

Review

Advanced Bioengineering Technologies as Alternative Methods in Pre-Clinical Research: A Future Perspective on Non-Animal Research

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Abstract

Background: Although the use of animals in various experimental activities is still necessary for the advancement of science, research for the development, validation, and recognition of new microchip technologies has increased in recent years, which characterizes a great advance for science. Alternative biotechnological methods aim to replace the use of animal models in scientific experiments or reduce their use when replacement is not possible, which corroborates the principle of the Replacement, Reduction, and Refinement (3Rs)—total replacement, reduction, refinement. Thereby, it is possible to overcome inaccurate predictions caused by distinct elements between species, significantly reduce research costs and time by promoting a comprehensive scientific advance in a more favorable regulatory environment. This review aims to highlight current and future perspectives of tissue bioengineering aimed at possible alternative or substitute methods to animal models in preclinical research in biomedical applications. **Methods:** The search strategy was based on the PubMed/Medline, SciELO, and Web of Science databases, as well as books on the respective topics, accessed in the period between October 2024 to March 2025. The keywords were “alternative biotechnologies”, “non animals”, “tissue bioengineering”, “organ on chip”, “biomaterials”, “tissue”, “engineering”, “bone” from the last five years, totalizing 4429 articles. The eligibility criteria, established as cutoff points, were described in the methodology. **Conclusions:** Thus, new tissue bioengineering tools and technologies are rapidly emerging to innovate *in vitro* practices. Examples include organs-on-a-chip and 3D biomimetic structures with tissue-like dynamic properties to enable in-depth investigation of disease mechanisms.

Keywords: Animal experimentation, biomimetic models, *in vitro* assays, organs-on-chip, translational medical research.

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Introduction

Regenerative medicine is known to aim to restore the functionality of tissues and organs resulting from injury, disease, or aging [1]. Tissues can repair themselves in isolation, including bone tissue. However, for critical lesions, biomaterials have a potential role in stimulating autologous cells for structural and functional repair with a quality cellular matrix in the injured tissue [2]. In this context, animal proxies have been used as experimental models.

In previous decades, there has been a global shift in awareness [3], resulting in scientific pressure to reduce, refine, and replace the use of animals in the safety assess-

ment of drugs and chemicals [4], as well as in biomedical research. Thus, the Food and Drug Administration (FDA) in the US has implemented next-generation risk assessment (NGRA). This is an exposure-focused, hypothesis-driven risk assessment that integrates chemical information from *in vitro* approaches without relying on animal data [5].

To investigate more complex scenarios of exposure and associated toxicity, the creation of organ models in a microenvironment was considered, thus characterizing the current technology known as organ-on-chip (OOC). This biotechnology meets the current demand for complex assays such as repeated dose, reproductive, and toxicokinetic

[6,7]. Communication between these multi-organ models requires not only the transfer of a culture medium from one organ chamber to another organ chamber, but also the simulation of reality *in vivo*, such that there are biological barriers that mimic those existing *in vivo* [8].

Tissue culture alone can provide significant information about some organ functions, but is not able to express the inherent systemic interactions that directly influence responses to injury, disease, and therapy [9,10]. Therefore, microphysiological systems (MPS) with bioengineered human tissues have been developed to mimic organ-level functions, allowing insights into *in vitro* physiology [11–13].

McKim *et al.* [14] developed an *in vitro* multi-organ MPS system capable of being used as a rapid cytotoxicity screen. Ronaldson-Bouchard *et al.* [15] developed a tissue chip system in which mature niches of human cardiac, liver, bone, and skin tissue were connected by recirculating vascular flow. Connected tissues maintained their molecular, structural, and functional phenotypes over four weeks of culture, recapitulated the pharmacokinetic and pharmacodynamic profiles of doxorubicin in humans, enabled the identification of early biomarkers of microRNA (miRNA) cardiotoxicity, and increased the predictive values of clinically observed miRNA responses relative to cultured tissues alone and fluidly connected tissues in the absence of barrier endothelial.

In this review, there are works by co-authors that provide examples to demonstrate how molecular biology tools can be used to deeply assess the compatibility of biomaterials. Among the main insights, there is the possibility of advanced characterization of biomaterials [16–18]; biomaterials with modified surfaces capable of inducing cytoskeletal reorganization and boosting osteoblast differentiation [19]; stimulation of adhesion and viability of osteoblasts on surfaces with TiO₂ with increased expression of osteogenic markers such as alkaline phosphatase (ALP), RUNX Family Transcription Factor 2 (RUNX2) and osteocalcin (OCN) [18]; increased lipid peroxidation activity in titanium-based biomaterials, inducing the reorganization of tissue matrix of bone tissues and tissues adjacent to alveolar tissue [20]; possibility of evaluating the efficiency of coupled mechanisms of osteogenesis-angiogenesis, providing predictive data on clinical outcomes [21]; in addition to *in vitro* analyses with biomaterials that allowed the exploration of epigenetic changes that occurred capable of promoting cytoskeletal remodelling and associated angiogenic responses [22,23].

Thus, new tissue bioengineering tools and technologies are rapidly emerging to support the innovation of *in vitro* practices. This narrative review aims to highlight the current and future perspectives of tissue bioengineering applied to alternative or substitute methods (partial or absolute) to animal models in preclinical biomedical research.

Methods

The search strategy was based on the PubMed/Medline, SciELO, and Web of Science databases, as well as on books on the respective topics, accessed between October 2024 and March 2025. The keywords used were “alternative biotechnologies”, “without the use of animals”, “tissue bioengineering”, “organ-on-a-chip”, “biomaterials”, “tissue”, “engineering” and “bone”, in articles published in the last five years, consisting of 4429 articles, 178 of which were from SciELO, 10 from PubMed and 4241 from the Web of Science. The inclusion criteria were: publication in the last five years; mention of one of the keywords described above in the abstract, title, and/or body of the text; and preclinical studies of biomedical applications, whose methodology should include a control group and a statistically significant sample size. Subsequently, studies that met the eligibility criteria based on traditional techniques and alternatives to animal experimentation were included in this review. Exclusion criteria were other biotechnologies not approved by the FDA or that are still in the evaluation phase. Exceptions to the eligibility criteria included articles that addressed these fundamental concepts, but that had been published before the five-year period.

Current Perspectives of Tissue Bioengineering on Alternative Methods in Biomedical Research

Alternative Methods in Tissue Bioengineering Biotechnology Research

The use of animal models (Table 1) in scientific research requires compliance with ethical and legal precepts [24]. *In vivo* testing was initially implemented in practice, both for its physiological similarity and for its possible functional replication at the organ and system level. However, such testing has also been affected by rising costs and growing ethical concerns, in addition to the challenges in selecting the ideal species to serve as an animal model [25].

In 1959, the ethical aspects of animal research were redefined based on the three “Rs” (Replacement, Reduction, and Refinement (3Rs)): “Replacement, Reduction, and Refinement” [24]. The great potential and availability of current alternative strategies and biotechnologies in animal-free research (Fig. 1) support a human research focus (e.g., New Alternative Methods, NAMs) and the ultimate clinical application to disease diagnosis and treatment.

An alternative method is intended to replace, either totally or partially, the use of animals in scientific research [29]. They are characterized by validated and accepted tests capable of guaranteeing similar and reproducible results to achieve, whenever possible, the same purpose as the replaced procedures [30]. They are classified as validated alternative methods, recognized alternative methods, and valid alternative methods [31,32].

Furthermore, a validated alternative method is one

Table 1. Summary of the advantages and limitations for current traditional *in vivo* mammalian models utilised for testing and experimentation.

Advantages	Limitations	Applications
Complex and dynamic system	Ethical considerations. Environment not controlled.	Preclinical studies (host interactions, pharmacology, and efficacy after <i>in vitro</i> assays).
Holistic view of interactions	Interspecies differences. Different immune responses compared to human physiology.	Chemical, drug toxicity testing.
Protocols and tools available	Requires trained personnel. High costs with maintenance and group controls required. Anatomic and dimensional differences between animal models to humans.	To reproduce systemic diseases. Microbiome studies.

Sources: Wouters *et al.* [26], Dias *et al.* [27], and adapted from Alonso-Roman *et al.* [28].

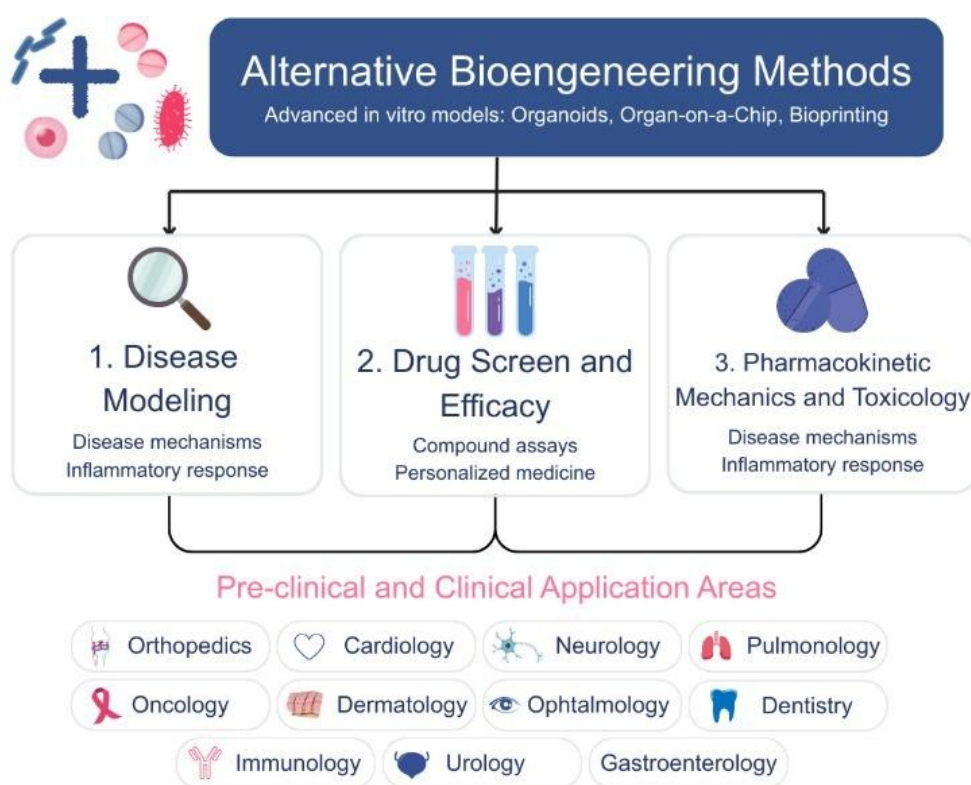


Fig. 1. Clinical and preclinical applications of alternative current strategies and biotechnologies on non-animal research. Created in Canva. Penteado dos Santos, M. L. (2026) <https://canva.link/1dzfsxrd58azgc2>.

whose reliability and relevance for a given experimental purpose have already been established, performed at different stages of its development, enabling it to obtain regulatory acceptance at the international level. A recognized alternative method is a validated alternative method that has been recognized by the National Council for the Control of Animal Experimentation (CONCEA) and is already mandatory throughout the country. A valid alternative method has not yet been validated, but for which there is sufficient data to demonstrate its reliability and relevance [31–33].

Bioethics in Tissue Bioengineering

These limitations have encouraged the search for alternative approaches to address fundamental aspects of global biomedical technical problems [34]. Thus, in Europe, there is the European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection of the European Commission's Joint Research Centre (JRC) in Ispra, Italy. ECVAM's main responsibilities are to finance and manage validation studies and to support other Commission services on issues related

to alternatives to animal testing [35].

Therefore, soon after, a growing social impact was observed regarding ethical aspects and research involving animal models. In 1978, the Universal Declaration of Animal Rights was promulgated by United Nations Educational, Scientific, and Cultural Organization (UNESCO) [36], sparking intense social debate about the real need for animal experimentation. And, the following year, in Brazil, Law No. 6638 was created, aiming to regulate animal experimentation in institutions so that animals were not subjected to situations that caused them suffering. In 2008, the Arouca Law, No. 1153/1995, was passed and transformed into Law No. 11,794/2008 [37]. Therefore, the breeding and use of animals in teaching and scientific research activities are currently regulated [24,36,37]. Similar laws have also been enacted in many countries, particularly those with sophisticated research activities.

The United Kingdom (UK) remains the third largest user of animals in Europe. Since the Cruelty to Animals Act of 1876, there have been more reports of animal abuse and new considerations about its applicability and reproducibility [24]. In light of this recognition of the differences between humans and animals, and the increased ethical focus that encourages the reconsideration of animal models, the UK government established the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) to promote innovation to replace, reduce, and refine the use of animals in science, both in industry and academia [38]. In the US, the Center for Alternatives to Animal Testing (CAAT), founded in 1981, was established as part of the Johns Hopkins University Bloomberg School of Public Health in recognition of public concerns about animal testing for the cosmetics industry (Henry Spira). They promote humane science by supporting the creation, development, validation, and use of alternatives to animals in research, product safety testing, and education [39].

In Brazil, this initiative was supported by the creation of the National Network of Alternative Methods (RENAMA) and, in 2013, the Brazilian Center for Validation of Alternative Methods (BRACVAM) [40,41]. Currently, CONCEA, RENAMA, and BRACVAM are focused on developing alternative methodologies to animal experimentation [42].

CONCEA is a regulatory, deliberative, and advisory body for animal experimentation in Brazil, responsible for recognizing alternative methods to the use of animals in the country [42]. The National Health Surveillance Agency (ANVISA) recognizes all alternative methods recognized by CONCEA [31]. There are 40 normative acts in force in the country in March 2025. Of these, 87.5% are Resolutions (35/40), 5% are Technical Guidelines (2/40), and the remaining 7.5% correspond to legislation related to Ordinance (1/40), Law (1/40), and Decree (1/40) [33]. Furthermore, among the methodologies that have been validated nationally or internationally, but not yet recognized

by CONCEA, these can be used if the introduction of such alternative techniques is respected and monitored [29].

Replacement Innovation: Alternative Current Strategies and Biotechnologies and Their Applications to Biomedical Research

Contemporary biomedical research is witnessing a crucial transition from two-dimensional (2D) cell culture models to more physiologically relevant three-dimensional (3D) platforms. This evolution is driven by the search for systems that more accurately mimic the complexity of tissues and organs *in vivo*. The models listed below—ranging from simple cell aggregates (spheroids) to high-tech, environmentally controlled systems (organs-on-a-chip and bioprinting)—differ fundamentally in their cell source (influencing their complexity and differentiation capacity), their mode of organization (whether through intrinsic cell self-organization or controlled microengineering), and their scalability (the feasibility of large-scale production for applications such as drug screening). The following table (Table 2) provides a comparative overview of these essential biotechnologies.

Spheroids, Organoids, and 2D Cultures

Currently, there is increasing consideration of technological strategy development for tissue bioengineering capable of increasing the relevance and precision of *in vitro* practices. Standard examples include 2D cultures, which depend on rigid plastic or glass culture plates to support cell adhesion and proliferation. Such methods are commonly used due to their practicality and low cost, in addition to their ease of observation and analysis [43,44]. However, monolayer cell cultures, although they are still effective approaches for preliminary screening to allow the determination of cytotoxic effects, are limited because they are usually cultivated in simplified and physiologically unrealistic conditions, restricting their expansion to *in vivo* systems [43].

Thus, 3D systems are currently available to complement previous 2D analyses and offer a more realistic biochemical and biomechanical microenvironment with the architecture and nature of realistic tissues [44]. For example, in human organ systems, cells can interact in a complex 3D microenvironment with an extracellular matrix that is continuously supplemented with nutrients and oxygen [45].

The self-organization of these 3D cultures, called organoids, occurs through the following mechanisms: (A) cell sorting, driven by differential intercellular adhesion between cell types, and (B) through the decision of restricted spatial cell fate, being influenced by local environmental signalling differences [46]. Thus, organoids allow an alternative form of study to animal models for investigating development processes, tissue regeneration, and homeostasis [47]. Therefore, it is known that there are at least some of the mechanisms involved in organogenesis in this process,

Table 2. Differentiation of biotechnology models: cell source, organization, and scalability.

Biotechnology	2D Cultures	Spheroids	Organoids	3D Cultures (General)	Microtissues	3D Bioprinting	OOC	Organoid-on-a-chip	Biosensors
Cell Source (Examples)	Immortalized cell lines or primary cells.	Cancer cells (Tumors), primary cells, or cell lines.	Induced pluripotent stem cells (iPSCs), Embryonic stem cells (ESCs), or adult stem cells.	Various types (primary cells, cell lines, iPSCs).	Primary cells or cell lines, derived from a tissue.	Bio-inks containing primary cells, cell lines, or iPSCs	Primary cells, cell lines (PSCs-epithelial organoids, ESCs/iPSCs, endothelial cells, immune cells).	Organoids (PSC-derived (iPSCs or ESCs) or adult stem cells).	Genetically modified cells or biological components (enzymes, antibodies) are immobilized.
Organization: Self-organization vs. Micro-engineering	Self-organization (Limited): Grow as a monolayer, without complex tissue structure. High: Standard, well-established method.	Self-organization (Moderate): Cell aggregates formed and maintained by aggregation in non-adherent conditions.	Self-organization (High): Stem/progenitor cells spontaneously organize into complex 3D structures, mimicking organ.	Variable: Can be self-organization (aggregate-based) or scaffold-based (scaffolds) with extracellular matrix (ECM).	Microengineering: Cell aggregates with controlled geometry and size, using microwell plates, free-floating scaffolds, or molds.	Microengineering (High): Positioning of cells and bio-ink cements (e.g., with extracellular matrix) in pre-defined architectures.	Microengineering (High): Use of microfabrication to design an organ interface (e.g., tissues, control of fluid flow, and mechanical forces).	Hybrid: Combines Organoid Self-organization with chip Micro-engineering to provide perfusion and environmental control.	Microengineering: Design of the biological recognition receptor/transducer and the electronic transducer.
Scalability (Production volume)	Easy automation and large scale.	High: Can be automated (e.g., low-adherence plates). Cost per spheroid is relatively low.	Moderate to Low: Greater complexity, long protocols, and variability. Requires more optimization for large-scale.	Moderate: More challenging than 2D, but methods exist to optimize production.	Moderate: Frequently produced in batches using microwell plates, allowing for some parallelism.	Low to Moderate: High initial cost and printing time, but with potential for future automation and customization.	Low: Low production capacity (throughput). Models are complex, requiring microfabrication.	Low: Combines the difficulties of the organoid (e.g., reproducibility) and microfluidics, being generally focused on research.	High: After development, the production of biosensors (electrodes or membranes/platforms) is highly scalable.

2D, two-dimensional; 3D, three-dimensional; OOC, organ-on-chip. Sources: Alonso-Roman *et al.* [28], Cullen *et al.* [43], Lancaster and Knoblich [46], da Silva Feltran *et al.* [47], Unger *et al.* [52], Fang and Eglen [53], Sung *et al.* [55], Wang *et al.* [56], Murphy and Atala [57], Panda *et al.* [58], Blundell *et al.* [59], Jackson and Lu [60].

in addition to organ and tissue regeneration [48].

A wide range of protocols has already been developed to generate organoids, allowing the production of several organ-like structures produced using this approach [46]. Several prototypes of biodegradable scaffolds have been developed using printed polymers, ceramics, and composites for bone tissue regeneration and implanted in the femur and tibia of animal models [49,50].

Among the limitations of this method, it is known that they are not exact replicas of the organs they model. Organoids do not incorporate critical factors observed *in vivo*, such as vascularization, nor are they exposed to mechanical stress and electrical stimulation, for example. Therefore, caution should be exercised when extrapolating such information to *in vivo* processes [51].

Spheroids, on the other hand, refer to a culture of tumour cells carried out under non-adherent conditions in low-adhesion plates that develop heterogeneous tumour-like structures *in vivo*, in response to specific biomolecular gradients. This method has been widely used for studies on tumour biology and drug screening [52,53].

Another important practical application in bone tissue repair is the use of chondrocytes cultured at high density, forming a 3D cartilage tissue. The use of such cells allows the formation of tissue that can be induced to form stratified cartilage and incorporate a zone of calcified cartilage, thus recreating the interface between the cartilage and the subchondral bone [54].

Therefore, the evolution from 2D culture, which is accessible and low-cost, to 3D models, such as spheroids and organoids, reflects the search for greater physiological relevance in *in vitro* research. While 2D offers simplicity for initial screening, its main limitation is its physiological unreality, failing to replicate the architecture and complex tissue interactions. On the other hand, 3D models provide a more realistic microenvironment, with organoids being crucial for developmental studies and spheroids being superior for tumor modeling and drug screening. The critical limitation of 3D, however, is the absence of essential *in vivo* factors, such as vascularization and mechanical stimulation, requiring caution in extrapolating results to the clinic.

Microtissues

Microtissues represent the combination of microfabrication and tissue engineering to provide *in vitro* models with tissue characteristics, as well as 3D architecture, multicellularity, cell-extracellular matrix interactions, and functionality close to that of physiology. To do this, they need to be cultured in conventional plates that, through passive diffusion, allow the nutrition of cells undergoing cell growth. Therefore, they can be used in high-throughput analyses, such as in drug development and toxicological tests [55], even in the process of analysing biomaterials in orthopaedics [61]. However, the main challenge has still been to apply this methodology, commonly used in denser

materials, to less dense materials and cells [55].

Among the techniques used to design such microtissues are hydrogels, which are extracellular matrices designed with a high-water content and mechanical properties to replicate the host's extracellular matrix [62,63]. Therefore, by creating microenvironments that mimic *in vivo* conditions, this technology opens new avenues by providing more reliable analyses in preclinical research, disease analysis, and drug testing. Currently, microtissues have been used in preliminary toxicological analyses of drug-induced liver and cardiac diseases, which are the main cause of drug withdrawals [64].

In addition, 3D cell culture has allowed advances in the differentiation and maturation of cells derived from stem cells, as well as their *in vitro* characterization [65]. The technique can also be used in epithelial tissues, which, for several ethical reasons, restrict animal experimentation [66]. Several models of human epidermal tissue have been approved and validated in Europe to replace animal testing [67]. In short, the process involves mixing human dermal fibroblasts with type I collagen until contraction is observed. Soon after, keratinocytes are added, and their proliferation is observed. Finally, the engineered tissue is cultured under air-liquid interface conditions to promote tissue maturation [66].

Among the main challenges in using microtissues are the expensive microscopy images and configurations required for automation, in addition to high throughput. In this sense, biosensors can be essential to assist in the application of microtissues [68].

Microtissue technology represents an advancement in 3D tissue engineering, using microfabrication to create high-fidelity, functional *in vitro* models of tissues. Its main advantage is its compatibility with high-throughput analysis for drug screening and toxicological testing (especially in liver and heart models), as well as serving as a validated ethical alternative to animal testing, using materials such as hydrogels to mimic the *in vivo* environment. However, its limitations include the technical challenge of application to low-density materials and the high cost of automation and advanced microscopy setups required—barriers that need to be overcome, possibly with the use of biosensors, for its widespread adoption.

Biosensors

Aiming to overcome the difficulties related to *in vitro* models, characterized by their dependence on image data, there is an excellent approach that is integrated into bioengineering platforms. Biosensors are analytical devices that perform molecular recognition of biological capture probes, such as antibodies, oligonucleotides, enzymes, and even cells, to measure the concentration of analytes. These recognition events will be translated into a signal that will be read, and this transducer can be electrochemical, optical, thermal, among others. This biotechnology can be used to

continuously monitor critical parameters of the cellular microenvironment and to study long-term drug response [56].

Zhang *et al.* [69] used a multi-organ platform on a chip for automated online monitoring with sensors that monitored pH, oxygen, and temperature, as well as electrochemical biosensors monitoring soluble protein biomarkers and mini microscopes for generating morphological images of organoids cultured within the platform. As an example, a tumour intravasation model was used for real-time monitoring of TNF- α that included tumour, endothelial, and macrophage cells, revealing the dynamics of the process with increased endothelial permeability and interactions between the tumour and endothelial cells [70].

In addition to soluble biomarkers, some tissues, such as skeletal, cardiac, and smooth muscle, require specific sensors to assess the main functional response, the contraction capacity. Such mechanical forces generated in these models are generally measured by monitoring the flexion of silicon cantilevers or micropillars [71]. Furthermore, electrical activity can also be a functional marker for myocardium and muscle, and nerve tissues. Such electrical signals can be recorded by microelectrode arrays integrated into micro-physiological systems [72].

Biosensors, therefore, represent an integrated and promising solution to overcome the reliance on imaging *in vitro* models, as they allow continuous, real-time monitoring of critical cellular microenvironment parameters, such as pH and O₂, as well as soluble biomarkers and functional responses (contraction and electrical activity). This advantage significantly increases the relevance of dynamic models for studying drug response. However, the main limitation lies in the technical complexity of miniaturization and integration of multiple transducers on microphysiological platforms, requiring high standardization, stability, and sensitivity for the technology to succeed.

3D Bioprinting of Tissues and Organs

Aiming to overcome the delay for functional organ transplants, 3D bioprinting has been promoted. It refers to several biotechnologies that rely on layer-by-layer assembly principles to precisely pattern cell mixtures and biomolecules in combination with bioinks to create living, anisotropic 3D structures that mimic human tissues and organs. Currently, it has been used mainly to generate complex 3D microenvironments for *in vitro* experimentation. Among the advantages is the ability to develop relatively large, engineered tissues, allowing insights into physiological processes and tissue responses to drugs, which would not be possible with other biotechnologies [57].

3D bioprinting, therefore, provides a potential solution to the shortage of organ transplants, allowing the manufacture of functional organs such as liver, heart, lung, kidney, cartilage, skin, and bone. Among the main advantages over traditional methods with implants and traditional transplants is the intrinsic similarity to biological systems, fur-

ther reducing the risk of rejection by the immune system [58].

One challenge is the difficulty of ensuring that cell viability is maintained during the process due, for example, to the shear force applied during the delivery of bioinks through capillaries, which can cause damage to the cells. The most used bioprinting technologies are based on the continuous extrusion of a bioink filament [73] or through the delivery of discrete bioink droplets by an inkjet printer head through a collector [74]. It is also possible through laser-assisted bioprinting, performed by applying a laser pulse to the top face of a metal tape to propel a bioink droplet from the layer attached to the opposite face of the tape to a collector [75]. In addition, there is another technique that uses light to crosslink bioinks [76].

Ng *et al.* [77] developed a stratified pigmented skin model that includes dermal and epidermal layers, including keratinocytes and melanocytes in the epidermis. This model matured more similarly *in vivo* tissue than other related techniques. An important feature is the ability to precisely pattern cells and biomolecules into 3D structures so that functional vascular networks can be incorporated into these tissue models. In the absence of these, without the supply of nutrients and metabolites, tissue necrosis can occur [78].

Current approaches design scaffolds with large pores to allow interconnected porosity to accommodate new vasculatures *in vivo*. However, even when associated with proangiogenic factors, this is a slow process that can lead to substantial loss of already resident viable cells [79]. There are currently several ways to integrate functional vascular networks into tissue engineering. Tubular structures that can serve as vascular networks can be produced through coaxial extrusion.

Attalla *et al.* [80] incorporated endothelial cells into this device with bioink to form the tubular structure of the vessels. The main limiting factor is the difficulty in fabricating branched networks [81]. An alternative approach incorporates bifurcation by depositing an ink characterized by reversible physical cross-links, such as alginate, agarose, or gelatin, within the 3D structure and during the printing process [82,83]. The ink patterns are temporary and liquefiable, revealing a network of channels that can be endothelialised and perfused with culture media or sutured to blood vessels after implantation. This prevents the long-term mechanical integrity of the channels, revealing the need for the use of bioinks that do not degrade rapidly [81]. Furthermore, with current bioprinting technologies, the idea of bioprinting multiscale vasculature down to submicron capillaries becomes unsustainable [84].

In fact, current bioprinting technologies, although they have achieved temporal control mechanisms within printed tissues and hydrogels, still have several challenges. It is still not possible to reach the level of complexity observed in living tissues, and it also does not allow the fab-

rication of many tissues on a large scale, due to the time required to print such structures, as well as the adverse bioprinting conditions that can compromise cell viability [57].

3D bioprinting is therefore promoted as a promising technology to overcome organ shortages and create complex *in vitro* models, with the advantage of manufacturing large, standardized structures with intrinsic similarity to human tissue, minimizing the risk of immune rejection. However, it faces critical limitations that prevent its full application: the difficulty in maintaining cell viability during the printing process, the inability to achieve the level of complexity of living tissues, and, fundamentally, the challenge of integrating a large-scale functional and branched vascular network to avoid tissue necrosis.

Organ-on-Chip

OOO technology is characterized by the reduction of complete human organs and/or organ systems to minimal functional units capable of developing fundamental aspects of *in vivo* physiology and maintaining key features of native tissue architecture. These are microchannels lined with various types of cells arranged to replicate organ-specific interfaces [59]. These micro-engineered systems, through a system of pumps, valves, and filters, allow precise control of the perfusion of microliters of fluids through the microchannel network in a laminar flow regime. In this way, it becomes possible to mimic specific aspects of the response of the entire organ, replicate *in vivo* connections between different organs, and investigate pharmacokinetic and drug toxicity mechanisms, for example [60].

In addition to the characteristics, it is necessary to assign mechanical actuators that can generate different states of stress, such as shear, compression, and/or traction forces [85]. There are several methods capable of manufacturing these microfluidic systems, from engraving techniques to laser photoablation. In their manufacture, it is necessary to combine soft lithography techniques and replicas with polydimethylsiloxane (PDMS) due to its low cost, ability to cross-link into intricate features, and transparency to allow microscopic observation [86,87]. Additional advantages include: ease of direct observation in real time of cellular processes of interest with conventional optical microscopes; the ability to control important environmental factors, such as temperature and gas concentration, delivering soluble factors (cytokines, drugs, etc.) and collecting metabolites; reduced cost associated with smaller volumes of reagents required; possibility of reproducibility, in addition to integration with other technologies; and the possibility of associating several organs on a chip in order to investigate interactions between different organs and recreate the complete human physiology, that is, the body on a chip [86,88,89].

Currently, all major organs have been reconstituted on OOO platforms, such as the liver, skin, lung, and placenta. Regarding drug responsiveness and toxicity, liver, lymph

node, bone marrow, intestine, and kidney on a chip have already been evaluated. Therefore, regardless of the tissue type, organs on a chip are mainly used for two purposes: (a) disease modelling; (b) advanced drug screening [90].

Despite its increasing evolution, the technology still requires improvements, such as: (a) specific scalability conditions and standard and large-scale manufacturing techniques; (b) better-performing materials that avoid unwanted formations on the chip that compromise its permeability; (c) cellular sources; (d) ideal perfusion process that avoids bubble formation and controls fluid recirculation; (e) more sensitive and synergistic imaging and analysis [86,90–92].

Khan *et al.* [93] described a protocol for developing a human bone marrow organoid that supports the growth of primary cells from patients with myeloid and lymphoid blood cancers. The organoids supported engraftment and survival of cells from patients with blood malignancies, including cancer types that are notoriously difficult to culture and study *ex vivo*. This model enables mechanistic studies of blood cancers in the context of their microenvironment and provides a necessary *ex vivo* tool for prioritizing novel therapeutics. Thus, the development and application of organoids, which are living, self-organized, 3D multilineage structures, can facilitate translational research. This allows for the pharmacological and pathological modulation of the disease, as well as facilitates analyses of genetic panels.

Among the limitations cited by the authors [93] are that distinct arteriolar and sinusoidal endothelial subtypes are not present, and no osteoid lineages, lymphoid cells, smooth muscle cells, or adipocytes are generated. Despite this, bone marrow organoids can be used to support a variety of bone marrow cancers and cell types, allowing possible customizations to support other relevant studies.

Suhito *et al.* [94] described several biotechnological approaches capable of generating and characterizing the physiological authenticity of models that mimic human organs *in vitro*. Among these are human bone marrow organoids (bone marrow-on-a-chip) [95–98]. With BM-on-a-chip, the functional haematopoietic niche *in vitro* and the possible retention of haematopoietic stem and progenitor cells in this scaffold are described. Among the main challenges are both the difficulty of maintaining haematopoietic stem and progenitor cells *in vitro* and the fact that the arteriolar and sinusoidal endothelium are distinct [94].

Fig. 2 summarizes some alternative current strategies and biotechnologies described in this paper, with the main advantages and limitations related.

OOO technology is characterized by the reduction of functional organ units into microchannels, offering the advantage of mimicking physiological and architectural aspects *in vivo*, with precise control of environmental factors and mechanical actuators, making it ideal for disease modeling, pharmacokinetic studies, and drug screening. How-

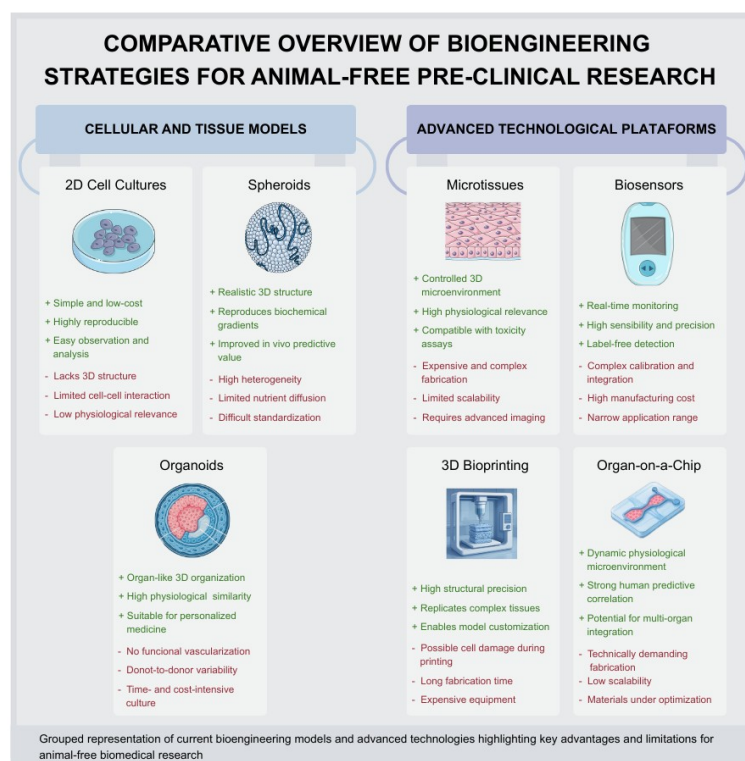


Fig. 2. Main alternative current strategies and biotechnologies with the most relevant advantages and limitations related. Created in Canva. Penteado dos Santos, M. L. (2026) <https://canva.link/14cex05y5m8ah43>.

ever, it faces significant limitations that require improvements, such as the need for scalability and standardization of manufacturing, the development of higher-performance materials, optimization of cell sources and the perfusion process (to avoid bubbles), as well as a lack of more synergistic imaging and analysis systems.

Organoid-on-Chip

Aiming to overcome the challenges of organoid systems related to vascularization, as well as their possible frequent contaminations, there is the organoid-on-a-chip. This combines the main benefits related to the OOC and organoids, such as: possibility of differentiation into organ-specific cell subtypes; it recapitulates human variability and has a more easily controllable environment than with organoids [28].

Some authors have already described the use of human gut-derived organoids-on-a-chip for various purposes. Bein *et al.* [99] studied the cellular and inflammatory responses of the host infected with coronavirus NL63. They therefore suggested its usefulness as a human preclinical model in studies related to infectious diseases caused by coronaviruses, as well as in the analysis of potential antiviral or anti-inflammatory therapeutics.

Beaurivage *et al.* [100] integrated intestinal epithelial cells (IEC) derived from human intestinal organoids with monocyte-derived macrophages in a gut-on-a-chip platform

to model the human intestine and key aspects of inflammatory bowel disease (IBD). Despite some challenges, they found that the multifactorial IBD platform can be expanded to evaluate the efficacy of compounds in early stages of drug development or in personalized medicine.

However, among the main challenges with this biotechnology are the difficulty of configuration and maintenance, in addition to the high costs required compared to organoids and OOC, as examples. In addition, there is intense variability between donors from organoids and the difficult analyses resulting from intracellular interactions [28].

Organoid-on-a-chip technology emerges as a high-biological-value hybrid system, integrating the complex self-organization and differentiation of cell subtypes of organoids with the microfluidic and environmental control of the OOC. Its main advantage is offering a model that recapitulates human variability and specific aspects of complex diseases (such as coronavirus infections or IBD), while allowing for a more controlled environment and minimizing the risks of contamination, overcoming the vascularization limitations of pure organoids. However, the technology presents critical limitations that restrict its adoption: the high cost and difficulty of configuration and maintenance compared to organoids and OOCs individually. Additionally, the intense variability among organoid donors and the complexity of the analyses resulting from intracellular in-

teractions within the chip remain challenges to be overcome to ensure standardization and reproducibility.

All these biotechnologies—from 2D and 3D cultures, spheroids and organoids, to microtissue platforms, 3D bio-printing and complex systems such as biosensors, OOC and organoid-on-a-chip—represent crucial advances in *in vitro* models aimed at replacing (eliminating), reducing and refining the use of animals in research and toxicological testing, aligning science with the ethics of the 3Rs.

Advanced *in Vitro* Models for Biomaterial-cell Interactions: Investigating Biocompatibility Through Cell Signalling

In response to the growing need to replace animal models in preclinical biomaterial research, several advanced *in vitro* platforms have been developed to assess the osteogenic potential of biomaterials [101]. These platforms enable high-resolution monitoring of biomaterial-cell interactions under controlled laboratory conditions, allowing for in-depth assessment of biocompatibility without relying on animal experimentation [102–104]. In addition to conventional viability assays, these analyses integrate molecular profiles to elucidate the mechanistic cellular behaviors underlying biomaterial biocompatibility, opening new avenues for advanced biomaterial characterization [47].

Over the last decade, these methodologies have been increasingly discussed and implemented, particularly in line with ISO 10993 standards, which emphasize prioritizing *in vitro* methods over animal-based assays [105]. In this sense, there are several significant examples of the application of molecular methodologies to better understand cell behavior during osteoblastic differentiation [19,101,106].

In strict accordance with the ISO 10993-5 guidelines, direct and indirect contact cytotoxicity assays (based on extract) have been validated, expanding conventional methods such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) and LDH (lactate dehydrogenase) to include comprehensive analyses of gene expression, kinase activity (quinome profile), and protein phosphorylation [20,107,108]. For example, investigations of nanostructured hydroxyapatite surfaces, such as those conducted by Bezerra *et al.* [19], revealed the early activation of critical signaling pathways such as FAK (focal adhesion kinase), Akt (serine-threonine kinase), and MAPK/ERK (mitogen-activated kinase/extracellular signal-regulated kinase), which together promoted cytoskeletal reorganization and boosted osteoblastic differentiation.

In the case of titanium-based biomaterials, ions released from TiO₂ surfaces have been shown to increase the expression of osteogenic markers (e.g., ALP (alkaline phosphatase), RUNX2, OCN), stimulate focal adhesion formation via the FAK/Src pathway, and, consequently, promote osteoblastic adhesion and viability [20]. Additionally, these cellular responses correlated with metabolic adaptations, indicating that biomaterials influence not only adhe-

sion processes but also cellular metabolism [22], particularly under conditions involving mechanical or oxidative stress [23].

More recently, Wood *et al.* [21] proposed a molecular coupling mechanism between angiogenesis and osteogenesis, elucidating the molecular basis of titanium as a gold-standard biomaterial. It was observed that dual cell culture models (osteoblasts co-cultured with endothelial cells) highlighted critical interferences mediated by angiocrine factors, notably VEGF-A (vascular endothelial growth factor A), secreted after exposure to titanium or hydroxyapatite-coated surfaces. These findings reinforce the potential of animal-free *in vitro* platforms to efficiently assess coupled osteogenesis-angiogenesis mechanisms, providing predictive data for clinical outcomes [21].

In another set of experimental approaches, *in vitro* methods were explored to characterize specific cellular phenotypes induced by interactions with biomaterials, applying advanced epigenetic analyses as a strategic tool [109]. Biomaterials were found to induce significant epigenetic changes in endothelial and osteogenic cells through the modulation of HDACs (histone deacetylases) and DNA methylation pathways [110]. These epigenetic alterations facilitated cytoskeletal remodeling and angiogenic responses, revealing a new dimension of biological responses beyond classical signaling cascades [111–114].

To further enhance the predictive power of these advanced platforms, OOC systems have recently been introduced as complementary tools. These microfluidic devices allow the mimicry of complex tissue-tissue interfaces and dynamic microenvironments, integrating various cell types into spatially organized 3D architectures and enabling precise control of perfusion and mechanical stimulation for a more accurate recapitulation of physiological conditions [115]. For example, bone-on-chip and osteovascular chip models are being used to simulate the interaction between vascular and osteogenic compartments, increasing the translational relevance of *in vitro* studies in the field of biomaterials [28]. In parallel, the integration of biosensors into these systems allows for real-time, marker-free monitoring of cellular responses, crucial factors such as pH variations, ionic flow, cytokine secretion, and metabolic activity [116]. This immediate feedback significantly increases the resolution of biomaterial evaluations and allows for the dynamic profiling of cellular behavior in response to environmental or material-induced stimuli.

In this context, the role of nanocarriers is noteworthy; these are important devices capable of modulating cellular signaling through the controlled delivery of key biomolecules [92]. Nanocarriers have been explored in pre-clinical models for bone, cartilage, neural, cutaneous, and cardiac regeneration. For example, poly (lactic-co-glycolic acid) (PLGA) nanoparticles loaded with Bone Morphogenetic Protein 2 (BMP-2) and bioactive peptides have demonstrated enhanced osteoinductive capacity when in-

egrated into bone scaffolds [117]. Similarly, they contribute to the viability and effectiveness of stem cell therapies by creating a protective microenvironment, improving cell survival, and facilitating their retention at the target site through the controlled release of growth factors [63].

Thus, advanced *in vitro* platforms represent a robust and comprehensive alternative to animal testing, integrating cell signalling pathways, gene expression profiling, and metabolic evaluation in biomaterial evaluations. These models are critical for the screening and development of next-generation biomaterials—including bioactive and ion release systems—in full alignment with the 3Rs (Replacement, Reduction, Refinement) principles and regenerative medicine goals.

The Power of Alternative Methods in Non-animal Research and Future Perspectives

Current Demand for Research with Biotechnology: Main Advantages and Challenges

These technologies in advanced *in vitro* platforms will undoubtedly change the way preclinical research is conducted, especially for more accurate disease modelling, mechanistic studies, and drug screening [118]. In addition, they can be integrated with existing technologies to achieve a major impact on health and economic benefits for society [119]. In general, these integrated or online monitoring biotechnologies are valuable tools in drug development and essential for adequate application in bioengineering models in the near future [120].

Furthermore, there is a global emergency in healthcare systems, such as addressing the growing number of emerging pathogens, antimicrobial resistance, and opportunistic infections. This leads to global research into potential new biotechnologies that can be safely used for optimal tissue repair. Similarly, interactions between the host and potential external pathogens need to be analyzed and studied using non-animal models, particularly human-focused models and systems. This is in line with the current trend of animal replacement in scientific research, supported by reduction and refinement [118,121].

However, there are still many challenges to overcome. Among the main challenges are the complexity of the systems and the resulting costs [120]. The complexity of the physiological system is mainly reflected in efforts to add microvasculature to these biotechnologies, in addition to the associated immune response [119]. It is relevant that the immune system has functions in each organism that are from the initial adaptive function in protecting the organism against external pathogens to its intrinsic relationship with the expectation related to the diagnosis and therapeutic efficacy achieved [25,119]. However, among the main challenges are its complexity, high interindividual variability, and low accessibility to primary immune tissues. Besides that, this may be assisted by combinations of other alterna-

tive (NAMs) methods, for example, human data analytics and access to bio-banked cells and tissues [122–124].

In addition, most preclinical studies involving immunological analyses are still with animal models, which may relate to possible imprecise patterns of immune response to these models due to species differences. And as a result, low success rates for translation to humans in clinical trials are among the main associated reasons, generating exponential costs involving millions to billions of dollars in the evaluation of new therapies and drug development [26].

Current efforts are focused on integrating different approaches into the same platform to achieve synergistic effects resulting from this combination of advantages of each technique and to overcome the challenges inherent to each technique. For example, several engineering strategies have aimed to integrate organoids and organ-on-a-chip into *in vitro* platforms that respect cellular fidelity and have high-throughput phenotypic readings [119]. Therefore, these biotechnologies could be integrated into 3D technologies to reconstruct the tissue microarchitecture [120,125].

The advancement in the area of New Approach Methodologies (NAMs) gains crucial regulatory endorsement with the April 2025 announcement from the USA. FDA. The agency revealed a plan to phase out the requirement for animal testing in the preclinical evaluation of monoclonal antibodies and other drugs, actively encouraging the replacement of *in vivo* data with data generated from NAMs. This paradigm shift represents a milestone in regulatory modernization, aligning FDA guidelines with the central theme of this article: the prioritization of methods that are more relevant to human physiology, such as OOC models, organoids, and advanced *in vitro* platforms, reinforcing the urgency and scientific value of replacing animal experimentation in the safety and efficacy assessment of biomaterials [126].

Organoid and OOC technologies represent crucial advances in *in vitro* human tissue modeling, offering more realistic 3D platforms for drug development and precision medicine by mimicking complex physiological functions. This technological evolution is significantly driven by synergy with Artificial Intelligence (AI), which, by analyzing large datasets of biological data, promises to dramatically accelerate and revolutionize the Drug Discovery and Development (DDD) process, overcoming the high costs and failure rates of conventional methods. In parallel, this transition is supported by a historic regulatory milestone, such as the FDA Modernization Act 2.0, which signals the FDA's move to move beyond animal testing mandates in favor of more human-relevant models. However, regulatory, technical, and ethical challenges persist that need to be addressed to ensure the reliability, scientific validity, and full applicability of these promising new approaches in the drug evaluation and approval process [127–129].

In this way, they can surpass the throughput of current

vitro assays, enabling personalized technological and bio-functional applications in highly complex diseases [63].

Conclusions

Although no current alternative biotechnology fully replaces animal models (especially for complex tissues like bone), they offer a triple benefit in science, ethics, and economics, driving translational research. Basic 2D and 3D cultures (spheroids) show the most immediate potential for high-throughput clinical trials due to their simplicity and cost-effectiveness. On the other hand, advanced technologies such as organoids and microtissues stand out in simulating specific physiological aspects. The technologies facing the greatest challenges due to their complexity and the need for systemic integration are OOC and 3D bioprinting, which aim to reconstruct complex systemic interactions, vasculature, and complete physiology, with the main overall challenge being the correct selection and integration of the ideal approach for each scientific objective.

List of Abbreviations

2D, two-dimensional; 3D, three-dimensional; 3Rs, Replacement, Reduction, and Refinement; AI, Artificial Intelligence; Akt, serine-threonine kinase; ALP, alkaline phosphatase; ANVISA, National Health Surveillance Agency; BM-on-a-chip, Bone Marrow-on-a-chip; BMP-2, Bone Morphogenetic Protein 2; BRACVAM, Brazilian Center for Validation of Alternative Methods; CAAT, Center for Alternatives to Animal Testing; CONCEA, National Council for the Control of Animal Experimentation; DDD, Drug Discovery and Development; DNA, deoxyribonucleic acid; ECM, extracellular matrix; ECVAM, European Centre for the Validation of Alternative Methods; ERK, extracellular signal-regulated kinase; ESCs, embryonic stem cells; FAK, focal adhesion kinase; FDA, Food and Drug Administration; HDACs, histone deacetylases; IBD, inflammatory bowel disease; IEC, intestinal epithelial cells; iPSCs, induced pluripotent stem cells; ISO, International Organization for Standardization; JRC, Joint Research Centre; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; miRNA, microRNA; MPS, microphysiological systems; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NAMs, New Approach Methodologies; NC3Rs, National Centre for the Replacement, Refinement and Reduction of Animals in Research; NGRA, next-generation risk assessment; OCN, osteocalcin; OOC, organ-on-chip; OoCs, organs-on-chips; PLGA, poly(lactic-co-glycolic acid); PSCs, pluripotent stem cells; RENAMA, National Network of Alternative Methods; RUNX2, RUNX Family Transcription Factor 2; SciELO, Scientific Electronic Library Online; Src, Src family kinase; TNF- α , tumor necrosis factor alpha; UK, United Kingdom; UNESCO,

United Nations Educational, Scientific and Cultural Organization; US, United States; USA, United States of America; VEGF-A, vascular endothelial growth factor A.

Availability of Data and Materials

The research data supporting this review are from previously reported studies and datasets, which have been cited. The processed data is available from the corresponding author upon request.

Author Contributions

TDTPM, BAL contributed to the planning of this work; AFCA, MLPS, BAL, MJS, WFZ, and VMVM contributed to the interpretation of the data; TDTPM, BAL, MLPS, and WFZ analyzed the data; TDTPM, AFCA, MLPS, and WFZ wrote the work; TDTPM, BAL, and WFZ critically reviewed the important intellectual content. All authors read and approved the final manuscript. All authors agree to be responsible for all aspects of the work, ensuring that issues related to the accuracy or integrity of any part of the work are properly investigated and resolved.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgments

To all colleagues that, even from the other side of the world, supported this vision and the relevance of this scientific topic in research with their expertise.

Funding

Not applicable.

Conflict of Interest

The authors declare no conflict of interest.

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Editor’s note: The Scientific Editor responsible for this paper was Martin Stoddart.

Received: 6th June 2025; **Accepted:** 10th April 2026;
Published: 30th September 2026