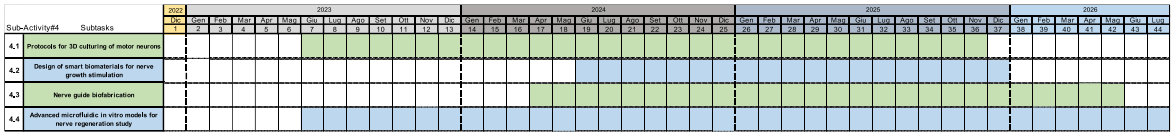

Fig. 2. GANTT of SubActivity#4.

In these months of project, ST 4.1 and ST 4.4 started and the scientific activities performed are described in the following.

ST 4.1. Protocols for 3D culturing of motor neurons.

The activity of ST 4.1. focuses on the development of Extra Cellular Matrix (ECM)-like hydrogels for motor neurons 3D culture. Polysaccharide-based polymers, i.e. chitosan (Ch), have been selected as starting materials because of their higher affinity to physiological ECM, their availability and low cost. In order to develop hydrogels suitable for bioprinting, we focused on stimuli responsive materials, and in particular, on thermoresponsive Ch-based hydrogels.

In a previous study [5], we proposed a thermosensitive chitosan-based hydrogel, prepared by implementing an innovative procedure that allowed to obtain a bubble-free system with optimal chemical–physical characteristics for use in 3D culture systems. Two formulations were assessed, and both allowed to reproduce the mechanical characteristics of “soft” biological tissues, a fundamental requirement for the application of interest. The biological analyses conducted with motor neuron-like NSC-34 cells revealed the ability of our systems to support not only cell growth and viability but also the changes in morphology induced by differentiation.

Within the present Subtask, with the aim of further improve the biological response of motor neuron-like cells embedded in the 3D hydrogel network, we designed a new chitosan-based thermoresponsive hydrogel by functionalizing Ch chains with bioactive motifs present in the ECM. Indeed, many bioactive motifs that are present

in the ECM can help cells anchor, migrate, and differentiate into mature cell types. Most of the peptide motifs that are used in neural tissue engineering are derived from laminin, which is one of the main structural support elements of the brain ECM and promotes growth of neural stem cells *in vitro*. Among the peptide motifs, the pentapeptide epitope consisting of IKVAV of laminin is found to be actively involved in different biological activities such as promoting cell adhesion, neurite outgrowth, angiogenesis and collagenase IV production.

In order to functionalize Ch with IKVAV, the following chemical reactions have been performed (**Fig. 3**). The IKVAV peptide and chitosan were conjugated by thiolation reaction using the cross-linking reagent N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP). 10.5 mL of 2 mg/mL chitosan solution (1% acetate buffer) were added to 700 µg of SPDP to react the NH_2 groups of the chitosan for 4 hours at room temperature. Afterwards, 500 µg of IKVAV were added to SPDP-activated chitosan solution for 24 hours at room temperature. After this reaction, dialysis was done for 48 hours to isolate conjugates.

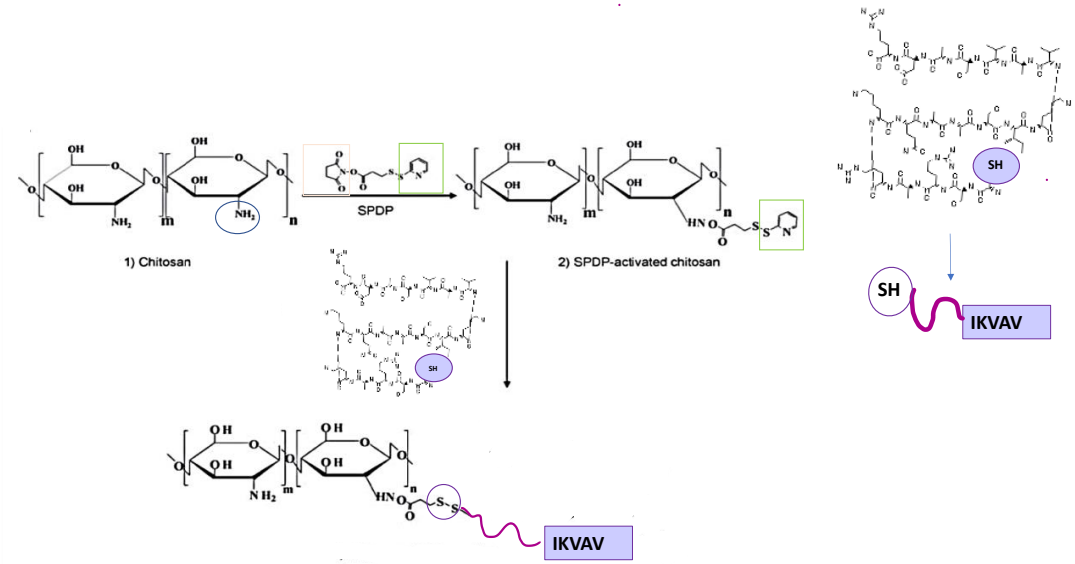

Fig. 3 Reaction mechanism of Chitosan functionalization with the IKVAV small peptide motif.

The successful functionalisation of Ch polymeric chains with IKVAV has been verified through Bicinchoninic Acid (BCA) Protein Assay. BCA assay mainly relies on two reactions. Firstly, the peptide bonds in the protein sample reduce Cu^{2+} ions, in a temperature dependent reaction, from the copper solution to Cu^{+}. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid (BCA) chelate with each Cu^{+} ion, forming a purple-coloured product that strongly absorbs light at a wavelength of 562 nm that is linear for increasing protein concentrations between the range of 0.02 to 2 mg/ml. The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing with protein solutions with known concentrations (**Fig. 4**).

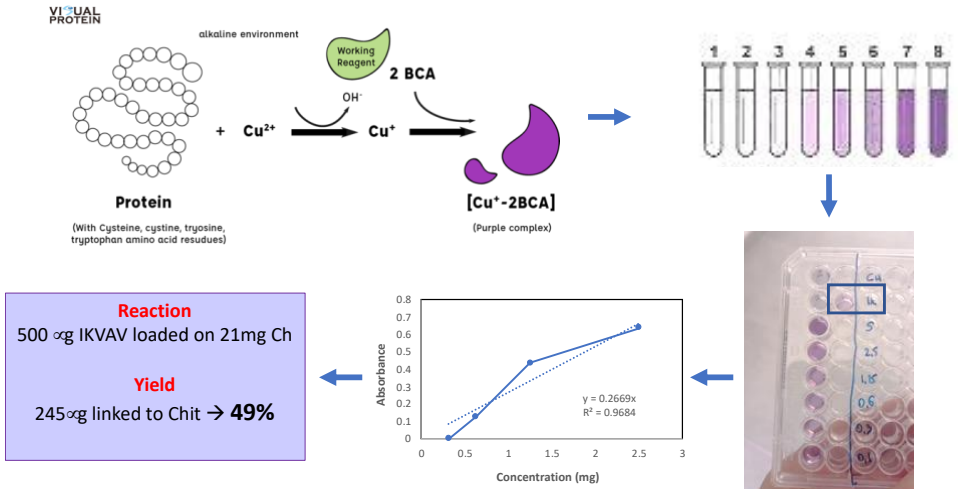

Fig. 4 Quantification of IKVAV loading on chitosan through BCA assay

This assay allowed to quantify the % of IKVAV linked to the chitosan chains that resulted equal to **49%** of the available bonding sites.

The Ch+IKVAV polymer resulted not able to perform the sol-gel transition by heating at 37°C, therefore a different strategy was implemented. The Ch+IKVAV has been mixed with not-modified Ch in different ratio and the beta-glycerophosphate solution (BGP) was used as gelling agent of the not-modified component (as reported in our previous works). The result is a semi-interpenetrating polymer network (semi-IPN) in which the Ch+IKVAV polymer chains are incorporated within the Ch+BGP crosslinked hydrogel, providing a double components system in which one component, Ch+BGP, becomes hydrogel at 37°C (thermosensitive component), while the other, Ch+IKVAV, provides a more neural-like 3D environment presenting the characteristic bioactive motifs of neural tissues.

The hydrogel was successfully prepared and the best performing ratio among Ch, Ch+IKVAV and BGP solution in terms of gelation was: 2.5 Ch solution/0.5 Ch+IKVAV solution/BGP solution.

Afterwards, the IKVAV amount effectively loaded within the semi-IPN hydrogel made of Ch/Ch+IKVAV/BGP could be easily evaluated knowing the amount of IKVAV for each gr of chitosan, equal to 11.6 µgr, and the amount of polymer in 1 mL of hydrogel (18 mg of Ch and Ch+IKVAV in the optimized ratio), and resulted equal to **0.194%**, in perfect accord to the results reported by other authors [6, 7].

The hydrogel will be characterized in terms of morphology (scanning electron microscopy, SEM), swelling and stability/degradation tests in physiological conditions (gravimetric measurements). Afterwards, biological test will be performed to evaluate the IKVAV effect on cell process and behaviour.

ST 4.4. Advanced microfluidic *in vitro* models for nerve regeneration study.

In order to develop advanced *in vitro* models useful to test the ability of nerve to regenerate both spontaneously and by means of different strategies, such as *(i)* nerve growth factor administration, *(ii)* iPSCs addition in the gap region, *(iii)* use of smart biomaterials, and once fully developed the efficacy of the multifunctional nerve here proposed, *(iv)* microfluidic platforms are powerful tools and will be used with this goal. Microfluidic devices allow for precise control over factors affecting distinct neuronal regions, compartmentalized by ad hoc designed microchannels and microchambers, making the study of neuronal injury (applied mechanically or chemically) and axon degeneration feasible [Methods Mol Biol. 2020:2143:133-144.]. These devices present several advantages over the conventional Campenot chambers. Among them, the use of transparent materials (i.e., PDMS) guarantees high efficiency, good biological compatibility, and ability to integrate with other characterization assays, such as electrophysiology and high-resolution microscopy. Although in vivo models (e.g., sciatic nerve transection, optic nerve crush, and lateral fluid percussion) represent established methods for recapitulating neuron degeneration after trauma, they fail in applying insults locally (i.e., soma versus axon) and accurately assessing the biochemical events associated with degeneration in specific subcellular portions. In the first 6 months of activity a three channels

device, able to allow the competitive administration of different stimuli, has been designed and microfabricated, starting from a preliminary device developed in our group (De Vitis E., et al, Sci Rep 11, 7019 (2021)).

Microfluidic device design and fabrication

Aiming at evaluating the nerve regeneration *in vitro*, as above-highlighted, microfluidic devices were designed and fabricated by optical lithography and polydimethylsiloxane (PDMS) replica molding [8], as summarized in **Fig. 5**. Three different perfusable compartments (500 µm wide, 6 mm long), having distinct inlets and outlets but interconnected through a series of narrow microchannels (10 µm wide, 250 µm long), were considered for culturing/administrating different cell types and/or biological molecules and physical stimuli.

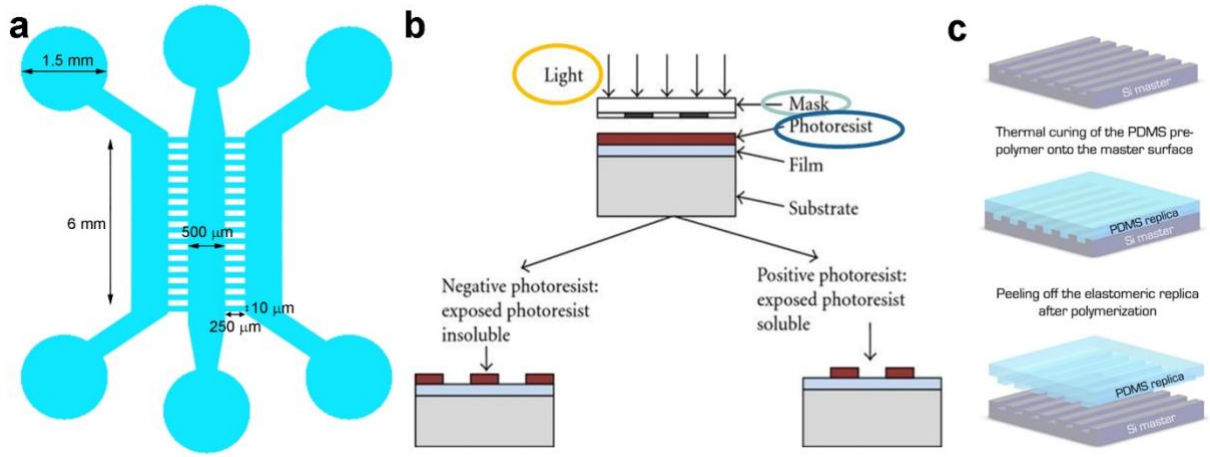

Fig. 5. Schematic of a preliminary 3-unit microfluidic device (a), fabricated by photolithography (b) and soft lithography (c) approaches.

The fabrication process consisted of two subsequent lithographic steps for obtaining a photoresist/silicon mold. First, SU-8 2005 was spin coated on silicon wafers and exposed in a mask aligner apparatus (SUSS MA6) in order to achieve a target thickness of 5 µm, corresponding to the height of the interconnecting microchannels in the final PDMS devices. As described in literature [9-12], height values in the range of 2.5-5 µm are highly desirable for allowing a good communication between adjacent compartments and avoiding cell migration across the microchannels at the same time. Subsequently, a SU-8 2050 lithography process was optimized to build the remaining larger channels and inlets/outlets chambers aligned to the pristine narrow microchannels. The final molds were used for generating PDMS replicas by soft lithography.

As widely reported, PDMS is intrinsically hydrophobic, but its surface can become hydrophilic by treatment with oxygen or air plasma: on its surface, PDMS has repeating units of $–O-Si(CH_3)_2$ groups that after plasma treatment become silanol (Si-OH) groups while methyl groups ($Si-CH_3$) are removed [13]. We treated PDMS replicas with oxygen plasma (200 sccm, 100 W, 30''), placed the patterned side on a glass microscopy slide to form covalent –O-Si-O- bonds between the two surfaces and finalized the bonding procedure by thermal treatment (30' at 75°C). This procedure led to the formation of an irreversible bonding between PDMS and glass surfaces (**Fig. 6**).

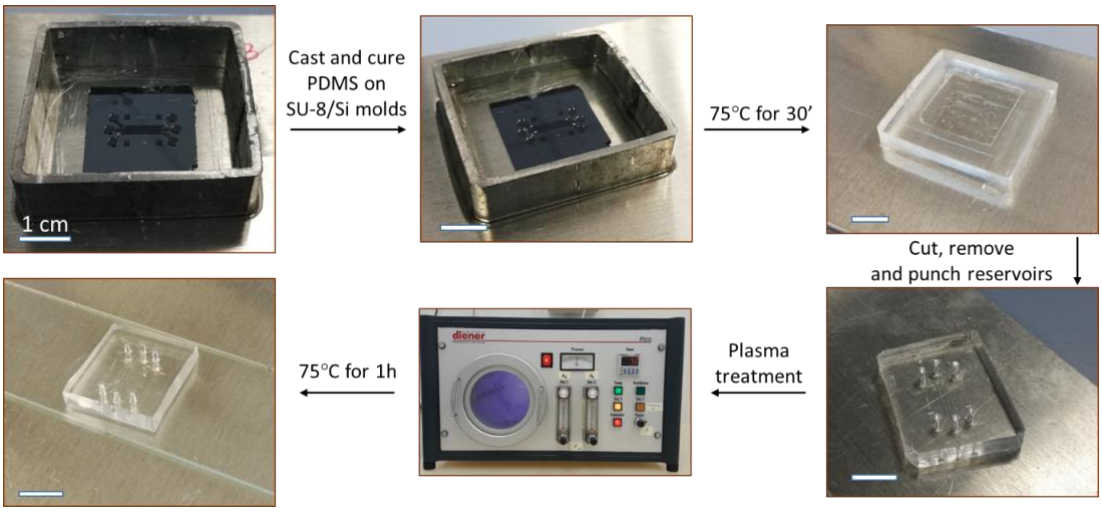

Fig. 6. Schematic of the different steps for the fabrication of PDMS microfluidic devices.

In the newly designed mold, the cross microchannel length was reduced to 50 μm, the minimum feature we could achieve with our approach. This was intended to have a shorter microchannel for axon extension without axon-Schwann cell interaction (in the microchannel there is indeed not enough space for such interaction) and favour such communication in the bigger channel. The devices made from such mold are currently under use in cell culture studies.

3. DISSEMINATION ACTIVITIES (SCIENTIFIC PAPERS, POP MAGAZINES, TV, ETC.)

- Canciani, B.; Semeraro, F.; Herrera Millar, V.R.; Gervaso, F.; Polini, A.; Stanzione, A.; Peretti, G.M.; Di Giancamillo, A.; Mangiavini, L. In Vitro and In Vivo Biocompatibility Assessment of a Thermosensitive Injectable Chitosan-Based Hydrogel for Musculoskeletal Tissue Engineering. Int. J. Mol. Sci. 2023, 24, 10446. https://doi.org/10.3390/ ijms241310446

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