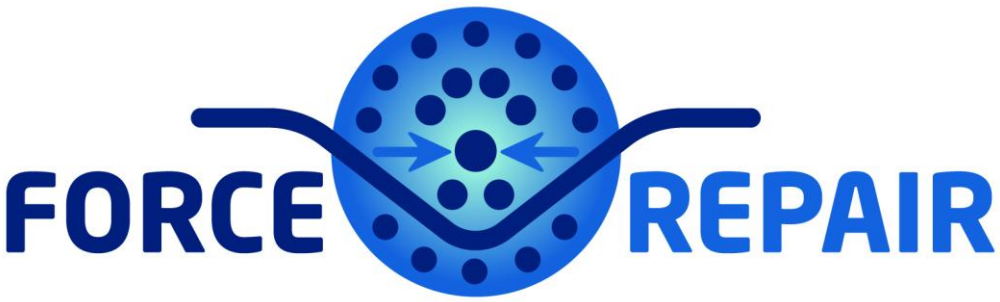
Call: HORIZON-CL4-2022-RESILIENCE-01
Topic: HORIZON-CL4-2022-RESILIENCE-01-13
Funding Scheme: HORIZON Research and Innovation Actions



Deliverable No. D3.7

Summary Report WP3

Grant Agreement no.: 101092243

Project Title: **smart and multiFunctional 3D printable prO-Regenerative biologiCal matrix modulating mEchanotRansduction as advancEd theraPy to treAt skIn chRonic wounds**

Contractual Submission Date: 31/12/2025

New Due Date (if delay): 31/03/2026

Actual Submission Date: 27/03/2026

Responsible partner: P2 - Universita degli studi del Piemonte orientale Amedeo Avogadro (UPO)

Funded by
the European Union

Grant agreement no.	101092243
Project full title	FORCE REPAIR - smart and multiFunctional 3D printable prO-Regenerative biologiCal matrix modulating mEchanotRansduction as advancEd theraPy to treAt skIn chRonic wounds

Deliverable number	**D3.7**
Deliverable title	**Summary Report WP3**
Type[1]	R
Dissemination level[2]	PU
Work package number	WP3
Author(s)	Davide Raineri (UPO), Giuseppe Cappellano (UPO), Annalisa Chiocchetti (UPO), Lia Rimondini (UPO), Ziba Najmi (UPO), Andrea Cochis (UPO), Hugo Oliveira (INSERM), Begoña Castro (HCELL), Eva González (HCELL), Giuseppina Sandri (UNIPV), Marta Pollini (UNIPV), Barbara Wolff-Winiski (AKR), Juliana de Souza Nunes (BIOG)
FORCE REPAIR reviewers	Damien Dupin (CID)
Keywords	3D-printed scaffold, Hydrogel-based bioinks, Antibacterial activity, Tissue regeneration, Immunomodulation, Biofilm inhibition, Neurite outgrowth, Angiogenesis, Bioadhesion, Pro-regenerative formulation

Funded by the European Union. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Health and Digital Executive Agency (HaDEA).

Neither the European Union nor the granting authority can be held responsible for them.

[1] **Type**: Use one of the following codes (in consistence with the Description of the Action):
R: Document, report (excluding the periodic and final reports)
DEM: Demonstrator, pilot, prototype, plan designs
DEC: Websites, patents filing, press & media actions, videos, etc.

[2] **Dissemination level**: Use one of the following codes (in consistence with the Description of the Action)
PU: Public, fully open, e.g. web
SEN: Sensitive, limited under conditions of the Grant Agreement

Table of Contents

Summary

The activities carried out within WP3 provided a comprehensive evaluation of the developed bioinks and hydrogel-based scaffolds, addressing their antibacterial efficacy, regenerative potential, biocompatibility, immunological profile, bioadhesive properties, and performance in clinically relevant conditions. The results demonstrated that the combined mupirocin–tobramycin formulations ensured broad-spectrum antibacterial activity, including effectiveness against complex biofilms, while maintaining compatibility with human cells. Advanced in vitro models confirmed the neuro-supportive properties of the bioinks, promoting neurite outgrowth, although angiogenic responses remained limited under the tested conditions. All hydrogel formulations proved to be non-cytotoxic and capable of promoting cell recruitment, with efficient cellular uptake of nanocapsules supporting their role as delivery systems. Immunological analyses highlighted a favourable safety profile, with no evidence of non-specific immune activation and preserved immune cell viability even under bacterial challenge. In parallel, the hydrogels exhibited strong and tuneable bioadhesive properties, particularly in pro-regenerative formulations, ensuring stable interaction with biological tissues. Finally, studies using human wound exudates demonstrated the ability of the bioinks to restore fibroblast proliferation in pathological conditions, although immunomodulatory effects were variable and dependent on the specific inflammatory microenvironment.

1 Introduction

Work Package 3 was designed to identify and validate the most effective formulations of the FORCE REPAIR 3D printed scaffold through a series of in vitro and ex vivo experimental approaches on the different bioactive inks developed in project, as well as the hydrogel alone as control, as defined in Annex I of the Grant Agreement. The overarching goal of this WP was to demonstrate the multifunctionality of the developed system, integrating antibacterial, regenerative, and immunomodulatory properties.

More specifically, WP3 aimed to select the most efficient nano-encapsulated bioactive compounds, assess their biological activity when incorporated into hydrogel-based bioinks, and evaluate their effects on human cells under physiologically relevant conditions. Particular attention was given to the interaction between the scaffold and the cellular components involved in wound healing, as well as to the influence of chronic wound microenvironments, including patient-derived wound exudates.

2 Description of Activities

The objective of this deliverable is to provide a comprehensive summary of the activities carried out within WP3, highlighting its main results and overall impact.

Specifically, this deliverable summarizes the outcomes of the following tasks:

Task 3.1: Antibacterial Properties & Biofilm interaction of bio-ink and combined product (UPO; M3-M30);
Task 3.2: Innervation and angiogenesis in vitro studies (INSERM, CID, BIOG; M3-M36);
Task 3.3: In vitro cytotoxicity and cell recruitment (HCELL, CID, BIOG; M3-M36);
Task 3.4: Immunological response of the 3D-printed scaffold and its components (UPO, CID, BIOG; M19-M30);
Task 3.5: Bioadhesive properties and contractile force on relevant tissue (UNIPV; M12-M36);
Task 3.6: Efficacy of the individual and combined biological Compounds on Human WE. (AKR; M6-M36).

For greater clarity, the following sections are organized into six main parts, each corresponding to one of the focus tasks listed above.

3 Results & Discussion

3.1 Antibacterial properties and biofilm interaction

A major component of the work focused on evaluating the antibacterial activity of nano-emulsions and hydrogel formulations incorporating different bioactive compounds. Initial experiments highlighted the limited efficacy of mupirocin against Gram-negative bacteria, which prompted the introduction of tobramycin as a complementary antibiotic. This strategic combination proved to be highly effective, enabling broad-spectrum antibacterial activity against both Gram-positive and Gram-negative pathogens.

Detailed *in vitro* analyses demonstrated that the combined mupirocin–tobramycin formulation significantly reduced bacterial metabolic activity and viability across multiple experimental conditions, including planktonic cultures and mature biofilms. Importantly, the system showed strong efficacy against mixed-species biofilms, which are highly representative of chronic wound infections and are typically more resistant to treatment. Co-culture experiments involving human mesenchymal cells and bacterial pathogens revealed that the antibacterial formulations not only reduced bacterial burden but also exerted a protective effect on human cells, preserving their viability under infectious conditions.

Furthermore, when incorporated into hydrogel matrices, these formulations maintained their antibacterial performance over extended incubation periods (up to 7 days), confirming their suitability for sustained local delivery.

These results enabled the identification of the most effective nano-encapsulated formulations and their biologically active concentration ranges.

3.2 Neurogenic and angiogenic in vitro studies

INSERM established optimized microfluidic chamber designs to support compartmentalized culture of human iPSC-derived sensory neurons and 3D co-culture of human endothelial cells (HUVEC) and fibroblasts.

Two microfluidic device generations were fabricated in PDMS using soft lithography: the initial circular–semicircular design was replaced with a linear two-chamber configuration to reduce hydrogel volume, improve PDMS–glass adhesion, minimize swelling, and allow reproducible hydrogel loading. SEM analysis confirmed accurate microchannel dimensions (width <7 µm, height 3–4 µm, length ~150 µm).

Human iPSCs were differentiated into sensory neurons and seeded into the microfluidic chambers. Immunocytochemistry at day 32 demonstrated expression of neuronal and nociceptive markers including βIII-tubulin, CGRP, TRPV1, and Substance P, confirming sensory neuron identity. Neurite outgrowth into hydrogel-filled compartments was visualized via Calcein AM staining and confocal microscopy and quantified using IMARIS software. Comparison of hydrogel formulations (HA alone, HA pro-regenerative) showed that the Pro-regenerative hydrogel significantly enhanced neurite extension relative to HA alone, supporting a higher number of extended neurites, suggesting improved network connectivity despite no significant increase in average neurite length over the scrambled control.

Angiogenic potential was assessed using GFP^+ HUVECs co-cultured with dermal fibroblasts on hydrogel surfaces, in 2D extract exposure assays, and in scratch wound-healing experiments. Unfortunately, confocal imaging microscopy and quantitative analysis revealed limited endothelial organization, migration, and capillary-like network formation in all HA-based hydrogels. Significant swelling of the hydrogels in aqueous culture likely impaired cell–matrix interactions, explaining the absence of angiogenic enhancement under growth factor–deprived conditions.

Overall, the task successfully validated advanced microfluidic and co-culture platforms for the evaluation of hydrogel bioinks. FORCE REPAIR bioactive inks demonstrated clear neuro-supportive properties, promoting neurite outgrowth and network formation of human iPSC-derived sensory neurons, while their angiogenic potential in vitro remained limited under the tested conditions. These results provide a robust foundation for further preclinical studies and highlight the suitability of the developed bioinks for neuro-regenerative applications.

3.3 In vitro cytotoxicity & cell recruiting

The evaluation of cytotoxicity and migration capacity of the antibacterial, anti-inflammatory, and pro-regenerative hydrogels demonstrated that all tested formulations are non-cytotoxic, preserving cell viability and maintaining optimal cell morphology. A decrease in cell viability

observed in MTT and live/dead assays at higher hydrogel concentrations (1:5 dilution) was attributed to a physical effect caused by the increased viscosity of the hydrogels, rather than to any intrinsic toxicity of their components. Moreover, the hydrogels exhibited favourable chemotactic properties towards human primary cells, particularly the pro-regenerative hydrogel, as well as the anti-inflammatory hydrogel. These formulations are expected to interact with the tissue microenvironment, promoting cell recruitment and potentially activating regenerative and healing processes. To further investigate the interaction of HA-based hydrogels with human cells, the uptake of fluorescently labeled HA-MA nanocapsules, used for drug encapsulation and their controlled release, by keratinocytes (HaCaT) and dermal fibroblasts (HDFs) was assessed using confocal laser scanning microscopy (CLSM). Nile Red (NR) and BODIPY™ 493/503 were used as fluorescent markers incorporated in the oil phase of the nanoemulsions, ensuring that dye incorporation did not alter particle size or stability. CLSM imaging revealed rapid internalization of the fluorescent nanocapsules, with detectable signals in both cell types as early as 1 hour after incubation, which increased over 24 hours. Z-stack imaging confirmed intracellular localization of the droplets, demonstrating successful internalization in both non-crosslinked and crosslinked emulsions. In contrast, no fluorescence was detected in control cells treated with non-encapsulated oil, confirming the specificity of uptake (Figures 1–4). These results indicate that the hydrophobic drug-loaded nanocapsules developed are efficiently internalized by skin cells, supporting their potential for targeted delivery of bioactive compounds in wound healing applications.

Overall, the combined qualitative, cytotoxicity, migration, and cellular uptake studies demonstrate that all tested hydrogel formulations are biocompatible, non-cytotoxic, and able to recruit cells effectively. The pro-regenerative and anti-inflammatory formulations, in particular, exhibited optimal chemotactic properties, suggesting that these bioinks can actively interact with the tissue microenvironment to promote wound healing and regeneration. The internalization of nanocapsules further confirms their suitability as carriers for localized therapeutic delivery, providing a promising strategy to enhance the efficacy of 3D printed hydrogels in chronic wound management.

3.4 Immunological response of the 3D-printed scaffold and its components

Task 3.4 focused on evaluating the immunological response elicited by the developed hydrogel formulations and their individual components, using human peripheral blood mononuclear cells (PBMCs) derived from healthy donors. The studies aimed to assess both the biocompatibility of the hydrogels and their potential immunomodulatory effects, particularly in the context of bacterial challenge.

PBMCs were cultured in direct contact with different hydrogel formulations, including HA alone (Control hydrogel), pro-regenerative hydrogel, as well as the antibacterial or anti-inflammatory hydrogels. Co-culture experiments were performed both in the presence and absence of mixed bacterial pathogens (Staphylococcus aureus and Pseudomonas aeruginosa).

Initial optimization of cell activation and washing procedures ensured reproducible PBMC viability across all tested formulations. In the absence of bacteria, PBMC viability was preserved for all hydrogel formulations, indicating that the hydrogels are intrinsically biocompatible and do not induce non-specific immune activation. When PBMCs were exposed to live bacteria, a time-dependent reduction in cell viability was observed, with maximal effects at 48 hours. Importantly, treatment with the antibacterial hydrogel or antibacterial nanocapsules effectively prevented bacterial-induced cytotoxicity, as confirmed by CFU enumeration and Live/Dead staining, demonstrating strong antibacterial activity while preserving immune cell viability.

The immunomodulatory profile of PBMCs was further characterized by multiparametric flow cytometry. In the $CD4^+$ T cell compartment, hydrogel exposure did not alter total $CD4^+$ counts, naïve, central memory, Th1, or Th17 subsets. Minor modulations were observed in effector subsets, such as an increase in $CD4^+$ TEMRA cells with soluble diclofenac, while incorporation into hydrogels mitigated this effect, likely due to controlled release. Pro-inflammatory subsets, including Th9 and Th22, were slightly reduced in specific conditions with the antibacterial and anti-inflammatory hydrogels, whereas Th2 cells increased under certain bacterial exposures. In the $CD8^+$ compartment, no major differences were observed across naïve, effector memory, or total $CD8^+$ T cells. These data indicate that the hydrogels themselves do not broadly activate T cells and that observed changes are largely driven by bacterial stimulation or soluble drug components.

Cytokine analyses performed on PBMC supernatants confirmed these findings. While most cytokines, including IL-1β, TNF-α, IL-6, IL-8, IFN-γ, and others, were unaffected by hydrogel exposure alone, the presence of bacteria induced specific changes. For instance, G-CSF levels were restored to baseline upon application of the antibacterial treatment, and IL-6 and MCP-1 levels reduced by bacterial exposure returned to control levels when the antibacterial hydrogel was administered. Notably, IFN-γ levels increased under hydrogel culture alone but normalized under bacterial co-culture with the anti-inflammatory hydrogel. Overall, these results confirm that the hydrogel matrices do not compromise immunocompatibility and that any immunological alterations are primarily mediated by bacteria or soluble drug components rather than by the biomaterial itself.

Collectively, the data from Task 3.4 demonstrate that the developed hydrogel formulations are biocompatible, do not induce broad non-specific immune activation**,** and maintain immune cell viability even in the context of bacterial challenge. These findings provide a critical validation of the safety and immunological performance of the FORCE REPAIR scaffolds, reinforcing their potential for clinical application in chronic wound environments.

3.5 Bioadhesive properties and contractile force on relevant tissue

An additional set of activities within WP3 focused on the evaluation of the bioadhesive properties of the developed hydrogel formulations, as well as their interaction with biologically relevant substrates. These investigations were conducted through ex vivo measurements using porcine ear skin, which represents a widely accepted model for human skin due to its comparable structural and mechanical characteristics.

Bioadhesion was quantitatively assessed by measuring the force required to detach the hydrogel from the biological substrate. In this experimental setup, a defined amount of hydrogel was brought into contact with the skin surface for a fixed period, after which the detachment force was recorded as a function of displacement. The maximum detachment force (Fmax) was used as the primary parameter to describe the adhesive performance of the material. The results clearly demonstrated that all tested hydrogel formulations exhibited significant bioadhesive properties, as indicated by a substantially higher detachment force compared to the blank condition, in which no hydrogel was applied. This confirms the intrinsic ability of the developed materials to establish stable interactions with biological tissues, a key requirement for their application in wound dressing. Among the tested formulations, the pro-regenerative hydrogel showed the highest bioadhesive performance. This enhanced adhesion is likely attributable to the presence of extracellular matrix-derived components and the special visco-elastic properties of this hydrogel compared to the other, as it is more defromable. These findings are consistent with previous results obtained in earlier work packages, further supporting the role of these bioactive components in improving material–tissue integration.

Comparative analyses were also performed between ex vivo measurements and previously obtained in vitro data using skin substitutes. Although minor quantitative differences were observed, the overall trends were highly consistent across the two models, confirming that the in vitro system represents a reliable surrogate for bioadhesion testing. Once again, the pro-regenerative hydrogel showed the highest bioadhesive properties This cross-validation strengthens the robustness of the experimental approach and supports its use for future material screening.

Further experiments were conducted to evaluate the bioadhesive behavior of different functional formulations, including antibacterial, anti-inflammatory, pro-regenerative, and control hydrogels. While all samples retained adhesive properties, the pro-regenerative formulation consistently exhibited the highest detachment force, whereas antibacterial formulations showed comparatively lower adhesion. Importantly, these trends were reproducible across different batches of hydrogels, demonstrating the consistency and reliability of the manufacturing process.

Overall, these results demonstrate that the developed hydrogels possess strong and tuneable bioadhesive properties, which are influenced by their biochemical composition. This characteristic is particularly relevant for ensuring stable positioning of the scaffold at the wound site, thereby enhancing its therapeutic efficacy and supporting prolonged interaction with the tissue.

3.6 Efficacy of individual and combined biological compounds on human wound exudates

A key objective of WP3 was to evaluate the biological activity of the developed bioinks and their individual components in a clinically relevant context, using wound exudates (WEs) derived from patients with chronic wounds. These experiments were designed to investigate both regenerative and immunomodulatory effects of the materials under conditions that closely mimic the pathological wound microenvironment.

A total of 69 wound exudate samples were collected from 13 patients at multiple time points, representing a heterogeneous cohort in terms of wound status and biological activity. The samples included both healing and non-healing exudates, with the latter defined based on their ability to inhibit fibroblast proliferation in vitro. Due to variability in sample volume and biological activity, a subset of exudates with sufficient volume and strong inhibitory properties was selected for detailed functional analysis, complemented by additional samples from previous collections to ensure adequate statistical power. The impact of wound exudates on tissue regeneration was first assessed using a fibroblast proliferation assay. Human dermal fibroblasts exposed to select non-healing exudates showed a marked reduction in proliferative capacity, confirming the pathological nature of these samples. When treated with FORCE REPAIR bioactive inks, a significant recovery of fibroblast proliferation was observed in a substantial proportion of cases. This rescue effect was consistently detected across multiple formulations and was particularly pronounced for the pro-regenerative ink. In contrast, individual components of the pro-regenerative hydrogel tested separately showed limited or inconsistent effects, highlighting the importance of the combined formulation in restoring cellular function. Importantly, the magnitude of the rescue effect was strongly dependent on the specific wound exudate, with significant improvement observed in 9 out of 15 samples. This variability suggests that patient-specific factors, such as the composition of cytokines, proteases, and microbial products, play a critical role in determining the response to treatment. These findings support the concept that the therapeutic efficacy of the scaffold may be influenced by the biological heterogeneity of chronic wounds.

In parallel, the immunomodulatory effects of the bioinks were evaluated by analyzing cytokine secretion in macrophages exposed to wound exudates. All tested exudates induced a pro-inflammatory response, characterized by the secretion of key cytokines including IL-1β, TNF-α, IL-6, and IL-8. The ability of the bioinks and their components to modulate this response was found to be highly variable and context dependent. A reduction in IL-1β and TNF-α

secretion was observed only in a subset of wound exudates, and in some cases this effect was also detected with the control hydrogel, suggesting that material-related factors may contribute to cytokine modulation. The anti-inflammatory hydrogel did not consistently outperform the other bioinks, indicating that drug encapsulation alone is not sufficient to guarantee a robust immunosuppressive effect in complex biological environments. For IL-6 and IL-8, the results revealed an even more complex scenario, with bioinks frequently inducing or enhancing cytokine secretion rather than suppressing it. Only limited inhibitory effects were observed, and these were restricted to specific combinations of exudates and treatments. This behaviour likely reflects the dynamic and multifactorial nature of macrophage activation in response to wound-derived signals, as well as the interaction between biomaterials and immune cells. Taken together, the macrophage data indicate that the immunomodulatory activity of the FORCE REPAIR bioinks does not follow a uniform pattern but is instead highly dependent on the inflammatory context defined by the individual wound exudate.

Overall, the combined analysis of fibroblast and macrophage responses provides important insights into the biological performance of the developed materials. While the bioinks demonstrate a consistent ability to promote fibroblast proliferation under inhibitory conditions, their effects on inflammatory pathways are more heterogeneous. Notably, only a limited number of wound exudates showed both a rescue of fibroblast proliferation and a reduction in pro-inflammatory cytokine secretion, and these effects did not always correlate across different cytokines.

These findings highlight the complexity of CW environment and underscore the importance of evaluating advanced therapeutic materials in patient-derived systems. At the same time, they suggest that the FORCE REPAIR bioactive inks hold significant potential for promoting tissue regeneration, while their immunomodulatory effects may require further optimization or stratification based on patient-specific profiles.

4 Conclusions

Overall, WP3 successfully validated the multifunctional performance of the FORCE REPAIR bioactive inks, demonstrating their safety, antibacterial efficacy, and regenerative potential across multiple experimental platforms. The results support their suitability as advanced therapeutic materials for chronic wound management, particularly due to their ability to combine infection control with tissue regeneration. However, the observed variability in immunomodulatory responses highlights the complexity of the wound microenvironment and suggests that future optimization or patient stratification strategies may be required to maximize therapeutic efficacy. These findings provide a strong foundation for further preclinical and clinical development of the technology.