Main tutor : Alice Zoso

Project for the course:
"Laboratory of Tissues and Physiological Processes' Models", A.A. 2024/25.

Project

Title:	Animal-free **mi**crofluidic **m**odel of Mult**i**ple S**c**lerosis-affected **B**lood **B**rain **B**arrier
Acronym:	**MIMIC – BBB**
Keywords:	*blood-brain barrier, multiple sclerosis, microfluidic model, animal-free model*

Students' team

Surname and name: D'Agostino Claudia
Badge number: 337584
E-mail: s337584@studenti.polito.it

Surname and name: Dasi Alessandro
Badge number: 349093
E-mail: s349093@studenti.polito.it

Surname and name: Attardo Carmen Maria
Badge number: 333803
E-mail: s333803@studenti.polito.it

Surname and name : Averardi Alessandra
Badge number: 333802
E-mail: s333802@studenti.polito.it

Surname and name : Biesso Giada
Badge number: 349048
E-mail: s349048@studenti.polito.it

Surname and name : Rossi Alessia
Badge number: 331666
E-mail: s331666@studenti.polito.it

Main tutor : Alice Zoso

Abstract

Multiple sclerosis (MS) is a chronic autoimmune disorder characterized by neuroinflammation and progressive demyelination. A pivotal early event in MS pathogenesis is the disruption of the blood–brain barrier (BBB), which normally regulates the access of immune cells into the central nervous system. Existing in vitro BBB models often rely on animal-derived extracellular matrices, lack multicellular organization, reducing their physiological relevance to the study of the disease, and/or offer limited scalability for drug testing. To address these limitations, we propose the development of a fully human, animal-free, microfluidic BBB model capable of mimicking the pathological permeability observed in MS.
The general aim of this project is to create a biomimetic and scalable in vitro BBB platform that enables mechanistic studies of barrier dysfunction in MS and supports drug screening and future patient-specific modeling.
This will be achieved by:
(i) engineering a three-lane microfluidic model with endothelial and glial components mimicking brain capillary structure,
(ii) inducing a pathological BBB phenotype by introducing into the model pro-inflammatory cytokines through the endothelial lane and disease-specific chemokines trough the bottom perfusion lane,
(iii) validating the model's responsiveness to known MS therapeutics (fingolimod and retinoic acid) and assessing novel candidates such as Imatinib,
(iv) integrating results across WPs into a statistical model to support robust drug screening.
The employed materials and platforms will include the Mimetas Organoplate®3-lane 40, chosen for its pump-free perfusion, membrane-free co-culture, ease of handling, and compatibility with automation (e.g., OrganoTEER), endothelial and glial (astrocytes and pericytes) cell lines to be cultured respectively on the coated microfluidic lane and in a cell line-derived human collagen I gel, with cell seeding densities and coating protocols optimized to support tight junction formation, and barrier integrity monitoring through TEER measurements, FITC–dextran assays and immunostaining.
The project strictly complies with the 3Rs principle by replacing animal products with human derived materials and reducing animal experimentation through the establishment of a reliable in vitro model and the maximization of its experimental efficiency.
The proposed platform will deepen the understanding of MS-related BBB dysfunction, accelerate therapeutic screening, and lay the groundwork for patient-specific models.
Team roles will be distributed as follows:

- Alessia Rossi: materials optimization, microfluidic platform preparation
- Alessandro Dasi: cell culture, continuous TEER registration
- Giada Biesso: pathological induction, advanced permeability assays
- Alessandra Averardi: drug treatment protocol refinement
- Carmen Maria Attardo: optical imaging, immunostaining assessments
- Claudia D’Agostino: planning, timeline verification and adaptations, data integration, statistical modeling, reporting

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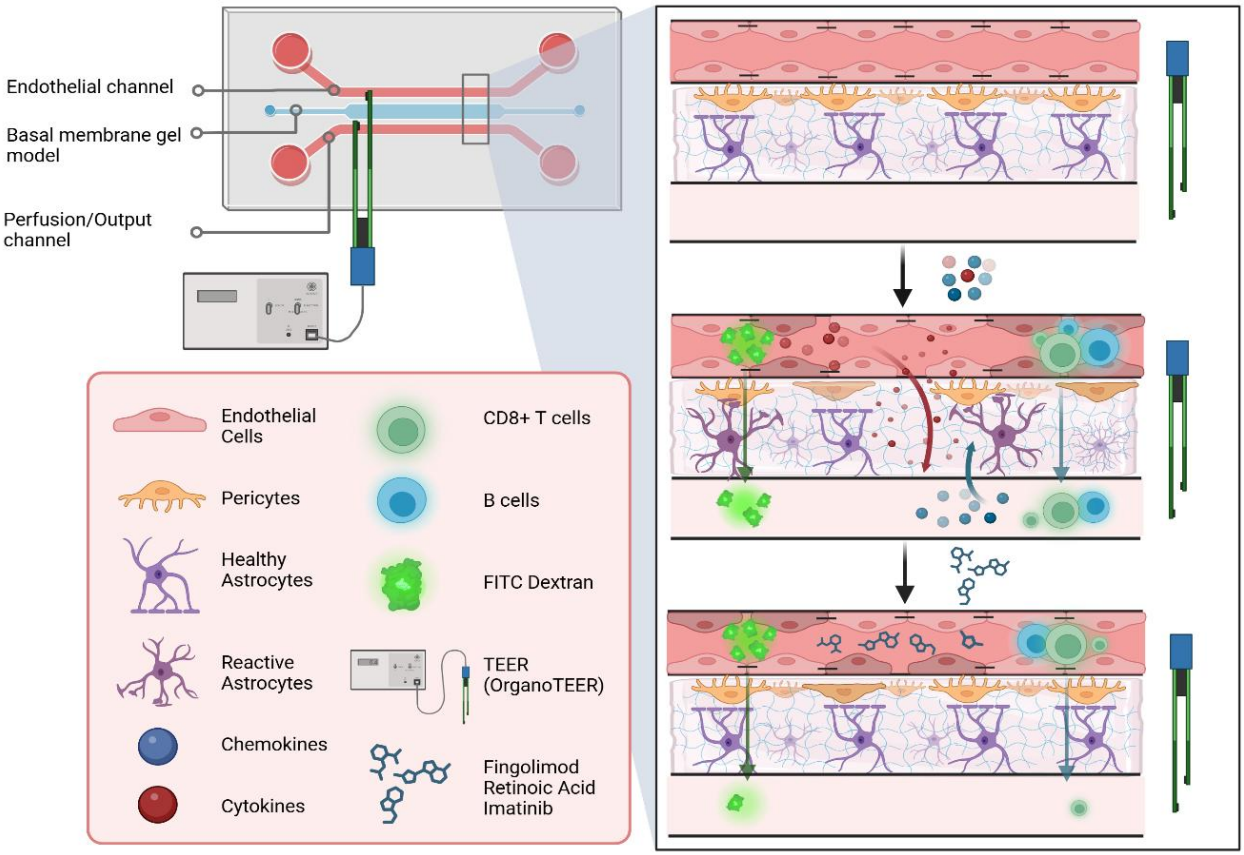
Graphical Abstract



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PROJECT WORKPLAN

1. RATIONALE OF THE PROJECT

Current in vitro blood–brain barrier (BBB) models are transitioning from traditional Transwell systems toward microfluidic “BBB-on-a-chip” platforms, in response to several known limitations. Transwell models lack physiological shear stress and dynamic fluid flow, features critical for endothelial cell function and barrier integrity. Additionally, they often fail to replicate the multicellular complexity of the in vivo BBB, resulting in oversimplified models with limited predictive value for drug screening or disease modeling [1]. Recent microfluidic systems address some of these issues by enabling continuous perfusion and closer spatial interaction between endothelial cells and perivascular components such as pericytes and astrocytes. Co-cultures of all three cell types in particular have been shown to be significantly superior in replicating the in vivo permeability and structural architecture of the BBB compared to monocultures of endothelial cells alone [2] [1].

Despite this progress, no standardized microfluidic architecture has yet been adopted across the field. Common geometries include vertical “sandwich” designs, which stack endothelial and glial compartments but introduce an unnatural gravitational-related component that can skew transport and signaling dynamics, and plane-parallel multi-channel systems, which offer a more biomimetic and controllable setup: they allow for lateral perfusion, reproducible cellular organization and the inclusion of additional channels (e.g., for sampling of permeated drugs), while minimizing physical artifacts [3]. However, most of these systems still rely on animal-derived matrices and suffer from low scalability and poor reproducibility across labs.

Moreover, a critical gap in current BBB models is the lack of validated platforms that specifically mimic pathological barrier breakdown as observed in neurological disorders, particularly Multiple Sclerosis (MS). MS is characterized by disrupted BBB integrity, increased permeability, and the infiltration of autoreactive immune cells into the brain tissue [4] [5] [6]. Studies have identified astrocytes with elevated CXCL12 expression and altered chemokine signaling as contributing factors [4]. Yet, there is no widely adopted in vitro model that mimics this pathological state, limiting our ability to investigate its mechanisms or screen therapeutic interventions.

To address these unmet needs, this project will develop a human-only, animal-free, parallel-plane multichannel microfluidic BBB model that will (i) eliminate the biological variability introduced by animal products, (ii) allow precise flow control and pathological induction, and (iii) support high-throughput, quantitative permeability evaluations throughout its whole development and drug testing after the validation of the basic model. This platform is designed to serve as both a research tool enabling dynamic studies of BBB integrity under pathological stimuli and a screening platform for candidate MS therapeutics testing in a controlled, reproducible setting, with future potential for patient-specific modeling using iPSC-derived cells.

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2. PROJECT DEVELOPMENT

2.1 Aims

The general aim of the MIMIC-BBB project is to create a biomimetic BBB platform simulating the pathological permeability induction mechanisms observed in MS patients to elucidate underlying mechanisms, accelerate drug discovery, and ultimately enable personalized in vitro modeling.

This will be achieved through the following objectives:

1. Engineering of a co-culture model incorporating an engineered human endothelial vessel adjacent to a glial compartment containing human astrocytes and pericytes in a cell line-derived collagen gel that would mimic the basal membrane found in the BBB.
2. Induction of pathological permeability via inflammatory stimulation (cytokines and CXCL12 chemokine perfusion) and quantitative assessment by TEER and tracer assays.
3. Validation of model responsiveness through administration of known barrier-restoring drugs (e.g., Fingolimod, retinoic acid) and establishment as a screening platform for novel compounds.

2.2 Methodology

Component	*Chosen Approach*	*Justification*
Microfluidic Platform	Mimetas Organoplate® 3-lane 40	Made with low compound-absorbing polymers as an alternative to PDMS, high parallelization potential, pump-free perfusion (reduces mechanical stress on cells), membrane-free co-culture allowing for direct cell–cell interactions, compatibility with automation tools such as OrganoTEER, straightforward handling compared to tubing-based systems
Matrix Material	Cell line-derived human collagen I (central channel); fibronectin coating (top perfusion lane)	Collagen I: major ECM component in the brain's basal membrane, allows for 3D encapsulation of astrocytes and pericytes. Using a recombinant human-derived form supports full xeno-free compliance and avoids batch variability typical of animal-derived gels. Fibronectin: improves endothelial adhesion
Cell Types	Immortalized human brain • microvascular endothelial cells (TY10) • vascular pericytes • astrocytes	High physiological relevance without need for stem cell differentiation, facilitating reproducibility; the TY10 line is a promising candidate for in vitro models due to exhibiting a leak-tight barrier function with comparable claudin-5 expression to primary human brain endothelial cells[1][7], IM-HBVP and IM-HA enable accurate mimicry of glial–endothelial interactions in the BBB.

TEER measurement	OrganoTEER platform with integrated electrodes	Non-invasive, real-time monitoring of barrier tightness compatible with the Organoplate®, enabling automated, parallel TEER tracking.
Permeability assays	FITC–dextran (10 kDa) diffusion; immune cell transmigration	Dextran diffusion: quantitative measure of paracellular permeability (10 kDa size approximates small biomolecule drugs) [3] [8]. Immune cell migration assays: evaluation of barrier selectivity in pathological and treated states, mimicking MS-relevant mechanisms [1].
Imaging and protein localization	Confocal immuno-fluorescence for ZO-1, occludin and claudin-5	Subcellular resolution imaging of hallmark indicators of BBB integrity known to correlate well with TEER changes and permeability shifts; occludin is more involved in regulating junction permeability while claudins are more directly involved in forming the tight junction barrier

2.3 Project Workplan

The project is structured into four sequential Work Packages (WPs), each building on previous data to refine model fidelity and functionality and support the overall goal of producing a robust, scalable, and ethically compliant BBB model relevant for MS research.

- **WP1: Healthy Blood-Brain-Barrier Model Establishment (Months 1–9)**
  The objective of WP1 is to construct a baseline, physiologically relevant BBB model using the Mimetas Organoplate® 3-lane 40, integrating endothelial, astrocytic, and pericytic human cell lines cultured in a human collagen matrix.
  - **Task 1.1: Microfluidic Channel Preparation & Gel Polymerization (Months 1-4)**
    The first channel will be coated with fibronectin (~ 50 mg/ml) [9] to support endothelial adhesion, while the central channel will be filled with a cell line-derived human collagen I gel, targeting a stiffness of 0.3–3.3 kPa [10] by optimizing its concentration, starting from 4 mg/mL [7], and crosslinking outside of the microfluidic device with iterative rheological characterization (amplitude and frequency sweep tests). This configuration will mimic the extracellular matrix (ECM) of the basal lamina.
    - *Deliverable D1.1*: Protocol for optimal collagen gel formulation and casting (M4)
    - *Milestone 1.1*: Achieving target collagen gel stiffness and consistency across replicates (M3)
  - **Task 1.2: Co-culture Conditions Optimization (Months 3–9)**
    Endothelial cells will be seeded into the fibronectin coated top channel through the relative inlet and cultured under oscillation until the creation of a vessel-like tubular structure, while astrocytes and pericytes will be embedded within the gel through suspension in the gel precursor and in-situ gelification. Cell densities will be optimized (target: ECs ~1.5×10^7 cells/mL [1]; astrocytes ~7.5×10^6 cells/mL; pericytes ~5×10^6

cells/mL [1]), the endothelial culture medium will be circulated in the top channel and an appropriate and iteratively optimized mix of the pericyte and astrocyte mediums in the bottom perfusion channel at a physiological flow rate (shear stress target value: 1-2 dyne/cm² [11]), and the co-culture will be monitored with daily TEER measurements using OrganoTEER to verify baseline barrier functionality.

- *Deliverable D1.2*: Standardized co-culture protocol with defined cell densities and media composition to ensure a consistent barrier formation (M9)
- Milestone M1.2: Stable TEER values ≈ 20 Ω·cm² [1] indicating intact barrier function (M8).

- **WP2: Induction of Pathological BBB Permeability through biochemical stimuli to Model MS-like Conditions (Months 10–18)**
  WP2 will aim to recreate the barrier dysfunction hallmarks of MS through inflammatory stimulation. Cytokines TNF-α and IL-1β will be perfused in the endothelial channel and chemokine CXCL12 in the bottom perfusion channel, establishing a chemokine gradient inside the gel, to induce pathological conditions. BBB integrity will be assessed via TEER and permeability assays.
  - **Task 2.1: Chemokine-Induced Inflammatory Stimulation (Months 10–16)**
    Dose–response studies will be conducted starting from 800 ng/mL of CXCL12 chemokine [12] and 50 ng/mL of TNF-α and 10 mg/mL of IL-1β cytokines [13] to establish the minimum effective concentration that produces a reproducible permeability increase between the treated and untreated state, while consistently monitoring TEER measurements. Cell viability and morphology will be monitored in parallel with resazurin-based perfusion assays and microscope imaging to avoid acute cytotoxicity.
    - *Deliverable D2.1*: Dose–response curve protocol for inflammatory cytokine and chemokine stimulation (M16)
    - *Milestone M2.1*: Reproducible ≥ 75% [12] reduction in TEER across at least three biological replicates (M16)
  - **Task 2.2: Functional Permeability Validation via Transmigration Assays (Months 13–18)**
    Fluorescent dextran (10 kDa) and immune cell transmigration assays using a Jukart CD8+ T cell line and a Lymphoblastoid cell line (LCL) will be used to validate barrier leakage [12]. These results will be compared with TEER data to confirm the correlation between electrical resistance and functional permeability.
    - *Deliverable D2.2*: Validated workflow for dual TEER/tracer permeability quantification (M18)
    - *Milestone M2.2*: Confirmed TEER/diffusion correlation ($R^2 > 0.85$) for pathological models (M18)

- **WP3: Model Validation through Pharmacological Modulation of BBB Integrity Using Established Therapeutics (Months 19–24)**
  WP3 will focus on validating the pathological BBB model's responsiveness to drugs known to reduce barrier permeability in vivo, demonstrating the platform's utility for preclinical drug testing.

- **Task 3.1: Administration of Fingolimod and Retinoic Acid (Months 19–22)**
  Pathological models will be treated with the introduction through the endothelial channel inlet of therapeutic amounts of Fingolimod and retinoic acid (around 100 nM each [14]), alone and in combination to assess potential additive effects. Drug dosing will be refined based on TEER response curves.
  - *Deliverable D3.1*: Dose-optimization protocol for therapeutic rescue (M22)
  - *Milestone M3.1*: Demonstrated TEER recovery ≥ 70% of baseline [12] following treatment (M22)
- **Task 3.2: Evaluation of Functional Recovery (Months 21–24)**
  Immunostaining for tight junction proteins (ZO-1, occluding, claudin-5) and post-treatment permeability assays will support recovery claims. Cytokine levels will also be measured to assess the local anti-inflammatory effects of the administered compounds.
  - *Deliverable D3.2*: Functional recovery dataset integrating TEER, tracer, and immunofluorescence data (M23)
  - *Milestone M3.2*: Recovery of both structural (tight junction expression) and functional parameters (M23)

- **WP4: Extended In Vitro Testing, Statistical Analysis & Scientific Dissemination (Months 1–24)**
  WP4 will evaluate the model's flexibility as a screening tool by applying a novel candidate drug (Imatinib) and will serve as a support to all WPs by analyzing the relative data and guiding technical decisions. A scientific manuscript integrating results from all work packages will be drafted.
  - **Task 4.1: Imatinib Efficacy Testing Compared to Standard Therapeutics (Months 22–24)**
    Imatinib will be tested under the same pathological induction conditions, at concentrations of 6-7 µg/mL [15]. Efficacy will be compared to Fingolimod and retinoic acid based on TEER improvement, permeability decrease, and cytokine expression.
    - *Deliverable D4.1*: Comparative dataset on permeability-modulating effects of Imatinib vs. standard drugs (M24)
    - *Milestone M4.1*: Statistically significant difference in permeability ($p<0.05$) between Imatinib and control (M24)
  - **Task 4.2: Statistical Modeling and Final Report Drafting (Months 1–24)**
    The correlation between input conditions (cell densities, cytokine doses) and readouts (TEER, tracer flux) will be assessed through multivariate statistical analysis. Final manuscript preparation will integrate all WP results.
    - *Deliverable D4.2*: Draft of scientific manuscript suitable for peer-reviewed submission (M24)
    - *Milestone M4.2*: Final validation of model robustness and manuscript submission readiness (M24)

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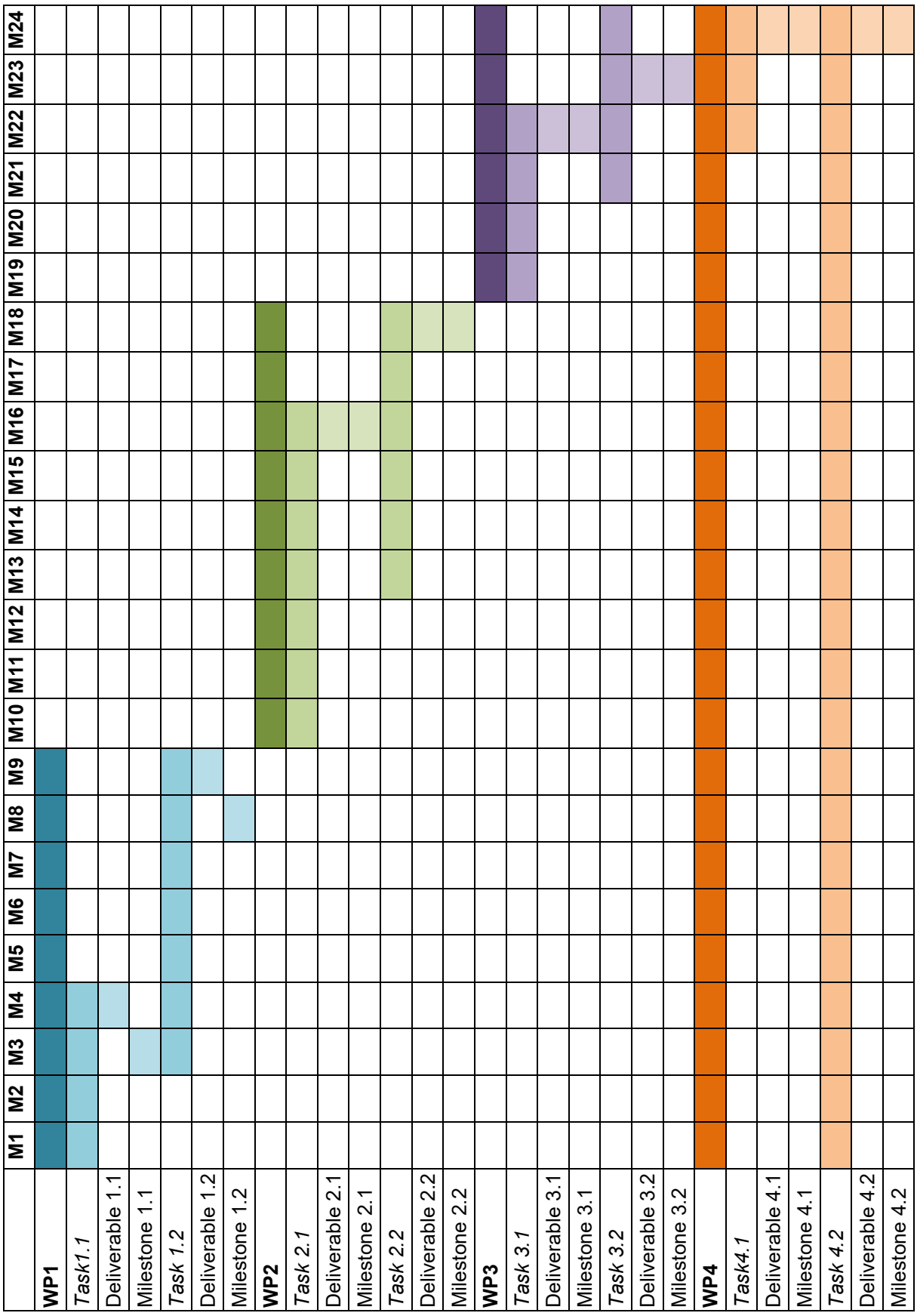
Gantt chart



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Contingency Plan

	Risk	Likelihood	Impact	Mitigation Strategy	Responsible
WP1	Variations in fibronectin coating or possible degradation of the protein over time, causing incomplete barrier formation and unreliable permeability data	Medium	Medium	Using a calibrated dispensing system, maintaining a constant ambient temperature; supplementing the fibronectin layer with additional ECM proteins	Alessia Rossi
WP1/2	Batch variability in collagen [16], leading to altered gel stiffness between samples and uneven distribution of astrocytes and pericytes	Medium	Medium	Adjust pH/gel ratio; conduct additional preliminary experiments to optimize gel properties; switch to alternative human cell line-derived or recombinant ECM	Alessia Rossi, Alessandro Dasi
WP1/2/3	Excessive or insufficient shear stresses and consequent endothelial cells detachment or diminished tight junction formation	Low	High	Flow parameters recalibration; use of the secondary optional pump perfusion method	Claudia D'Agostino
WP1	Low baseline TEER values leading to unreliable barrier measurement	Low	Medium	Increase cell density; test Transwell® backup system to exclude cell or materials defects	Alessandro Dasi
WP2	Cytokine-induced cytotoxicity [17]	Medium	Medium	Reduce applied cytokine and CXCL12 doses, shorten the exposure time, perform single exposure instead of joint	Giada Biesso
WP2	Inhomogeneous cytokine delivery and localized inflammatory responses, resulting in inconsistent alterations in permeability	Medium	Low	Prepare cytokine solutions with precise concentrations and thorough pre-mixing to ensure a homogeneous suspension	Giada Biesso, Carmen Maria Attardo
WP3	Drug solubility issues leading to incomplete therapeutic exposure	High	Low	Use of cosolvents or encapsulated forms of the drugs; perform solubility pretests	Claudia D'Agostino, Alessandra Averardi

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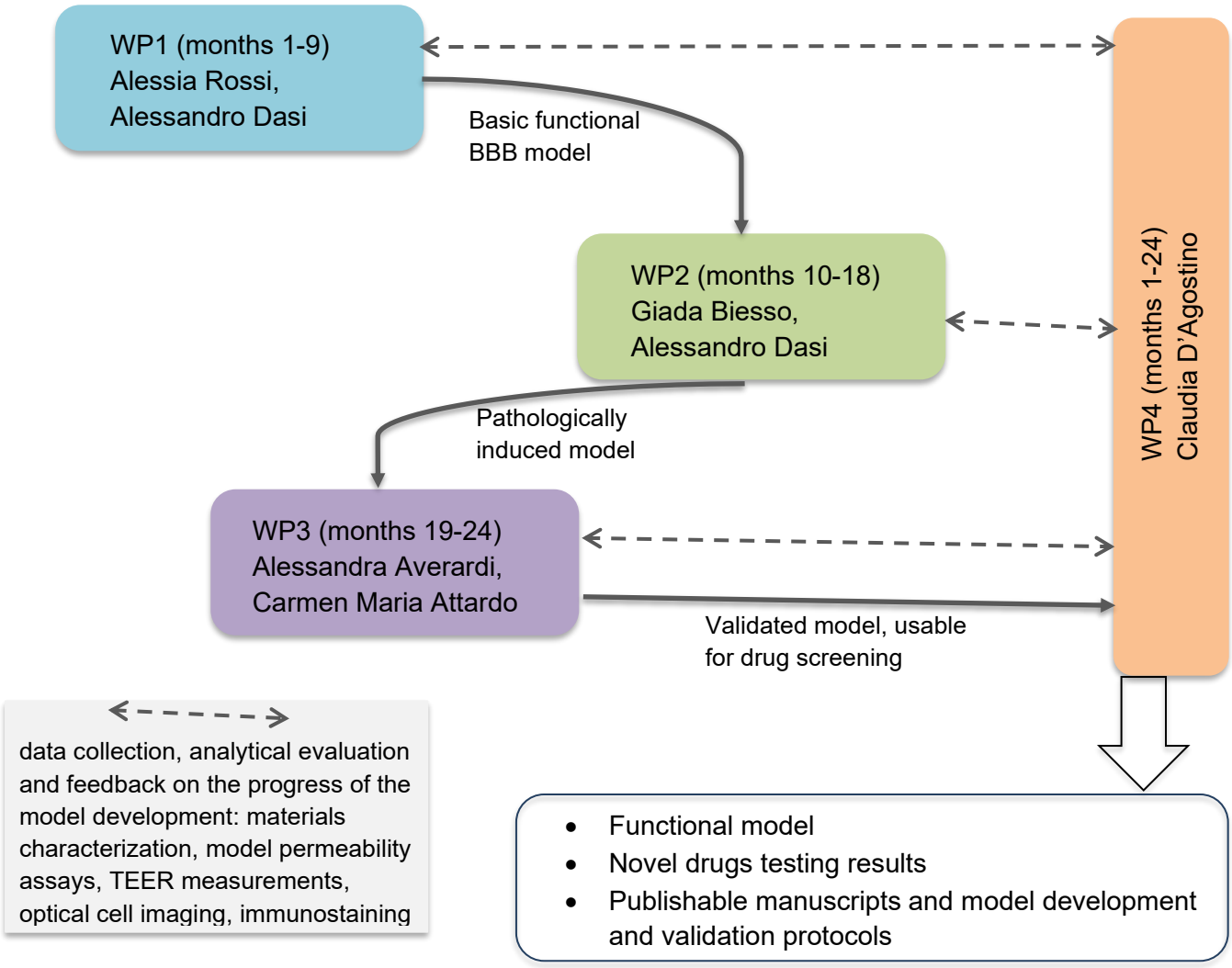
2.4 Team role and interactions



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4. COMPLIANCE TO 3Rs PRINCIPLE

This project demonstrates strong adherence to the principles of Replacement, Reduction, and Refinement (3Rs) at every stage of its design and implementation. Replacement is achieved by employing a fully human-only in vitro system, entirely eliminating the need for animal-derived components. In particular, the extracellular matrix component traditionally composed of or including animal-derived products is replaced with cell line-derived human collagen I, which not only addresses animal usage concerns but also ensures a higher physiological relevance and lower batch variability. Furthermore, all cellular components of the model, including endothelial cells (TY10), astrocytes (IM-HA), and pericytes (IM-HBVP), are established human cell lines, selected for their robust characterization and compatibility with microfluidic systems, and contributing towards the goal of replacing in vivo animal experimentation with species-specific modeling.
Additionally, the use of a miniaturized, multi-chip microfluidic platform (Organoplate® 3-lane 40) aims to enable a higher throughput for preliminary drug screenings in vitro, which could be used to refine the design of subsequent in vivo experiments, if necessary, and potentially reduce the number of animals required for later-stage validation by narrowing down drug candidates and optimizing dosage ranges.
Overall, this project exemplifies a comprehensive implementation of the 3Rs, offering an ethical, reproducible, and scientifically robust alternative to animal-based BBB research and preclinical drug testing.

5. IMPACT OF THE PROJECT

The proposed project holds substantial scientific, technical, ethical, and socio-economic impact by addressing persistent limitations in current in vitro models of the blood–brain barrier, particularly in the context of neuroinflammatory diseases such as Multiple Sclerosis (MS).
(1) Scientific Advancement: This model provides a physiologically relevant, multicellular human BBB system capable of recreating pathological permeability changes observed in MS. By incorporating endothelial cells, astrocytes, and pericytes in a structured microfluidic environment and inducing inflammation through targeted cytokine and chemokine exposure, the model enables mechanistic investigations that are currently unfeasible with animal models or more simplified in vitro systems.
(2) Methodological Contribution: Leveraging the Mimetas Organoplate® 3-lane 40 platform enables scalability, reproducibility, and integration with automated readout systems, minimizing manual intervention. This, together with the standardization of the materials used and of the protocols developed over the course of the project, will allow a reasonably straightforward replication of the key experiments and results and a possible future application of derived models to similar contexts.
(3) Translational and Therapeutic Potential: By validating the system with established MS therapeutics (e.g., fingolimod, retinoic acid) and testing emerging candidates (e.g., Imatinib), we aim to propose this model as an innovative preclinical evaluation platform for identifying and refining barrier-targeted therapies. It also lays the foundation for a future incorporation of iPSC-derived patient cells in the place of

general human cell lines, supporting the development of personalized treatment strategies.
(4) Ethical and Economic Benefits: The elimination of animal-derived materials and the replacement of animal experiments with a fully human in vitro model significantly reduces ethical concerns and regulatory barriers. This, in turn, can accelerate the approval process for drug candidates, lowering development costs and improving time-to-market efficiency.
In conclusion, the project will not only help fill a significant gap in MS-related BBB research but also introduce a versatile platform that supports ethical, personalized, and cost-effective innovation in biomedical science.